STUDIES OF
ERYTHROCYTE MEMBRANE ALTERATIONS
IN DUCHENNE MUSCULAR DYSTROPHY

by

MICHAEL ANTHONY BRIDGES
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Michael Anthony Bridges

Department of Pharmacology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date November 25, 1976

ABSTRACT

Duchenne Muscular Dystrophy (DMD) appears to be a generalized plasma membrane disorder involving many body tissues of affected individuals, including erythrocytes. Investigation of the myopathic aspects of this disease has suffered from difficulties in distinguishing between the immediate effects of the primary lesion in muscle and the sequelae of muscle fiber necrosis. However, since there are indications that erythrocytes may also be abnormal in DMD, it may be possible to characterize this primary lesion in these cells. Furthermore, examination of erythrocytes of DMD patients and their female "carrier" relatives may reveal convenient biochemical markers of the disease which may aid both in early patient diagnosis and in carrier detection.

The present investigations comprise a screening study in which a variety of chemical and biochemical techniques were employed in order to compare the structural and functional characteristics of DMD patient and carrier erythrocytes with those of normal control erythrocytes. A number of red cell abnormalities were found to be present in erythrocytes from patients with DMD or from their female carrier relatives: alterations in erythrocyte membrane phospholipid contents, in membrane-bound enzymatic activities associated with active sodium and potassium transport, as well as in those believed to be related to active calcium extrusion, and in the osmotic fragility characteristics of intact red cells. Although these findings are still tentative, they provide evidence supporting the generalized membrane defect hypothesis of DMD, as well as suggest promising avenues for further investigation of the molecular basis of DMD pathogenesis utilizing red cells. Recognized

mechanisms of cellular injury are discussed in the attempt to reconcile the experimental findings of these studies with those of other investigators, and parallels are drawn between the alterations observed in DMD erythrocytes and those exhibited by erythrocytes in various other disorders and in experimental models.

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INTRODUCTION

General Considerations

Attempts to conceptually define the term "muscular dystrophy" have been frustrated by the lack of precise information regarding the homogeneity of the various clinical myopathies placed in this group and their etiological bases. Despite this lack of basic knowledge regarding the nature of the muscular dystrophies in man, operational definitions have been utilized in order to permit the differential diagnosis of individual cases and to distinguish these from the non-dystrophic myopathies. These operational definitions take into consideration factors such as the mode of transmission of the disorder, its age of onset, rate of progression and the like [1]. This ability to distinguish between the various clinical myopathies is crucial to both the investigator, who seeks information on the basic disorder, and the clinician who must properly manage the myopathic patient. Unfortunately, the dystrophic myopathies are as yet incurable, and the clinician must content himself with mere palliation of these syndromes [1].

Duchenne muscular dystrophy (DMD), the focus of the present investigation, is a progressive, crippling disease of young males characterized by recessive sex-linked inheritance and an early onset of symptoms; these symptoms include proximal muscle weakness and atrophy, hypertrophy of the calves in most cases, muscular contractures, myocardial involvement and a high incidence of mental retardation, with death usually resulting from respiratory or cardiac failure by the second or third decade [1]. In British Columbia, the Health Surveillance Registry estimates the province-wide frequency of DMD as one case per 6000 live male births [2]. One third of DMD cases are believed to be new mutants; one third have a previous family history of the disease, and one third are born to unknowing and often mutant carriers [3].

A great deal of research has been directed towards characterization of the primary lesion underlying DMD. It should be noted here that much confusion in the literature has been generated by researchers who apparently assume that the etiologies of all muscular dystrophies are the same and who therefore seek to characterize the basic defect in DMD by extrapolation from experimental data derived from the study of other human myopathies and dystrophic animal models. As will become more evident, there is no firm evidence that these various muscular dystrophies are pathophysiologically identical to DMD [4].

Investigators have advanced four different hypotheses regarding the possible etiology of DMD: (a) the myogenic hypothesis suggests that the primary defect in muscular dystrophy lies within the muscle fiber; (b) the neurogenic hypothesis seeks to implicate abnormal neurotrophism of defective motor neurons in myopathy; (c) the vascular hypothesis maintains that the primary lesion resides in the microcirculation, leading to skeletal muscle necrosis; an extension of this hypothesis implicates faulty vasoactive amine handling in the production of DMD; and (d) the membrane defect hypothesis suggests that muscular dystrophy is an inherited molecular disorder of cellular membranes with widespread tissue involvement. The experimental evidence upon which these various hypotheses rest warrants some discussion.

The Myogenic Hypothesis

Although many structural and functional disturbances have been identified in muscle, the chief problem remains: how does one distinguish the primary lesion from secondary and tertiary effects arising from muscle fiber necrosis, lipogenesis and connective tissue infiltration? Some of the more compelling findings in studies involving muscle are presented here.

Hughes has analysed lipid extracts of skeletal muscle minces by thin layer chromatographic methods [5] and found no alteration in total muscle lipids of DMD patients. However, the content of individual lipid fractions was greatly altered and bore a marked similarity to the pattern observed in human fetal muscle: the cholesterol and sphinogomyelin lipid fractions were increased and the phosphatidyl choline fraction was decreased relative to normal. Hughes therefore suggested that DMD might represent a maturation failure of skeletal muscle. Similar results had previously been obtained for genetically dystrophic mice [6]. Studies in myopathic chickens [7] and hamsters [8], however, do not reveal the same pattern of lipid fraction alterations.

Dhalla et al. have quantified the adenosine triphosphatase (ATPase) activities of sarcolemmal preparations obtained from patients with DMD and dystrophic hamsters [9]. In both DMD patients and dystrophic hamsters, the basal and Ca^{2+}-dependent ATPase activities of muscle sarcolemma were observed to be increased; the Na^{+}, K^{+}-dependent enzyme activity was found to be depressed in the human myopathy but elevated in the hamster. Skeletal muscle sarcolemmal preparations from dystrophic mouse have revealed a pattern almost identical to that observed in DMD when assayed for the three ATPase enzyme activities [10, 11]. Dhalla et al. have suggested that the sarcolemmal abnormalities seen in DMD might result from an alteration in the chemical composition of the muscle cell membrane [9]. Such an alteration might profoundly affect the functional integrity of the muscle cell and be at the root of the dystrophic process.

Skeletal muscle sarcoplasmic reticulum (SR) has been the object of extensive investigation in the search for the primary lesion in DMD in view of the importance of this organelle in governing Ca^{2+} availability in the

process of muscle contraction. Takagai et al. have identified compositional abnormalities in protein and lipid fractions of SR vesicles in DMD [12], the lipid alterations resembling those observed by Hughes in whole muscle extracts [6]. Functional abnormalities were also found in SR by these and other investigators: decreased Ca^{2+}-uptake by SR vesicles was noted in in vitro studies [12, 13], as was decreased Ca^{2+}-dependent ATPase activity [13]. Similar functional alterations have also been shown to occur in myopathic hamsters [14] and dystrophic chickens [15], but not in human Myotonic muscular dystrophy [13]. Takagai et al. have advised caution in the interpretation of these findings, because SR vesicles are frequently contaminated with microsomal material from adipose and connective tissues which have infiltrated muscle as part of the dystrophic process [12]. The presence of these contaminants in SR vesicle preparations might well alter the compositional and functional parameters evaluated to a considerable degree.

Investigators have also turned their attention to the study of protein synthesis in DMD muscle. Monckton and Nihei have demonstrated a three-fold increase in protein synthesis in heavy polyribosome fractions of DMD muscle [16]. Ionasescu et al. have confirmed these observations and have shown that heavy polyribosomes from DMD muscle produce four to five times as much collagen as normal heavy polyribosomes [17]. Whether these findings are indicative of the primary defect underlying DMD (e.g., a defective genetic mechanism for terminating collagen synthesis [4, 16]), or whether the observed alterations merely represent a non-specific response to injury, is impossible to judge without further investigation.

Studies of the contractile apparatus of DMD muscle have been equally inconclusive. Gel electrophoretic analysis of contractile proteins reveal normal protein profiles in DMD material [18], but troponin activity (expressed

as trypsin-sensitive Ca^{2+}-binding capacity of natural actomyosin) was found to be depressed by at least 50% of normal in muscle obtained from biopsy of DMD patients [19]. This could reflect a basic defect in excitation-contraction coupling or could be a consequence of the presence of excessive proteolytic enzyme activity, often observed in dystrophic muscle [20, 21]. Actomyosin ATPase activity from dystrophic human muscle has also been shown to be depressed relative to normal muscle activity [22].

Strickland and Ellis have found an interesting abnormality in DMD muscle which also extends to other body tissues. These investigators had previously demonstrated an abnormally high conversion of glucose to fructose in dystrophic muscle, with a corresponding drop in glucose-6-phosphate production [23]. This shunting of substrate away from the energy-producing glycolytic pathway was shown to result from the presence of a modified hexokinase isoenzyme II in the DMD muscle fiber [24]. Strickland and Ellis demonstrated this enzyme alteration by gel electrophoretic methods in skeletal muscle, liver and brain tissues obtained post mortem from DMD patients. Such a defect in hexokinase isoenzyme II could arise from an inheritable lesion of protein synthesis and could produce widespread damage in many body tissues by depriving them of efficient glycolytic machinery for ATP production and by subjecting these tissues to noxious levels of fructose and sorbitol, implicated by some in the production of diabetic neuropathy [25].

The foregoing discussion serves to illustrate a number of important points: (a) a large number of disturbances of structure and function have been observed in Duchenne dystrophic muscle; (b) there are great difficulties associated with the search for the primary lesion in this disorder, since this pathogenic factor could easily be obscured by secondary and tertiary alterations arising from the dystrophic process; (c) the use of animal models

of human muscular dystrophy in the search for the DMD pathogenic lesion is fraught with peril, because in the absence of prior knowledge of this basic defect, there can be no valid criterion for deciding which animal model best approximates the human disorder; and (d) some abnormalities observed to occur in dystrophic muscle can also be demonstrated in certain other non-muscular tissues [23]. This last point suggests that the primary lesion of DMD may not necessarily reside exclusively in the muscle cell; in fact, some investigators believe that the lesion may not reside in muscle at all.

The Neurogenic Hypothesis

The neurogenic hypothesis originated with studies on murine muscular dystrophy. Conrad and Glaser examined the fatiguability of neuromuscular transmission in normal and dystrophic mice and found evidence of a slower rate of fatigue in dystrophic murine muscle which could not be explained by increased muscle responsiveness [26]. To account for this phenomenon, Conrad and Glaser postulated the existence of an alteration in neuromuscular transmission independent of any primary muscle dysfunction.

This suggestion of abnormal neural activity in murine dystrophy provoked great interest in myopathic animal models. Transplantation studies in dystrophic mice [27, 28] and hamsters [29] led to the suggestion that some extramuscular factor governing skeletal muscle regeneration is abnormal in the dystrophic host. In an attempt to identify the abnormal extramuscular factor, Gallup and Dubowitz grew various combinations of normal and abnormal murine nerve and muscle together in tissue culture [30]. These investigators found: (a) murine dystrophic muscle behaved normally with respect to myotube formation and contractile activity when regeneration occurs in the presence of normal spinal cord cells, but (b) regeneration of both normal and dystrophic

muscle was severely affected when coupled with spinal cord cultured from mice with muscular dystrophy. Since these tissue culture preparations were free from humoral, vascular and higher central nervous system influences, Gallup and Dubowitz conjectured that neurons in the spinal cord are by themselves capable of producing myopathic manifestations in murine muscle. Similar experiments conducted by Hamburgh et al. failed to reproduce Gallup and Dubowitz's results [31]. However, other investigators have shown alterations in the normal functioning of neurons in murine dystrophy. For example, there is evidence of abnormal axoplasmic flow of lipids [32] and proteins [33] in sciatic nerve of animal dystrophy models, as well as a report of defective central and peripheral cholinergic neurons occurring in these animals [34].

Although unequivocal evidence to support the assertion that neurogenic influences underlie the production of murine dystrophy is still lacking, the results obtained from animal dystrophy studies have stimulated much interest in the possible neural etiology of progressive muscular dystrophy in man. The results of some of these investigations of DMD are discussed next.

McComas, Sica and Currie were the first to make the case for a neurogenic etiology of DMD [35]. They observed a 75% reduction in the number of functioning motor units in the extensor digitorum brevis muscle of Duchenne dystrophy patients, while recording normal action potential amplitudes for any given motor unit in these affected children. This suggested that the process of denervation occurring in DMD is highly selective, destroying individual motor units in their entirety. On the basis of these and subsequent experiments, McComas et al. postulated that DMD involves a chronic dysfunction of motor neurons, leading to their physiological failure [36]. Other researchers have been less fortunate in their attempts to demonstrate this selective loss of motor units in DMD. Their reports reveal no significant difference in the

number of motor units between children with DMD and age-matched normal controls [37, 38, 39].

The neurogenic hypothesis of DMD pathogenesis is attractive, especially since the existence of neurotrophic influences on skeletal muscle is well established [40], but the present evidence is not sufficiently compelling to convince one that this is the whole story. In this form of muscular dystrophy, pathological alterations extend beyond nerve and skeletal muscle. Perhaps the etiological factors responsible for this myopathy arise elsewhere in the body. The next section discusses other possible loci for the Duchenne defect.

The Vascular/Vasoactive Amine Hypotheses

According to Engel, the earliest histological changes seen in skeletal muscle of patients with pre-clinical and early clinical DMD are small foci of grouped muscle fibers undergoing necrosis or regeneration -- all fibers of the group being at about the same stage [41]. Engel believes these focal abnormalities to be so characteristic of DMD that they may be considered to be diagnostic of this disorder [42].

Most hypotheses of DMD pathogenesis cannot adequately explain why abnormal muscle fibers occur in clusters surrounded by fibers of histologically normal appearance. The vascular hypothesis offers a solution to this problem. It suggests the possibility that the blood supply to these small groups of affected fibers is compromised, since the size of a given focus could correspond to the area serviced by a terminal arteriole [42]. Skeletal muscle lesions similar to those reputed to occur in DMD have been produced in rabbits by occlusion of the animal's muscle microvasculature with small doses of intra-arterially injected dextran particles [42].

Recent reports have disputed Engel's characterization of DMD myopathy and the relevance of dextran-embolus model. For example, O'Brien et al. have suggested that a random distribution of necrotic fibers may be more representative of Duchenne muscular lesions [43]. Furthermore, morphometric studies of Duchenne muscle microvasculature have failed to demonstrate any abnormality except the replication of the basement membrane of capillaries, an alteration found to occur in a number of systemic disorders [44]. These data cast some doubt upon the validity of the vascular occlusion hypothesis.

Investigators, unable to demonstrate gross morphological changes or occlusion of microcirculation in DMD, have sought evidence for functional ischemia in the pathogenesis of this disorder, reasoning that if elevated levels of circulating vasoactive amines (e.g., catecholamines and indoleamines) could be demonstrated, then the vascular hypothesis might yet solve the mystery of DMD etiology. Murphy, Mendell and Engel detected an abnormality of vasoactive amine handling associated with platelets obtained from Duchenne dystrophy patients. These authors observed a marked reduction in the initial rate of serotonin (5-HT) uptake by DMD platelets, as well as a lowering of platelet 5-HT content [45]. Murphy et al. have suggested that an analogous abnormality of vasoactive amine uptake may also occur in the autonomic nerve terminals of the intramuscular vasculature, since similarities have been described for amine transport mechanisms in platelets and in nerve endings in brain and in the periphery [46]. Because neuronal re-uptake is the major mechanism for termination of the action of certain neurotransmitters in the synaptic cleft [47], a defect in this re-uptake apparatus might lead to a prolongation of the effects of these vasoactive amines, with resultant damage being produced either by a direct toxic action upon muscle fibers or by rendering them ischemic through intense vasoconstriction [45]. The credibility

of this modification of the vascular hypothesis will depend upon the demonstration of such a neuronal uptake defect in patients with DMD.

It is clear from the foregoing sections that there are many tissues and organelles in which DMD patients show deviations from the normal situation: compositional and enzymatic abnormalities have been described for practically every organelle of the skeletal muscle cell; defects in glycolysis of skeletal muscle, liver and brain have been reported; mental retardation of affected children and functional loss of motor neurons have also been observed; alterations in the capillary basal lamina and abnormal vasoactive amine handling by platelets and muscle have been described. Such a diversified pattern of alterations clearly makes identification of the primary lesion in DMD a difficult problem. But this complex pattern may also be supplying an important clue: it might be suggesting a more basic, generalized defect than has been supposed underlies the production of this disorder. The following section continues to elaborate upon the diffuse pathology encountered in DMD and presents evidence for a generalized cellular membrane defect in the pathogenesis of this syndrome.

The Membrane Defect Hypothesis

It has long been recognized that myopathy in DMD is not restricted to skeletal muscle. Cardiac muscle involvement is usually associated with this syndrome [1]. In a recent retrospective study of autopsy findings covering a thirteen-year period in Denmark, Leth and Wulff showed histological evidence of cardiomyopathy in 80% of DMD patients investigated [48]. Goto, using gel electrophoretic analytical methods, was able to demonstrate abnormally high levels of the cardiac isoenzyme of skeletal muscle creatine phosphokinase (CPK) in the serum of DMD patients [49]. This may suggest that the

same pathological processes, which produce "leaky" skeletal muscle plasma membranes and the loss of intracellular enzymes (e.g., CPK), may also be operative in cardiac muscle.

Disturbances of cardiac rhythm, especially persistant tachycardia, are common in DMD [1]. Moreover, a number of investigators have claimed that the very characteristic electrographic pattern observed in this disorder is of diagnostic value in distinguishing between DMD and other juvenile forms of progressive muscular dystrophy in man [50]. Sudden death from cardiac failure frequently occurs in DMD [1]. In the Danish study previously cited [48], 41% of the Duchenne patients who died between 1960-1973 succumbed from cardiac complications of their disease. These reports clearly document that muscle lesions in DMD are not restricted to skeletal muscle but also extend to cardiac muscle.

Recently, evidence has accumulated that erythrocytes from DMD patients exhibit compositional, structural and functional abnormalities. Kunze et al. have demonstrated a significant increase in the sphingomyelin content, as well as alterations in the fatty acid composition of phosphatidyl ethanolamine and sphingomyelin, of DMD red cell membranes [51]. Matheson and Howland have reported that saline-washed erythrocytes from patients with DMD appear drastically deformed when viewed by scanning electron microscopy [52]. Although these structurally modified erythrocytes, termed echinocytes, were observed in both normal and dystrophic children, the proportion of these distorted cells to normally appearing cells in whole blood was very different for the two groups. In normal children the percentage of echinocytes ranged from 3-7%, but DMD children showed a range of 20-98% echinocytes in whole blood. Other investigators have confirmed Matheson and Howland's findings, but a considerable degree of overlap exists between the

normal and affected groups [53]. Furthermore, an increased incidence of echinocytes in whole blood has also been shown to occur in a number of other clinical conditions, including some myopathies [52, 54]. Thus, although the morphological alteration described here is not specific to DMD, it further supports the idea that the pathological process at work in this disease is widespread, involving even red blood cells,although in a stereotyped way.

Abnormalities in K^+ fluxes have been reported for Duchenne erythrocytes. In a recent study, Howland has shown that red blood cells from DMD patients exhibit an abnormally high permeability to potassium; he has also observed this alteration in brain and liver mitochondria of genetically dystrophic mice [55]. But Howland could find no evidence to suggest that intracellular K^+ levels are depleted in DMD erythrocytes. This very high passive K^+ permeability may be at least in part compensated by the reported increase in active K^+ transport into red cells in DMD [56].

Recently, Roses and Appel have demonstrated abnormal activity of erythrocyte membrane protein kinase in both DMD and Myotonic muscular dystrophy [57]. Endogeneous protein kinase is an enzyme system capable of catalyzing the phosphorylation of certain membrane protein components. Roses and Appel found that in DMD red cell membranes, the phosphorylation of a component of spectrin (gel electrophoretic band II) is increased, while in the myotonic erythrocyte membrane, phosphorylation of component "a" of band III is decreased [58]. It has been suggested that the state of phosphorylation of membrane components may be important in determining the structural and functional characteristics of the erythrocyte membrane. Consistent with this hypothesis is the report by Kury and McConnell that the state of phosphory-

lation of the erythrocyte membrane, which is modified in the presence of adrenaline or prostaglandins, influences the configurational state of membrane lipids, as monitored using the spin-label techniques [59].

The foregoing discussion of the many and varied tissue alterations reported in DMD including those described in red cells leads one to suggest that the primary lesion underlying this disorder may well be a generalized one involving cellular plasma membranes. This proposed membrane defect could involve alterations in the structural interrelationships between plasma membrane protein and lipid components, thereby giving rise to myriad secondary changes in cellular functional parameters, including alterations in the properties of subcellular organelles. Thus the membrane defect hypothesis of DMD could unify the previously discussed cellular abnormalities into a coherent whole. However, these experimental findings are still inconclusive: the pathogenic lesion in DMD has yet to be unequivocally identified, and the secondary and tertiary alterations which accompany this defect have yet to be rationalized relative to this primary lesion.

The Red Blood Cell in the Investigation of Duchenne Muscular Dystrophy

The use of erythrocytes in the study of DMD pathogenesis has much to recommend it: (a) the pathological process underlying DMD markedly influences red cell membrane structure and function; (b) erythrocyte membranes share many features in common with more complex plasma membrane systems, such as sarcolemma; these features include compositional similarities [60], a ouabain-sensitive active Na^+-transport system [61], and an active Ca^{2+}-extrusion mechanism [62]; furthermore, there is good evidence that erythrocytes possess an actomyosin-like system (spectrin associated with red cell actin)

which is located at their inner membrane surfaces and may be responsible for regulation of red cell shape [63, 64]; (c) erythrocytes are not subject to the same degenerative cellular processes which may obscure the pathogenic lesion in skeletal muscle; finally, (d) red cells from normal and affected subjects may be obtained by a minimally invasive procedure (venipuncture), and their membranes may be easily isolated in a very pure state and in high yield by routine methods (see Materials and Methods). In short, the red blood cell membrane obtained from patients affected with DMD offers a nearly ideal model membrane system for investigative purposes.

In addition to supplying information potentially useful in the identification of the molecular basis of DMD, the red cell may also be of value in the early diagnosis of this disorder. At present, the establishment of a definitive diagnosis of DMD in the affected child is an arduous and time-consuming affair for the patient and his family. There is often great difficulty in distinguishing between DMD, other muscular dystrophies and certain non-dystrophic disorders which produce proximal muscle weakness [1]. The diagnosis invariably involves long-term observation of the progression of the patient's symptoms, the performance of numerous clinical diagnostic tests for exclusion of the non-dystrophic myopathies, serial determination of serum levels of skeletal muscle enzymes (e.g., CPK), biopsy of affected musculature and electromyography [1]. This tends to work great hardships upon the patient and his family and poses problems in advising the parents about the risk of DMD in future pregnancies. Therefore, it would be of great value to develop a rapid, convenient and minimally invasive clinical test, which would unambiguously identify DMD in the child presenting with this disorder. If a biochemical DMD marker could be found in the erythrocytes of affected

children and if this marker were unique to this disease, then such a diagnostic tool would be a reality.

This hypothetical red cell DMD marker might even prove to be detectable years before the onset of the clinical signs of dystrophy, perhaps even as early as the neonatal or even the fetal stages. Diagnosis of DMD in utero by amniocentesis [65, 66] would allow the possibility of aborting affected fetuses, while neonatal screening could permit early institution of therapeutic measures in the pre-clinical stage, if research into DMD pathogenesis were ultimately to reveal ways to cure or ameliorate the dystrophy.

Analysis of erythrocytes may also provide a means of unequivocally determining carrier status in female relatives of Duchenne muscular dystrophy patients. A variety of indirect indices of carrier status have been used [65]: (a) the physical examination -- true carriers often have a slight degree of myopathy which occasionally can be detected as muscular hypertrophy or weakness [1], but physical examination rarely reveals these abnormalities; (b) skeletal muscle biopsy -- in some carriers, histological examination of muscle biopsy material will detect abnormalities, but the difficulty in obtaining a large enough sample for a definitive judgment on carrier status limits the value of this procedure; (c) quantitative electromyography -- EMG detects abnormalities in something less than 50% of definite or probable carriers; since the technique is laborious and often non-definitive, its popularity as a detection method is small; (d) determination of serum levels of muscle enzymes -- the most successful method to date for detection of carrier status is estimation of serum creatine phosphokinase (CPK) levels; CPK determinations are easy to perform, but this test only detects 60-70% of definite carriers. Clearly, more convenient and unequivocal means for detecting carrier status in DMD are needed.

When a woman with an affected son seeks genetic counselling, the probability of future affected offspring can only be discerned if the source of her son's disease can be identified as either familial or sporadic. It should be noted that sporadic cases of DMD are thought to occur with a high frequency. If the mother does not show myopathy, an estimation of this probability must be made by examination of the family history. This will usually allow the assignment of the woman to one of three categories described by Walton [1]: "Definite carriers" are those mothers of an affected son who have also an affected brother, maternal uncle, sister's son or other male relative in the female line of inheritance; also mothers of affected sons by different, non-consanguineous fathers. "Probable carriers" are the mothers of two or more affected sons, who have no other affected relatives. "Possible carriers" are the mothers of isolated cases and the sisters and other female relatives of affected males.

The accepted theory of a high incidence of sporadic DMD has recently been questioned by Roses and Appel [67]. They investigated 21 mothers of DMD patients (3 definite carriers, 4 probable carriers and 14 possible carriers), using three different methods of evaluating the carrier status in these women: (a) detailed physical examination for detection of muscle weakness, (b) electron microscopic examination of erythrocyte morphology to estimate the incidence of echinocytes, and (c) measurement of the extent of endogeneous phosphorylation of erythrocyte membrane spectrin. They demonstrated detectable muscle weakness in 19 of the 21 mothers. All 21 showed an abnormally high incidence of red cells with shape distortions. The mean values for spectrin phosphorylation were identical for the three carrier types and were statistically higher than the mean value for female control subjects. These

results suggest that many mothers of children showing apparently sporadic DMD are actually carriers of the defective gene.

Unfortunately, the alterations in red cell morphology and spectrin phosphorylation observed by Roses and Appel do not constitute unambiguous markers of DMD carrier status, since individual values for the women in the three carrier classes often fell well within the normal range [53, 67]. Therefore, the quest for reliable indices of carrier status continues.

The studies of red cells described in this thesis are a continuation of the search for the pathogenic lesion underlying DMD and for unambiguous markers of the affected and carrier states of this disorder. The work reported here comprises a screening study; erythrocytes obtained from patients with DMD and their female relatives were analysed by a wide variety of biochemical techniques to evaluate red cell structural and functional characteristics. Statistical comparison of these parameters to those obtained for age and sex-matched normal controls has revealed promising areas for future in-depth investigation.

MATERIALS AND EXPERIMENTAL METHODS

Materials

The following reagents were obtained from Sigma Chemical Company: Tris (hydroxymethyl) aminomethane (Trizma Base, reagent grade), imidazole (grade III), disodium adenosine-5'-triphosphate, p-nitrophenyl phosphate (104 phosphatase substrate), acetylthiocholine hydrochloride, Triton X-100, sodium dodecyl sulfate (SDS), 5,5'-dithiobis-(2-nitrobenzoic acid), picryl sulfonic acid (trinitrobenzenesulfonic acid), N-acetyl neuraminic acid (type III from egg), cholesterol standard, phosphorus standard solution (20 micro-

grams inorganic P/ml as $KH_2(PO_4)$), p-nitrophenol standard solution (10 micromoles/ml). Bovine serum albumin (fraction V) was supplied by Armour Pharmaceutical Company. Ninhydrin ("Baker TLC Reagent") was purchased from Baker Chemical Company. Cyanogum-41, ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TMED) were obtained from E-C Apparatus Corporation. Bromophenol blue (indicator pH 3.0-4.7) and Coomassie brilliant blue stain were supplied by Eastman-Kodak Company. All other chemicals were of analytical reagent quality. The water used in all experiments was prepared by double distillation using a Corning glass still.

Preparation of Erythrocyte Membranes

Blood for investigation was obtained from patients with muscular dystrophy, their families and age/sex-matched normal controls. Blood samples of approximately 20 ml were drawn from each subject into anticoagulant [1 ml 3.8% (w/v) sodium citrate per 9 ml blood] and kept at or below 4° C during the preparation of hemoglobin-free erythrocyte membranes [68]. The buffy coat fraction was removed during two initial isotonic saline washes, and the erythrocyte pellets were exposed to stepwise osmotic lysis in 0.08, 0.06, 0.04, 0.02 and 0.009 M NaCl, the pellets being recovered by centrifugation following each lytic step. After a final wash with 10 mM Tris buffer (pH 7.4), erythrocyte membranes were quick-frozen using dry ice and acetone and stored at -20°C. Chemical and enzymatic analyses of membranes followed a rigid time schedule such that a given determination was always performed after the same interval following preparation. This was of particular importance in the analysis of membrane enzymatic parameters, some of which vary with the period of storage.

Chemical Characterization of Erythrocyte Membranes

Protein content of membrane preparations was determined by the method of Lowry et al. [69], using bovine serum albumin as standard. Protein contents of membrane suspensions averaged between 3 and 4 mg protein per ml. Phospholipid was estimated by Bartlett's modification of the Fiske-SubbaRow assay for inorganic phosphorus [70]. Membrane cholesterol content was determined by the method of Zak et al. [71].

Sialic acid was estimated, following hydrolysis of 0.2 ml of membrane suspension in 0.1 N H_2SO_4 at 80°C for 30 minutes, by the method of Warren [72].

Amino group titers of intact and detergent-disrupted erythrocyte membranes were measured using trinitrobenzenesulfonic acid as described by Godin and Ng [73]. Membranes (0.2 ml) were incubated at 37 $\pm$ 0.5°C in the presence of 1.0 ml 20 mM Tris buffer (pH 8.0) in a total volume of 2.9 ml. In experiments on detergent-disrupted membranes, 0.2 ml of 3% (v/v) Triton X-100 detergent was added to the incubation mixture. The reaction was initiated by addition of 0.1 ml 10 mM trinitrobenzenesulfonic acid (TNBS) solution (pH 8.0) and terminated after 1 hour by addition of 1:1 (by volume) mixture of 1 M HCl and 10% (w/v) sodium dodecyl sulfate. The extent of trinitrophenylation was expressed as the absorbance at 335 nm per mg membrane protein.

The surface sulfyhydryl group titer of erythrocyte membranes was estimated by incubating membranes at 37 $\pm$ 0.5°C in the presence of 1.0 ml 0.15 M imidazole buffer (pH 7.4) in a total volume of 2.9 ml. Determination of total sulfhydryl group titer required disruption of membranes by addition of sodium dodecyl sulfate at a final concentration of 1% (w/v) to the reaction

mixture. Reactions were initiated with 0.1 ml 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Free sulfhydryl group labelling with DTNB is complete within 30 minutes and involves disulfide bond formation between a free sulfhydryl group of the membrane and one 5-thio-2-nitrobenzoic acid (TNB) group DTNB, with simultaneous release of the other molecule of TNB into the medium. The absorbance of the liberated TNB at 412 nm gives a measure of the number of membrane sulfhydryl groups modified. Membrane sulfhydryl titers are expressed in units of nanomoles sulfhydryl per mg protein (TNB Molar Extinction Coefficient is 1.36×10^4) [74].

Kinetic Analyses of Erythrocyte Membrane Enzymatic Activities

1. Adenosine-5'-triphosphatase

Erythrocyte membrane adenosine-5'triphosphatase (ATPase) activities were assessed under two different sets of chemical conditions.

a) Basal (Mg^{2+}-dependent) and
Total (Mg^{2+}, Na^+, K^+-dependent) ATPase

The reaction mixture for the determination of total Na^+, K^+-ATPase, which is the sum of the Mg^{2+}-ATPase and Na^+, K^+-ATPase, consisted of the following components at the final (mM) concentrations shown in parentheses: Tris-HCl buffer, pH 7.4 (55 mM); MgCl (3 mM); disodium adenosine-5'-triphosphate (ATP) (3 mM); NaCl (80 mM); KCl^2(20 mM); ethylene glycolbis-(B-aminoethyl ether)-N,N'-tetra-acetic acid (EGTA) (0.1 mM); 0.6-0.8 mg membrane protein, all in a final volume of 3 ml. The Mg^{2+}-ATPase component of total ATPase activity was determined as above except that Na^+ and K^+ were omitted from the reaction mixture. In this analysis as well as that described below (section b), corrections were made for non-enzymatic hydrolysis of the ATP substrate.

b) Mg^{2+}, Ca^{2+}-dependent ATPase

The reaction mixture for the kinetic analysis of Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase contained: Tris-HCl buffer, pH 7.4 (55 mM); $MgCl_2$ (6.4 mM); disodium ATP (2 mM); EGTA (0.1 mM); $CaCl_2$ (0.067, 0.080, 0.093, 0.100, 0.117, 0.133, 0.167, 0.200, 0.233 and 0.300 mM, corresponding to final free Ca^{2+} concentrations of 0.166, 0.326, 1.03, 2.69, 18.0, 29.7, 60.0, 89.4, 119.0 and 179.0 micromolar, respectively)*; 0.3-0.4 mg membrane protein, all in a final volume of 3 ml. The basal (Mg^{2+}-stimulated) ATPase activity represented that activity obtained by omitting Ca^{2+} from the reaction mixture.

ATPase reactions were initiated with membrane protein, incubated for one hour at 37 $\pm$ 0.5°C and terminated by addition of 1 ml ice cold 20% (w/v) trichloroacetic acid. Mixtures were centrifuged (30,000 x g, 10 minutes) to remove membrane material, and a 3.0 ml aliquot of supernatant was assayed for inorganic phosphate by the method of Fiske and SubbaRow [75]. Specific activities were expressed as micromoles of inorganic phosphate liberated per hour per mg membrane protein.

As described previously [76], the kinetics of Ca^{2+} stimulation of ATPase are complex. The existence of two distinct classes of Ca^{2+}-stimulated ATPase activities was deduced from the biphasic character of Eadie plots produced by plotting the reaction rate of Ca^{2+}-ATPase (total Mg^{2+}, Ca^{2+}-ATPase activity less basal Mg^{2+}-ATPase activity) as a function of rate/Ca^{2+} concentration. The Ca^{2+} concentration producing half maximal stimulation ($K_{.5}$) and the maximal reaction velocity (V_{max}) were evaluated for each activity;

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*The concentrations of free calcium in the EGTA-Ca^{2+} buffer system were determined by means of a computer program provided by Dr. B. Roufogalis, Faculty of Pharmaceutical Sciences, The University of British Columbia.

these kinetic parameters were determined from the slope and Y-intercept, respectively, of the best fitting lines describing linear segments of Eadie plots (See Results). Lines of best fit were computed using a Compucorp 140 "Statistician" calculator.

2. Basal (Mg^{2+}-dependent) and
Total (Mg^{2+}, K^{+}-dependent) p-Nitrophenyl Phosphatase

The reaction mixture for the kinetic analysis of K^{+}-stimulated p-nitrophenyl phosphatase (NPPase) consisted of the following components at the final (mM) concentrations shown in parentheses: Imidazole-HCl buffer, pH 7.4 (50 mM); $MgCl_2$ (3 mM); p-nitrophenyl phosphate (3 mM); KCl (2, 4, 6, 8, 10, 20, 30 mM); 0.6-0.8 mg membrane protein, all in a final volume of 3 ml. The basal (Mg^{2+}-stimulated) component of total NPPase activity was determined as above except that KCl was omitted from the reaction mixture.

NPPase reactions were initiated with membrane protein, incubated for one hour at 37 $\pm$ 0.5°C and terminated by addition of 1 ml ice cold 20% (w/v) trichloroacetic acid. Mixtures were centrifuged (30,000 X g, 10 minutes) to remove membrane material, and a 3.0 ml aliquot of supernatant was rendered alkaline with 1 ml 1.5 M Tris solution. The amount of p-nitrophenol in the basified supernatant was quantified spectophotometrically by measuring the absorbance of the solution at 412 nm. Specific activity of membrane NPPase was expressed as micromoles p-nitrophenol liberated per hour per mg membrane protein. In all cases, correction was made for the non-enzymatic hydrolysis of substrate.

Kinetic parameters describing the K^{+}-stimulated component of NPPase activity were evaluated using Eadie plot analysis. The K^{+} concentration producing half maximal stimulation ($K_{.5}$) and the maximal reaction

velocity (V_{max}) were determined from the slope and Y-intercept, respectively, of the linear Eadie plot representation of the data.

3. Acetylcholinesterase

Erythrocyte acetylcholinesterase (AChE) activity was determined in reaction mixtures containing the following components in final concentrations shown in parentheses: Tris-HCl buffer, pH 8.0 (90 mM); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), pH 8.0 (0.33 mM); acetylthiocholine (0.05, 0.10, 0.20, 0.50 and 1.00 mM); 30-40 micrograms membrane protein, all in a final volume of 3.0 ml. The reaction was performed in spectrophotometric cuvettes at room temperature and initiated by addition of substrate.

The increase in optical density at 412 nm due to the reaction of liberated thiocholine with DTNB was linear for at least two minutes, and reaction rates were calculated from the average absorbance increase per minute during the first two minutes of the reaction. AChE activity is expressed as micromoles thiocholine liberated per minute per mg membrane protein with correction being made for the non-enzymatic breakdown of acetylthiocholine. The kinetic parameters $K_{.5}$ and V_{max} for the reaction were determined from the slope and Y-intercept of the best fit line computed for AChE Eadie plot data.

Because NaF inhibition of erythrocyte membrane AChE activity has been reported to be a useful probe of membrane fluidity [77], the kinetics of inhibition of AChE by NaF were assessed in the same fashion as described above for the substrate kinetic experiments except that enzymatic activities were determined in the presence of increasing concentrations of NaF (0.5-2.5 mM) at a saturating concentration (1 mM) of acetylthiocholine. Kinetic data were analysed using Hill plots [$Log_{10}(v_o/v_i-1)$ plotted as a function of Log_{10} NaF

concentration, where v_o is the reaction velocity in the absence of inhibitor and v_i is the velocity at any given concentration of inhibitor]. The kinetic parameters, "n" (which has been reported to be sensitive to the state of fluidity of the erythrocyte membrane [77]) and $K_{.5}$ for inhibition, were determined from the slope and the negative $antilog_{10}$ of the X-intercept, respectively, of the line computed to best fit the plotted Hill data.

Other Techniques Used to Characterize Erythrocyte Membranes

1. Thin Layer Chromatography

Erythrocyte membrane pellets, obtained by centrifugation and containing 1.5-2.0 mg protein were extracted twice with 2 ml of a 2:1 (by volume) chloroform-methanol mixture. The extract was washed three times with 1 ml 0.75% (w/v) NaCl solution; the chloroform phases were pooled, and the aqueous phases discarded. Next, the pooled chloroform phases were evaporated to dryness and quantitatively spotted on an activated (30 minutes at 110°C) silica gel F-254 plate (0.25 mm thickness, Brinkmann). The plate was run in a solvent mixture containing chloroform, methanol and ammonia (14:6:1, by volume). Phospholipid spots were identified by their R_f values, which were highly reproducible in this solvent system. Visualization of resolved phospholipid spots was accomplished by treatment of the plate with ninhydrin reagent (for phosphatidyl ethanolamine and phosphatidyl serine) and iodine vapour (for phosphatidyl choline and sphingomyelin). The various phospholipid classes were quantified by extraction from the silica gel (with 1 ml methanol, three times), evaporation of the extract to dryness and analysis of the residue for inorganic phosphorus as described previously [70].

2. Gel Electrophoresis

Erythrocyte membranes were solubilized and subjected to gel electrophoretic analysis by the method described by Fairbanks et al.[78] as outlined below. One hundred microliter aliquots of membrane suspensions (containing 3 to 4 mg protein per ml) were combined with 50 microliters of a solution containing the following components: Tris-HCl buffer, pH 8.0 (30 mM); sodium dodecyl sulfate (SDS) (3%, w/v); sucrose (10%, w/v); disodium ethylenediaminetetraacetic acid (EDTA) (3 mM); bromophenol blue (20 micrograms/ml), used as tracking dye; and 2-mercaptoethanol (0.3%, v/v). This mixture was then incubated for 20 minutes at 37°C to reduce membrane sulfhydryl groups and achieve membrane solubilization. Next, aliquots of this mixture, containing 50 micrograms of protein material (to insure optimal resolution), were applied to a 5% polyacrylamide gel containing 1% SDS. Electrophoresis was performed in a circulating Tris-HCl buffer, pH 8.0 (50 mM), containing 0.1% SDS, with an E-C Corporation gel electrophoresis apparatus (150 volt setting). Resolution of membrane protein components was allowed to proceed until the tracking dye had migrated 10 cm or more from the origin. The gel was extensively washed with a mixture of methanol, water and glacial acetic acid (5:5:1, by volume) to remove residual SDS and stained with Coomassie brilliant blue dye.

Assessment of the Osmotic Fragility of Intact Erythrocytes Subjected to Hypotonic Stress

Two milliliters of whole blood were drawn into citrate anticoagulant (as described in "Preparation of Erythrocyte Membranes") and washed twice with cold isotonic saline. Half a milliliter of these packed erythrocytes were washed with a cold 0.15 M NaCl solution made up in Tris-HCl buffer, pH 7.0 (15 mM). The erythrocyte pellet, obtained by centrifugation, was resuspended

in 8 ml of this cold NaCl-Tris solution. A 0.2 ml aliquot of these resuspended erythrocytes was then added to 3.8 ml of each of several salt solutions [0.080, 0.075, 0.070, 0.065, 0.060, 0.050 and 0.040 M NaCl, made up in Tris-HCl buffer, pH 7.0 (15 mM)]. Following incubation for 15 minutes at room temperature, samples were centrifuged at 40,000 X g for one minute, and the absorbance at a of the supernatant was read at 540 nm. The percent hemolysis of erythrocytes at a given NaCl concentration was expressed relative to total hemolysis in distilled water.

Statistical Analysis

Erythrocytes obtained from clinical and control subjects were exhaustively analysed by the biochemical methods outlined in the preceeding sections. Data on a variety of red cell membrane parameters were generated in the hope of finding in the clinical subjects areas of significant departure from normality which might warrant future in-depth investigation. The time required to completely process clinical samples was such as to limit somewhat the total number of blood specimens that could be handled. Further, the relatively low incidence of DMD in the general population, compounded with the physical difficulties involved in drawing blood from affected subjects, placed some degree of constraint on the acquisition of clinical material for analysis. In addition, parental reluctance limited the number of sex/age-matched control blood samples which could be obtained. For these reasons, sample size became an important consideration in the selection of the most appropriate statistical method for group comparison of clinical and control subjects.

The "independent samples t-test" is often the statistical method of

choice when independent samples are drawn from two different populations and compared on the basis of a given single criterion to determine whether or not the populations differ. However, selection of this classical parametric method entails a number of assumptions regarding the nature of the populations from which the samples are drawn; in particular, the populations are assumed to be normally distributed and to exhibit homogeneous variances [79].

When the "t-test" is applied to sampled populations which seriously violate the assumptions underlying this classical parametric method, no great confidence may be placed in the statistical result [80]. Procedures, which have been developed to test the applicability of parametric methods to given sampled populations, depend upon sample sizes large enough to permit the adequate characterization of these populations. When the number of observations is relatively small, as in the present study, group comparison may be safely made using methods described as "non-parametric" and "distribution-free" [81]. These methods entail far fewer assumptions regarding the nature of the sampled populations and therefore are ideal for comparison of groups of small sample size. These tests are called "distribution-free" because they never assume that the population distribution of variate magnitudes is precisely defined, as do the classical parametric tests; generally, their only assumption is that the sampled population is continuously distributed [80]. Since these tests concern themselves with known sample-linked characteristics (e.g., the rank relationships within a set of pooled observations), and not with estimated population-linked parameters (e.g., variance), they are described as "non-parametric" methods [80].

The Wilcoxon rank sum test is a distribution-free, non-parametric analogue of the independent samples "t-test" [79], and therefore is considered

to be one of the most useful of the non-parametric methods. According to Armitage, when distributions are normal and variances are equal, the Wilcoxon rank sum test has an asymptotic relative efficiency of 96% of that of the "t-test", and if the distribution are not normal, the efficiency of the rank test is never less than 86% and may be infinitely high [81]. Thus, little is sacrificed when performing this test upon data which fulfills the more stringent requirements of the classical parametric methods. An added advantage of the Wilcoxon method is that calculations are made with ease and rapidity. A sample calculation is offered elsewhere (see Appendix) to illustrate the use of the Wilcoxon rank sum method of statistical analysis.

According to Bradley, the median and the interquartile range (i.e. the interval containing the median and the middle-most half of the experimental observations) are better indices of data location and dispersion than the mean and the standard deviation when the sampled population is non-normal, and these population indicators may even be employed to advantage when normality is assured [80]. Since the precise nature of the underlying populations sampled in this study remains obscure for the reasons previously stated, the mean and the median have both been used to characterize the experimental data for purposes of comparison (see Results). Unfortunately, the interquartile range is not well defined when dealing with samples of relatively small size. Therefore, in place of the interquartile range, the range of all sample observations has been chosen, along with the standard deviation, to describe the dispersion of experimental data (see Results).

RESULTS

A variety of chemical and biochemical techniques were employed in order to compare structural and functional characteristics of erythrocytes obtained from DMD patients or their female relatives with those of erythrocytes obtained from normal controls. With regard to the selection of appropriate controls, it should be noted that all clinical and control blood specimens examined were procured from either female adults, ranging in age from mid-adolescence to middle-age, or children primarily in their pre-adolescent years. The female relatives of DMD patients under study were categorized by the method of Walton [1] as definite, probable or possible carriers of DMD. In this study, definite and probable carriers were considered as a single group due to the limited availability of these subjects. The possible carriers, on the other hand, were more plentiful, and the decision was taken to subdivide these subjects on the basis of their relationship to the DMD patients. Thus for purposes of group comparison, three groups of clinical subjects have been compared with normal adult females: (a) definite and probable DMD carriers, (b) possible carriers who are mothers of DMD patients and (c) possible carriers who are sisters of DMD patients (see Tables I-XII).

Compositional Analyses of Erythrocyte Membranes

Abnormal plasma membrane sialic acid levels have been reported to occur in denervated rat skeletal muscle [82] and in erythrocytes obtained from patients with muscular dystrophy associated with severe hemolytic anemia [83]. In this latter case, Balduini *et al.* were unable to detect the presence

of any sialic acid in the erythrocyte membrane material sampled from two middle-aged brothers with this syndrome. These reports suggested that determination of erythrocyte membrane sialic acid content might be of value in the study of DMD red cells. Table I presents the results of this investigation. The sialic acid contents of erythrocyte membranes of DMD patients and their female relatives did not significantly differ from those of appropriate normal controls.

It has been reported in various animal models of muscular dystrophy that abnormally high levels of cholesterol are present in skeletal muscle plasma membranes [8, 84]. The data presented in Table II suggests that a similar alteration does not occur in the erythrocyte membranes of patients affected with DMD or of their carrier relatives.

Phospholipids have also been reported to be subject to considerable modification in animal muscular dystrophy. In myopathic hamster cardiac muscle, total cell phospholipids are decreased [8], while the opposite is seen in skeletal muscle of dystrophic chickens [7]. When erythrocyte membranes of DMD patients and carrier relatives were analysed for gross phospholipid content, no statistically significant departure from normality was observed (see Table III).

The decision to examine the sulfhydryl content of DMD erythrocyte membranes was prompted by the observations of Chou et al. who demonstrated that treatment of chickens suffering from hereditary muscular dystrophy with penicillamine partially alleviated the symptoms of the avian disease and appeared to protect muscle cell membranes, as evidenced by a decrease in plasma CPK activity [85]. Since pencillamine is a thiol compound with

TABLE I

Erythrocyte Membrane Sialic Acid Content

(units: nanomoles/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	98	95	9	4	88-110	-
Children with DMD	10	113	107	18	6	93-144	N.S.
Normal Female Adults	7	105	101	14	5	85-120	-
Definite and Probable DMD Carriers	6	106	107	14	6	80-122	N.S.
Possible Carriers: Mothers of DMD Patients	5	113	111	10	5	103-130	N.S.
Possible Carriers: Sisters of DMD Patients	7	108	105	16	6	85-134	N.S.

TABLE II

Erythrocyte Membrane Cholesterol Content

(units: micrograms/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	193	195	22	9	168-221	-
Children with DMD	9	216	216	26	9	178-275	N.S.
Normal Female Adults	8	234	228	48	17	170-328	-
Definite and Probable DMD Carriers	6	247	241	30	12	217-289	N.S.
Possible Carriers: Mothers of DMD Patients	4	228	229	13	6	213-244	N.S.
Possible Carriers: Sisters of DMD Patients	8	206	207	16	6	186-225	N.S.

TABLE III

Erythrocyte Membrane Phospholipid Content

(units: milligrams phospholipid/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	.528	.526	.031	.013	.484-.574	-
Children with DMD	6	.523	.484	.087	.036	.448-.639	N.S.
Normal Female Adults	8	.596	.599	.068	.024	.478-.721	-
Definite and Probable DMD Carriers	5	.531	.530	.074	.033	.439-.623	N.S.
Possibl Carriers: Mothers of DMD Patients	3	.618	.615	-	-	.585 .615 .654	N.S.
Possible Carriers: Sisters of DMD Patients	8	.577	.566	.071	.025	.489-.685	N.S.

reducing properties, these authors suggested that sulfhydryl groups of the muscle plasma membrane might play some important role in avian muscular dystrophy. By analogy, elevated plasma CPK levels observed in human DMD may at least in part be determined by a defect in muscle plasma membrane structure involving membrane sulfhydryl groups -- a defect which if sufficiently generalized might be detectable in erythrocyte membranes. When intact and detergent-disrupted erythrocyte membranes are reacted with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), the quantity and distribution of membrane sulfhydryls can be estimated. In intact membranes, only surface sulfhydryl groups are accessible to the reagent, while in detergent-disrupted membranes all membrane sulfhydryls react, allowing an estimation of latent or buried sulfhydryl groups in the membrane. The data displayed in Tables IV (A and B) reveal no significant differences between clinical and control groups with respect to these parameters.

Trinitrobenzene sulfonic acid (TNBS) is a group-specific reagent which introduces a chromophoric trinitrophenyl label onto primary amino groups. Since both protein and phospholipid components of membranes contain primary amino groups, the rate of labelling of intact and detergent-disrupted erythrocytes by TNBS provides a convenient means of assessing the structural integrity of these two important membrane components [73]. As in the DTNB studies, comparison of intact and detergent-disrupted membranes allows one to examine the properties of both surface and latent residues. If gross structural abnormalities occur in DMD erythrocyte membranes, these may alter the characteristics of TNBS incorporation into surface or latent primary amino residues. Examination of Tables V (A and B) will show that in DMD

TABLE IV (A)

Erythrocyte Membrane Surface DTNB Titer

(units: nanomoles sulfhydryl groups/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	4	28	30	5	2	21-32	-
Children with DMD	7	25	28	7	3	14-32	N.S.
Normal Female Adults	7	27	25	6	2	22-38	-
Definite and Probable DMD Carriers	5	25	24	9	4	13-36	N.S.
Possible Carriers Mothers of DMD Patients	3	23	25	-	-	20, 25, 26	N.S.
Possible Carriers: Sisters of DMD Patients	7	24	24	3	1	19-29	N.S.

TABLE IV (B)

Total Erythrocyte Membrane DTNB Titer

(units: nanomoles sulfhydryl groups/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	4	53	55	5	3	46-57	-
Children with DMD	8	51	54	15	5	28-69	N.S.
Normal Female Adults	7	54	51	9	3	42-69	-
Definite and Probable DMD Carriers	5	46	43	7	3	40-57	N.S.
Possible Carriers Mothers of DMD Patients	3	50	48	-	-	48, 48, 55	N.S.
Possible Carriers: Sisters of DMD Patients	7	51	49	8	3	43-70	N.S.

TABLE V (A)

TNBS Titer of Intact Erythrocyte Membrane

(units: absorbance at 335 nm/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	4	.125	.126	.004	.002	.119-.128	-
Children with DMD	7	.128	.128	.019	.007	.104-.161	N.S.
Normal Female Adults	7	.126	.124	.029	.011	.096-.187	-
Definite and Probable DMD Carriers	4	.143	.146	.019	.009	.119-.162	N.S.
Possible Carriers Mothers of DMD Patients	3	.137	.132	-	-	.129 .132 .149	N.S.
Possible Carriers: Sisters of DMD Patients	7	.135	.133	.023	.009	.113-.183	N.S.

TABLE V (B)

TNBS Titer of Detergent-disrupted Erythrocyte Membranes

(units: absorbance at 335 nm/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	4	.728	.734	.039	.020	.675-.767	-
Children with DMD	7	.779	.746	.077	.029	.698-.894	N.S.
Normal Female Adults	8	.813	.785	.115	.041	.693-1.042	-
Definite and Probable DMD Carriers	4	.777	.805	.139	.069	.586-.910	N.S.
Possible Carriers Mothers of DMD Patients	3	.783	.779	.017	.010	.768 .779 .802	N.S.
Possible Carriers: Sisters of DMD Patients	7	.786	.767	.062	.023	.700-.861	N.S.

patients and their female relatives, these parameters do not differ significantly from normal.

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids

There is a great deal of evidence that some muscular dystrophies are associated with alterations in membrane phospholipid components. For example, increases in skeletal muscle membrane sphingomyelin fractions have been reported in avian muscular dystrophy [84], vitamin E-deficiency myopathy in calf [86], and human DMD [5]; furthermore, this same alteration has been described by Kunze et al. for DMD erythrocyte membranes [51]. Phosphatidyl choline is another membrane phospholipid reported to deviate from normal in muscular dystrophy. Depression of skeletal muscle membrane phosphatidyl choline levels occurs in DMD [5], and certain animal dystrophy models [7, 84].

Kunze et al. have also observed highly significant changes in the fatty acid patterns of phosphatidyl ethanolamine and sphingomyelin in DMD erythrocytes [51]. Alterations, such as decreases in linoleic (18:2) fatty acid components of sphingomyelin with concomitant increases in stearic (18:0) components, might modify phospholipid polarity greatly, thereby affecting fractional migration parameters (R_f's) of phospholipids on thin layer chromatographic (TLC) analysis.

Membrane phospholipid alterations have also been shown to be associated with erythrocyte morphological abnormalities. Thus, erythrocytes from patients with beta-Thalassemia Major possess well characterized morphological alterations associated with an increase in membrane phosphatidyl choline [87]. Since morphological abnormalities have also been reported to occur in erythrocytes of DMD patients and possibly in female carriers of the dystrophic trait

as well, it was felt that detailed analysis of erythrocyte membrane phospholipids in DMD patients and their female relatives might provide valuable information on possible membrane alterations in these individuals.

Tables VI and VII present the TLC data for clinical and control groups. Tables VI (A-D) summarize the relative mobility (R_f) findings for the various phospholipid fractions (phosphatidyl serine, sphingomyelin, phosphatidyl choline and phosphatidyl ethanolamine), while Tables VII (A-D) describe a quantitative analysis of these erythrocyte membrane phospholipid fractions, expressed as a percentage of total phospholipid. No significant differences could be found between the mobilities of the erythrocyte membrane phospholipids of DMD patients and those of normal children [Tables VI (A-D)]. Similarly, DMD sisters in the possible carrier category show the same TLC migration patterns as age and sex-matched normal controls. Unfortunately, insufficient data are available on two other groups: definite/probable carriers and possible carriers who are mothers of affected children; without a larger number of observations in these data-poor groups, one is unable to assess whether or not the erythrocyte membrane phospholipids of these subjects exhibit normal fractional migration patterns. However, as may be noted in Tables VI (A-D), both data-poor clinical groups show at least one R_f observation falling within the normal range. On the basis of inspection alone, one might argue that significant differences between such subjects and their control counterparts are unlikely. The rationales behind this "over-lap argument" and some of its shortcomings will be considered in the Discussion section.

Despite the need for further assessment of fractional migration parameters for data-poor carrier groups, the data presented in Tables VI (A-D)

TABLE VI (A)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Phosphatidyl Serine R_f

(units: fractional migration relative to solvent front)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	.138	.136	.008	.003	.132-.152	-
Children with DMD	6	.125	.118	.025	.010	.102-.161	N.S.
Normal Female Adults	6	.151	.145	.021	.009	.134-.189	-
Definite and Probable DMD Carriers	2	-	-	-	-	.143 .159	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	.111 .145	?
Possible Carriers: Sisters of DMD Patients	8	.135	.124	.056	.020	.071-.240	N.S.

TABLE VI (B)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Sphingomyelin R_f

(units: fractional migration relative to solvent front)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	.219	.220	.013	.005	.200-.240	-
Children with DMD	6	.182	.174	.029	.012	.148-.226	N.S.
Normal Female Adults	6	.221	.211	.037	.014	.187-.278	-
Definite and Probable DMD Carriers	2	-	-	-	-	.221 .229	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	.175 .230	?
Possible Carriers: Sisters of DMD Patients	8	.200	.200	.069	.025	.106-.304	N.S.

TABLE VI (C)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Phosphatidyl Choline R_f

(units: fractional migration relative to solvent front)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	.353	.346	.020	.008	.333-.386	-
Children with DMD	6	.324	.319	.043	.018	.273-.391	N.S.
Normal Female Adults	6	.362	.351	.046	.019	.310-.438	-
Definite and Probable DMD Carriers	2	-	-	-	-	.368 .378	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	.310 .339	?
Possible Carriers: Sisters of DMD Patients	8	.340	.316	.087	.031	.224-.444	N.S.

TABLE VI (D)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Phosphatidyl Ethanolamine R_f

(units: fractional migration relative to solvent front)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	.494	.488	.023	.010	.467-.526	-
Children with DMD	6	.457	.451	.049	.020	.398-.528	N.S.
Normal Female Adults	6	.484	.473	.042	.017	.439-.556	-
Definite and Probable DMD Carriers	2	-	-	-	-	.493 .521	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	.444 .461	?
Possible Carriers: Sisters of DMD Patients	8	.478	.450	.077	.027	.382-.585	N.S.

TABLE VII (A)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Relative Quantity of Phosphatidyl Serine

(units: percent total phospholipid fraction)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	20.3	21.0	2.2	0.9	16.2-22.1	-
Children with DMD	6	21.3	20.5	3.2	1.3	18.8-27.5	N.S.
Normal Female Adults	6	14.9	15.1	4.0	1.6	8.9-20.1	-
Definite and Probable DMD Carriers	2	-	-	-	-	13.9 18.7	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	17.3 26.0	?
Possible Carriers: Sisters of DMD Patients	8	22.4	21.5	3.4	1.2	18.3-29.0	Significant ($p < 0.01$)

TABLE VII (B)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Relative Quantity of Sphingomyelin

(units: percent total phospholipid fraction)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	14.1	10.7	7.6	3.1	7.9-26.0	-
Children with DMD	6	11.9	11.7	2.1	0.8	9.4-14.5	N.S.
Normal Female Adults	6	10.1	6.2	7.5	3.1	4.0-22.5	-
Definite and Probable DMD Carriers	2	-	-	-	-	7.4 10.8	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	7.1 19.0	?
Possible Carriers: Sisters of DMD Patients	8	12.7	13.5	5.9	2.1	5.0-21.0	N.S.

TABLE VII (C)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Relative Quantity of Phosphatidyl Choline

(units: percent total phospholipid fraction)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	28.4	28.5	2.0	0.8	26.0-30.8	-
Children with DMD	6	27.0	27.3	1.4	0.6	25.1-28.3	N.S.
Normal Female Adults	6	29.1	28.4	2.6	1.1	25.7-32.7	-
Definite and Probable DMD Carriers	2	-	-	-	-	31.5 34.1	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	27.7 32.5	?
Possible Carriers: Sisters of DMD Patients	8	26.5	26.3	3.1	1.1	22.1-31.5	N.S.

TABLE VII (D)

Thin Layer Chromatographic Analysis of Erythrocyte Membrane Phospholipids: Relative Quantity of Phosphatidyl Ethanolamine

(units: percent total phospholipid fraction)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	6	37.2	38.0	4.8	1.9	30.6-43.2	-
Children with DMD	6	38.9	37.7	3.5	1.4	36.1-45.6	N.S.
Normal Female Adults	6	44.1	47.6	7.7	3.2	33.6-51.7	-
Definite and Probable DMD Carriers	2	-	-	-	-	37.9 38.7	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	34.5 36.0	?
Possible Carriers: Sisters of DMD Patients	8	37.0	36.9	4.3	1.5	30.6-43.8	N.S.

seems hardly supportive of the findings reported by Kunze et al. However, the tabulated data presented here does not reflect the whole story. Upon TLC analysis of erythrocyte membrane material obtained from six DMD patients, three of the affected children's specimens revealed two phospholipid spots migrating on the TLC plate with nearly the same mobility characteristics as normal sphingomyelin. These two spots were in fact supposed to be compositionally-modified sphingomyelin, although the truth of this assertion must await further investigation. Attempts to correlate the appearance of the "twin sphingomyelin-like spots" with the source of these children's dystrophy were unsuccessful: two of the three DMD cases were diagnosed as sporadic, one hereditary.

This sphingomyelin anomaly has also been observed in two other subjects: one the sister of a young adult male with Becker's muscular dystrophy (a more benign form of sex-linked, pseudo-hypertrophic muscular dystrophy than DMD), the other the autosomal recessive (carrier) mother of a child with limb-girdle muscular dystrophy. It is interesting to note that this woman's affected child did not show this particular phospholipid pattern, nor did an unrelated male limb-girdle carrier. These results indicate that TLC analysis of erythrocyte membranes, derived from patients with different types of muscular dystrophies and their carrier relatives, may prove to be a fruitful research tool.

The TLC data concerning the relative quantities of the four main phospholipid species have proven to be of interest and suggest continued utilization of this technique in DMD investigation. Tables VII (A-D) indicate that while no significant quantitative differences seem to occur in the various

phospholipid fractions of DMD and control children, possible carriers who are sisters of DMD patients clearly differ from normality ($p < 0.01$) with respect to the phosphatidyl serine content of their red cell membranes. Other phospholipid species appear to be present in normal quantities in this group's erythrocytes. Again the small number of experimental observations precludes definitive statements regarding the probability that definite/probable carriers and possible carrier mothers are normal with respect to their erythrocyte membrane phospholipid contents. Examination of Table VII (B) reveals a point of some interest: the sphingomyelin content of erythrocyte membranes of clinical and control subjects (adults as well as children) shows a high degree of variability.

Kinetic Analyses of Erythrocyte Membrane Enzymatic Activities

1. Basal and Total p-Nitrophenyl Phosphatase

There is very good evidence that the Mg^{2+}-dependent, K^+-stimulated p-nitrophenyl phosphatase (NPPase) activity observed in some membranes reflects the K^+-stimulated dephosphorylation step in the hydrolysis of ATP by Mg^{2+}-dependent, Na^+, K^+-stimulated adenosine-5'-triphosphatase (ATPase), an enzyme intimately associated with the regulation of intracellular sodium and potassium [88, 89]. Analysis of basal (Mg^{2+}-dependent) and total (Mg^{2+}, K^+-dependent) NPPase activities provides a means of focussing on the K^+-sensitivity of the membrane sodium-potassium pump's enzymatic machinery without the complication of the prior Na^+-dependent phosphorylation step.

Brody and Brody have reported NPPase abnormalities in certain muscular disorders: these investigators have observed elevated levels of basal

NPPase (but not total NPPase or Na^+, K^+-ATPase) activity in sarcolemmal preparations of denervated rat limb [90] and in the microsomal fractions of skeletal muscle obtained from rats rendered myotonic by treatment with (2,4-Dichlorophenoxy) acetic acid (2,4-D) and similar monocarboxylic acids [91]. Recent studies of DMD carriers conducted by Thomson et al. [92] have provided evidence of reduced total body levels of intracellular potassium in these subjects. Such findings prompted the investigation of NPPase function in erythrocyte membranes of DMD patients and carriers.

Reference to Tables VIII (A and B) will show that only DMD children differ significantly from normal controls with respect to basal and total NPPase activity levels. The finding that there is a statistically significant difference (at the 0.05 level) between total NPPase activities of DMD and control red cell membranes requires comment. Total NPPase activity represents the sum of basal NPPase and a K^+-stimulated component. This K^+-stimulated component, when evaluated for each subject by subtracting the basal level from the total level of enzymatic activity, exhibited no significant difference (at the 0.05 level) between clinical and control groups. However, these groups are found to differ at the 0.10 level of statistical significance.

Figure 1 illustrates a typical example of the kinetic analysis of K^+-stimulation of erythrocyte membrane NPPase. Here the pooled data for DMD patients and age-matched controls are displayed together in both Michaelis-Menten-type plots and Eadie plots (see insert). The kinetic parameters for K^+-stimulation of this enzyme, presented in Tables VIII (C and D), represent the means of individual values obtained by Eadie analyses as previously described (see Materials and Methods).

TABLE VIII (A)

Activity of Erythrocyte Membrane Mg^{2+}-dependent (Basal) p-Nitrophenyl Phosphatase

(units: micromoles p-nitrophenol/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	.120	.112	.019	.008	.103-.140	-
Children with DMD	10	.092	.096	.020	.006	.055-.118	Significant
Normal Female Adults	9	.098	.095	.026	.009	.057-.139	-
Definite and Probable DMD Carriers	6	.106	.104	.034	.014	.069-.164	N.S.
Possible Carriers Mothers of DMD Patients	5	.087	.090	.018	.008	.068-.104	N.S.
Possible Carriers: Sisters of DMD Patients	8	.092	.088	.020	.007	.059-.120	N.S.

TABLE VIII (B)

Activity of Erythrocyte Membrane Mg^{2+}-dependent, K^{+}-stimulated p-Nitrophenyl Phosphatase

(units: micromoles p-nitrophenol/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	.211	.202	.028	.013	.181-.248	-
Children with DMD	10	.164	.169	.039	.012	.089-.214	Significant
Normal Female Adults	9	.166	.156	.043	.014	.100-.234	-
Definite and Probable DMD Carriers	6	.170	.165	.034	.014	.136-.226	N.S.
Possible Carriers Mothers of DMD Patients	5	.172	.173	.045	.020	.113-.217	N.S.
Possible Carriers: Sisters of DMD Patients	8	.160	.151	.041	.015	.102-.220	N.S.

FIGURE 1: Concentration Dependence of K^+-stimulation of Erythrocyte Membrane Mg^{2+}-dependent p-Nitrophenyl Phosphatase in Normal and DMD Erythrocytes. Inset Eadie Plot Representation of the Same Data.

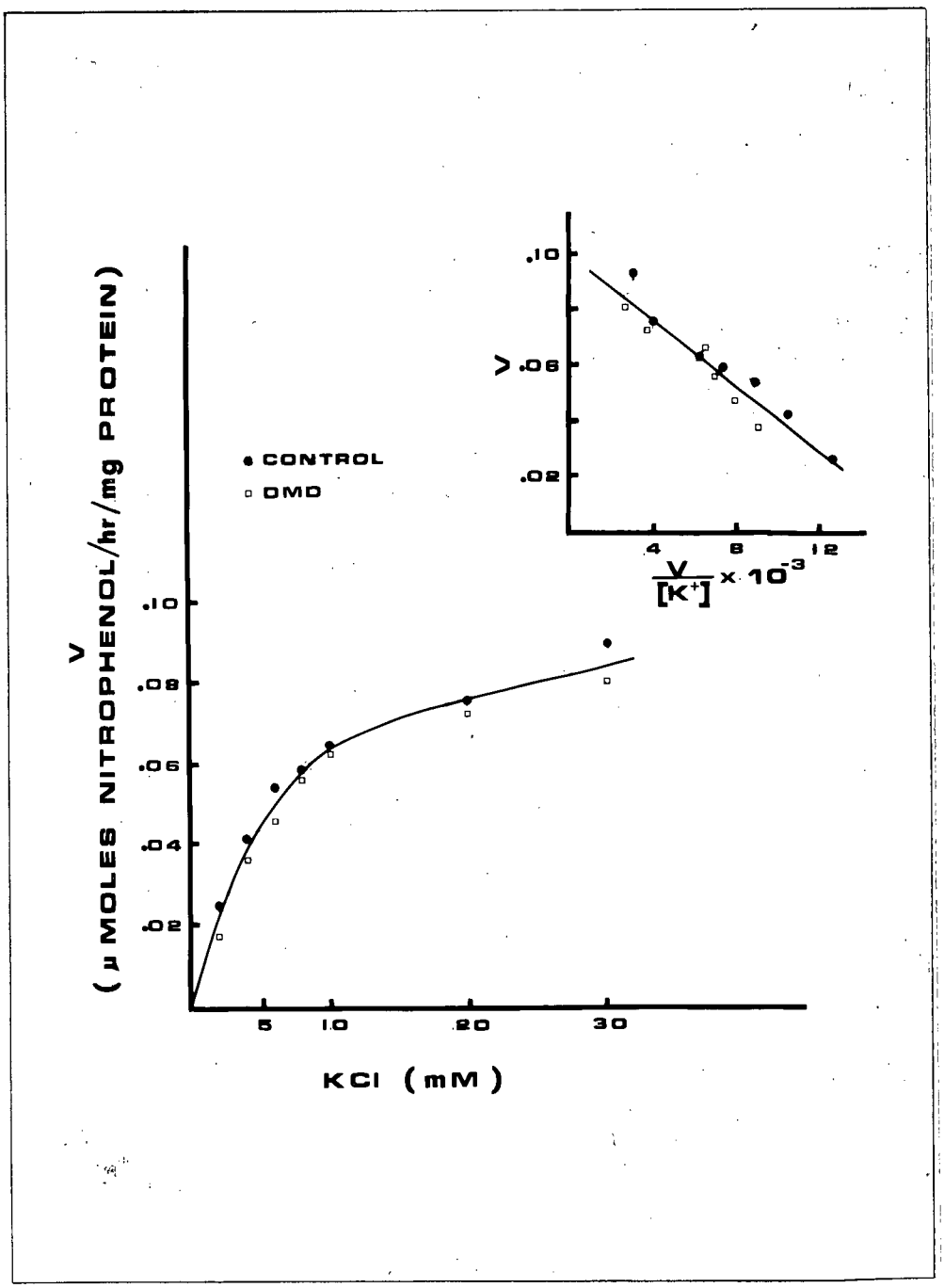

V
(μ MOLES NITROPHENOL/hr/mg PROTEIN)
.10
.08
.06
.04
.02
5
10
20
30
KCl (mM)
● CONTROL
□ DMD
V
.10
.06
.02
4
8
12
V/[K+] x 10-3

Table VIII (C) reveals that no significant differences are detectable between the clinical and control preparations with respect to the maximal velocity of K^+-NPPase activity. The suspicion that the definite/probable carrier group is heterogeneous, composed of two sub-populations differing with respect to this parameter, is raised by the marked difference between the two tabulated observations (0.109 and 0.195 micromoles p-nitrophenol/hour/milligram membrane protein). Further experimentation will be required to assess this possibility.

Although not shown in Table VIII (C), the V_{max} for K^+-NPPase of erythrocyte membrane material was found to be elevated in three limb-girdle subjects: one affected child and two carrier adults (one male and one female, both of whom were unrelated). Therefore, kinetic analysis of the K^+-stimulation of red cell NPPase may prove to be a useful tool in the characterization of erythrocytes in limb-girdle muscular dystrophy.

Of special interest are the kinetic data summarized in Table VIII (D) concerning the $K_{.5}$ for K^+ of erythrocyte membrane K^+-NPPase. While tendering the now-familiar reservations regarding data-poor groups, one also observes one carrier group which deviates very significantly from normality with respect to $K_{.5}$ for K^+: possible carriers who are sisters of DMD patients. The probability is less than 0.01 that this particular clinical population and the control population are identical with respect to the $K_{.5}$ parameter and that the observed differences in group sample means a rise merely by chance sampling from population extremes.

2. Adenosine-5'-Triphosphatases (ATPases)

There is much evidence in the literature that in muscular dystrophy

TABLE VIII (C)

K^+-stimulated Component of Erythrocyte Membrane p-Nitrophenyl Phosphatase: V_{max}

(units: micromoles p-nitrophenol/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	.096	.103	.026	.012	.054-.122	-
Children with DMD	6	.091	.086	.020	.008	.076-.128	N.S.
Normal Female Adults	6	.096	.098	.033	.013	.054-.134	-
Definite and Probable DMD Carriers	2	-	-	-	-	.109 .195	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	.051 .144	?
Possible Carriers: Sisters of DMD Patients	8	.083	.079	.027	.009	.046-.133	N.S.

TABLE VIII (D)

K^+-stimulated Component of Erythrocyte Membrane p-Nitrophenyl Phosphatase: $K_{.5}$ for K^+

(units: millimolar)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	4.51	4.52	0.69	0.31	3.59-5.47	-
Children with DMD	6	5.90	6.36	1.67	0.68	3.53-7.50	N.S.
Normal Female Adults	6	5.09	5.13	1.39	0.57	3.61-7.03	-
Definite and Probable DMD Carriers	2	-	-	-	-	5.88 6.74	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	4.33 4.64	?
Possible Carriers: Sisters of DMD Patients	8	3.40	3.49	0.50	0.18	2.46-4.18	Significant ($p < 0.01$)

alterations occur in plasma membrane Na^+, K^+-ATPase from a variety of tissues. Abnormal activities of this enzyme have been reported in skeletal muscle sarcolemma of dystrophic mice [11], hamsters [14, 93] and chickens [94], as well as in DMD muscle biopsy material [9]. The activity of this enzyme is also altered in hepatocyte membrane preparations from dystrophic avian liver [94]. That erythrocyte membrane levels of Na^+, K^+-ATPase activity may be aberrant in DMD is suggested by Sha'afi et al., who determined that red cells obtained from affected children and female carriers show increased active influx of potassium [56].

Tables IX (A and B) summarize respectively the clinical and control data for the basal (Mg^{2+}-dependent) and total (Mg^{2+}-dependent, Na^+, K^+-stimulated) ATPase activities found in erythrocyte membranes. Examination of these two tables reveals no significant differences between DMD patients and normal children with respect to these enzyme activities. However, the possible carriers who are DMD sisters do show a significant decrease in erythrocyte total Na^+, K^+-ATPase activity. This finding and the single high value for the definite/probable DMD carrier group in Table IX (B) suggest that red cell membrane ATPase function should receive more detailed study in the future. With larger sample sizes, this parameter may even prove to be significantly different from normal in DMD patient material.

The important role of Ca^{2+}-stimulated ATPase activity in skeletal muscle function is well known. Therefore, recent reports of abnormal activities of this enzyme in skeletal muscle organelles in muscular dystrophy are worthy of note: for example, this enzyme activity is said to be elevated in skeletal muscle sarcolemma procured from myopathic hamster [14], dystrophic

TABLE IX (A)

Activity of Erythrocyte Membrane Mg^{2+}-dependent (Basal) Adenosine Triphosphatase

(units: micromoles inorganic phosphate/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	.170	.178	.028	.013	.125-.196	-
Children with DMD	3	.182	.172	-	-	.165 .172 .210	N.S.
Normal Female Adults	6	.197	.199	.035	.014	.137-.242	-
Definite and Probable DMD Carriers	2	-	-	-	-	.210 .232	?
Possible Carriers Mothers of DMD Patients	1	-	-	-	-	.172	?
Possible Carriers: Sisters of DMD Patients	6	.159	.162	.030	.012	.121-.191	N.S.

TABLE IX (B)

Activity of Erythrocyte Membrane Mg^{2+}-dependent, Na^{+}, K^{+}-stimulated Adenosine Triphosphatase

(units: micromoles inorganic phosphate/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	.415	.417	.067	.030	.340-.504	-
Children with DMD	3	.343	.323	-	-	.307 .323 .399	N.S.
Normal Female Adults	6	.436	.435	.075	.030	.331-.559	-
Definite and Probable DMD Carriers	1	-	-	-	-	.621	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	.393 .424	?
Possible Carriers: Sisters of DMD Patients	5	.279	.240	.090	.040	.224-.439	Significant

mouse [10] and DMD patient [9] biopsy specimens, while the reverse alteration has been reported for avian muscular dystrophy [94]. Sarcoplasmic reticular (SR) activities of Ca^{2+}-stimulated ATPase have been reported to be depressed in dystrophic hamster [14], and SR calcium-uptake has been observed to be substantially lower in skeletal muscle of dystrophic chickens than in that of normal controls [15]. Furthermore, the density of 90 angstrom membrane particles in freeze-fractured SR, believed to be ATPase protein, is significantly reduced in avian dystrophy [15].

Red blood cells also have Ca^{2+}-ATPase activity present in their plasma membranes [95]. In fact, a number of reports have documented the existence of not one, but two such activities, each having different kinetic characteristics and perhaps different locations and/or functional roles in the erythrocyte membrane. These activities are often referred to as "high"- and "low"-affinity Ca^{2+}-ATPases because of their differing requirements for calcium [76, 96, 97, 98, 99]. Ca^{2+}-ATPase activity in the red cell membrane is believed to be associated with an active calcium-extruding mechanism, energized by ATP and serving to maintain low concentrations of calcium in the intact cell [98, 100]. There is some controversy, however, as to the relative roles of the "high"- and "low"-affinity components of the enzyme in active Ca^{2+}-transport [76, 101]. Experiments with erythrocytes using a divalent cation ionophore to raise intracellular levels of calcium have shown that such increases are associated with morphological alterations in the cells [102, 103]. A defect in calcium extrusion, with resulting accumulation of intracellular calcium, might explain the morphological changes reported in erythrocytes of patients with DMD [52, 53, 54]. The properties of erythrocyte

membrane Ca^{2+}-ATPases were therefore examined in both DMD patients and their female relatives.

The data in Figure 2 illustrate the biphasic nature of erythrocyte membrane Ca^{2+}-ATPase activity in both DMD patients and controls and is seen even more clearly when data are expressed in the form of Eadie plots (see Figure 3), from which the kinetic parameters ($K_{.5}$ for Ca^{2+} and V_{max}) could be calculated. The results for all groups examined are presented in Tables IX (C-F).

It is apparent from Table IX (C and D) that the three DMD patients examined with respect to their erythrocyte membrane high-affinity Ca^{2+}-ATPase activity showed significantly lower maximal rates of ATP hydrolysis than controls, but apparently normal $K_{.5}$ values for calcium. This was not the case with the DMD female relatives examined, although the single subject in the possible carriers who are mothers of DMD patients group exhibited an abnormally low V_{max} but a high $K_{.5}$ parameter. These results are far from conclusive, but they strongly suggest that this enzymatic activity warrants more intensive investigation in DMD. Although the low-affinity Ca^{2+}-ATPase activity [Tables IX (E and F)] does not appear to depart significantly from normal, the data from possible carriers, both mothers and sisters of patients, does indicate the need for further study of this ATPase.

3. Acetylcholinesterase (AChE)

Investigations into the synthesis and metabolism of acetylcholine (ACh) in tissues of muscular dystrophy animal models have revealed this to be another area of abnormal enzymatic function. For example, Trabucchi et al.

FIGURE 2: Concentration Dependence of the Stimulation of Erythrocyte Membrane Mg^{2+}-dependent ATPase by Calcium.

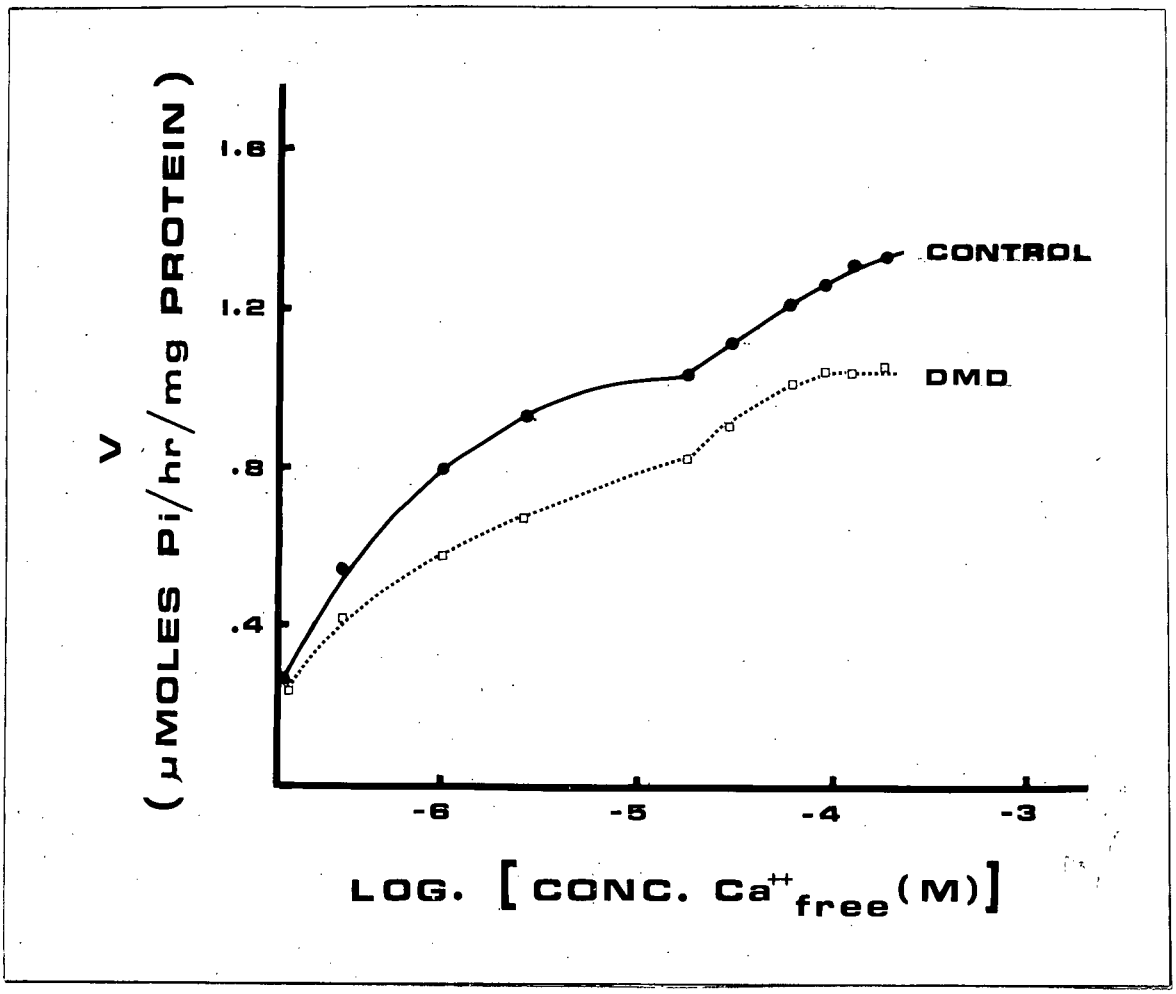

V
(μMOLES Pi/hr/mg PROTEIN)
1.6
1.2
.8
.4
CONTROL
DMD
-6
-5
-4
-3
LOG. [CONC. Ca++free (M)]

FIGURE 3: Eadie Plot Analysis of the Data in Figure 2.

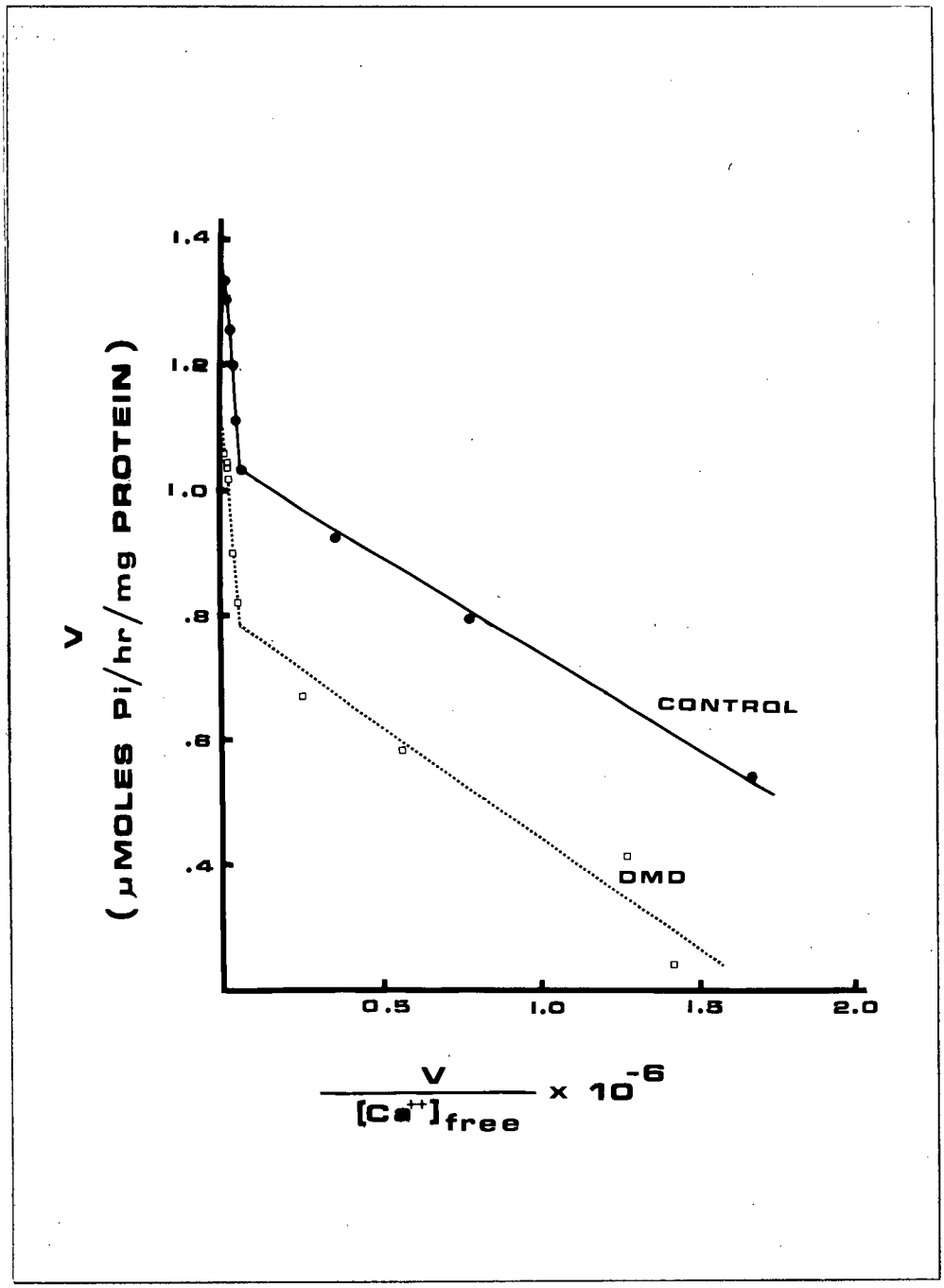

V
(μMOLES Pi/hr/mg PROTEIN)
1.4
1.2
1.0
.8
.6
.4
CONTROL
DMD
0.5
1.0
1.5
2.0
V / [Ca++]free x 10-6

TABLE IX (C)

High Affinity Ca^{2+}-stimulated Component of Erythrocyte Membrane Adenosine Triphosphatase: V_{max}

(units: micromoles inorganic phosphate/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	1.05	1.06	0.17	0.08	0.81-1.28	-
Children with DMD	3	0.73	0.76	-	-	0.65 0.76 0.79	Significant
Normal Female Adults	6	0.92	0.89	0.24	0.10	0.58-1.25	-
Definite and Probable DMD Carriers	2	-	-	-	-	0.77 1.21	?
Possible Carriers Mothers of DMD Patients	1	-	-	-	-	0.46	?
Possible Carriers: Sisters of DMD Patients	6	0.80	0.82	0.22	0.09	0.48-1.01	N.S.

TABLE IX (D)

High Affinity Ca^{2+}-stimulated Component of Erythrocyte Membrane Adenosine Triphosphatase: $K_{.5}$ for Ca^{2+}

(Units: micromolar free Ca^{2+})

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	.328	.364	.077	.035	.207-.393	-
Children with DMD	3	.261	.230	-	-	.205 .230 .350	N.S.
Normal Female Adults	6	.222	.212	.145	.059	.032-.412	-
Definite and Probable DMD Carriers	2	-	-	-	-	.207 .211	?
Possible Carriers Mothers of DMD Patients	1	-	-	-	-	.574	?
Possible Carriers: Sisters of DMD Patients	6	.307	.283	.140	.057	.133-.537	N.S.

TABLE IX (E)

Low Affinity Ca^{2+}-stimulated Component of Erythrocyte Membrane Adenosine Triphosphatase: V_{max}

(units: micromoles inorganic phosphate/ hour/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	1.35	1.29	0.19	0.09	1.12-1.63	-
Children with DMD	3	1.15	-	-	-	0.96 1.14 1.36	N.S.
Normal Female Adults	6	1.15	1.15	0.25	0.10	0.80-1.48	-
Definite and Probable DMD Carriers	2	-	-	-	-	1.09 1.53	?
Possible Carriers Mothers of DMD Patients	1	-	-	-	-	0.68	?
Possible Carriers: Sisters of DMD Patients	6	0.78	0.70	0.38	0.16	0.36-1.24	N.S. (significant at $p \leq 0.10$)

TABLE IX (F)

Low Affinity Ca^{2+}-stimulated Component of Erythrocyte Membrane Adenosine Triphosphatase: $K_{.5}$ for Ca^{2+}

(units: micromolar free Ca^{2+})

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	6.74	5.98	2.08	0.93	5.11-10.31	-
Children with DMD	3	7.21	8.23	-	-	4.77 8.23 8.62	N.S.
Normal Female Adults	6	5.35	3.99	3.61	1.47	2.66-11.91	-
Definite and Probable DMD Carriers	2	-	-	-	-	4.80 7.52	?
Possible Carriers Mothers of DMD Patients	1	-	-	-	-	12.10	?
Possible Carriers: Sisters of DMD Patients	6	5.41	5.16	1.33	0.54	3.63-7.33	N.S.

have found some evidence for a possible defect in the synthesis of ACh in central and peripheral cholinergic neurons of muscular dystrophic mice [34]. Other investigators have detected alterations in ACh handling in muscular dystrophy: AChE has been found to exhibit embryonic characteristics in avian myopathy [104]; the activity of AChE has been shown to be depressed in sarcolemmal preparations from dystrophic mouse skeletal muscle [10], and a three-fold increase has been reported to occur in the $K_{.5}$ for substrate for red cell AChE in murine dystrophy [105].

When the kinetics of erythrocyte AChE were investigated in DMD patients and carriers utilizing Eadie analysis (see Materials and Methods and Figure 4), no significant departures from normality were observed in either $K_{.5}$ for acetylthiocholine (ATCh) or V_{max}. These results are summarized in Tables X (A and B).

Butterfield et al. have conducted spin label studies of erythrocytes obtained from patients suffering from myotonic muscular dystrophy; their findings suggest that the red cell membrane matrix in this disorder is less polar and more fluid than the matrix of normal erythrocytes [106, 107]. Such polarity and fluidity alterations in myotonic red cells may reflect either gross differences in the membrane composition or more subtle abnormalities in the molecular arrangement of membrane lipid and protein constituents. Butterfield et al. have interpreted their erythrocyte spin label data as evidence that myotonic muscular dystrophy is a generalized membrane disorder, affecting even red blood cells [106, 107]. Similar arguments might also be advanced in the case of DMD, if erythrocyte membranes could be shown to display such matrix modifications.

FIGURE 4: Concentration Dependence of Acetylthiocholine Hydrolysis by Erythrocyte Membrane Acetylcholinesterase. Inset Eadie Plot Analysis of the Same Data.

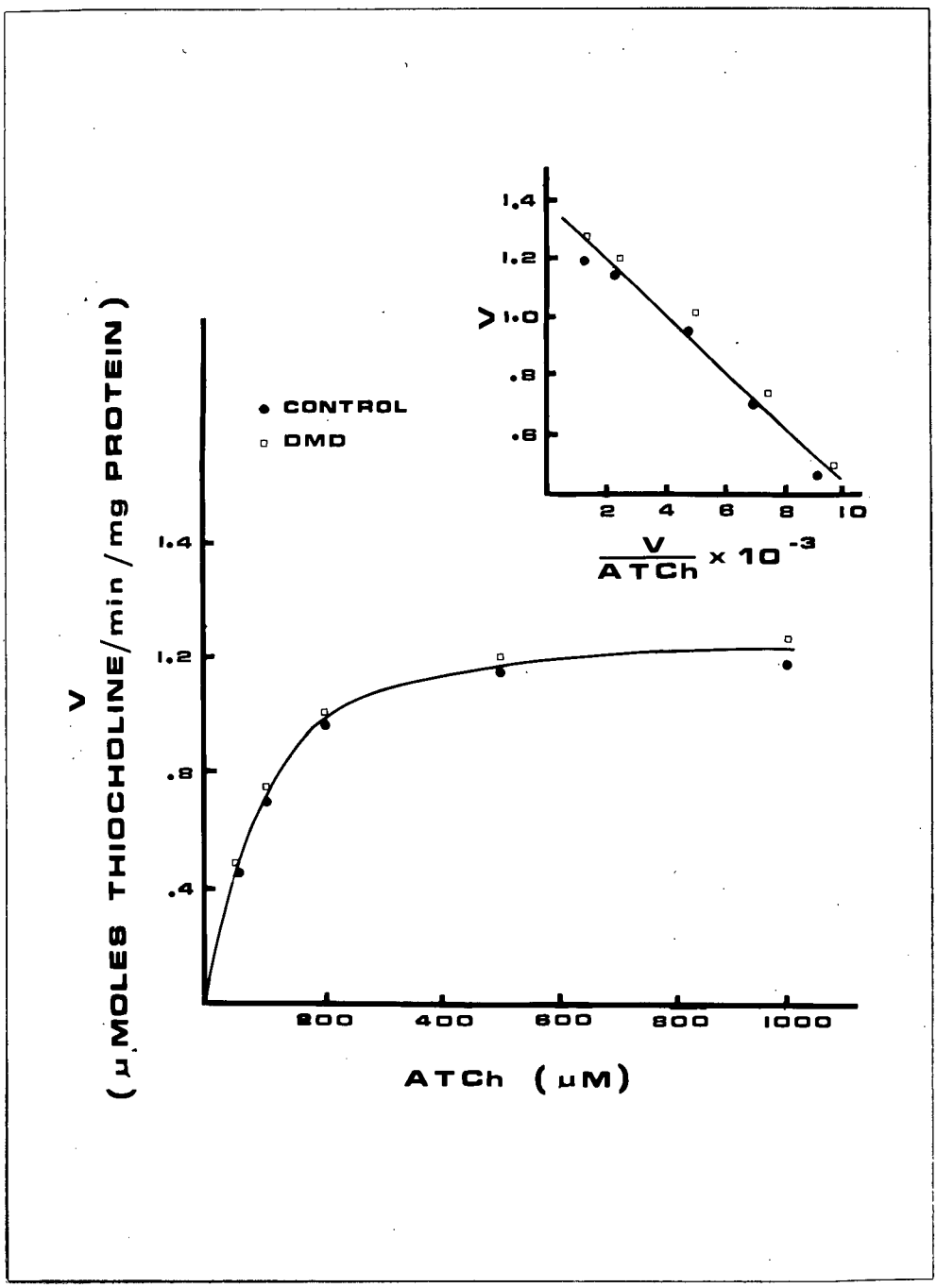

V
(μ MOLES THIOCHOLINE/min / mg PROTEIN)
CONTROL
DMD
.4
.8
1.2
1.4
200
400
600
800
1000
ATCh (μM)
V
.6
.8
1.0
1.2
1.4
2
4
6
8
10
V/ATCh x 10^-3

TABLE X (A)

Substrate Kinetics of Erythrocyte Membrane Acetylcholinesterase: V_{max}

(units: micromoles thiocholine liberated/ minute/ milligram membrane protein)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	1.34	1.33	0.12	0.05	1.22-1.53	-
Children with DMD	10	1.44	1.43	0.22	0.07	1.04-1.82	N.S.
Normal Female Adults	9	1.48	1.49	0.20	0.07	1.16-1.81	-
Definite and Probable DMD Carriers	6	1.56	1.56	0.13	0.05	1.39-1.74	N.S.
Possible Carriers Mothers of DMD Patients	5	1.42	1.32	0.36	0.16	1.05-1.88	N.S.
Possible Carriers: Sisters of DMD Patients	8	1.24	1.28	0.29	0.10	0.80-1.72	N.S.

TABLE X (B)

Substrate Kinetics of Erythrocyte Membrane Acetylcholinesterase: $K_{.5}$ for Acetylthiocholine

(units: micromolar)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	93	93	7	3	84-104	-
Children with DMD	10	97	93	10	3	88-112	N.S.
Normal Female Adults	9	99	98	13	4	84-129	-
Definite and Probable DMD Carriers	6	101	100	12	5	82-116	N.S.
Possible Carriers Mothers of DMD Patients	5	105	106	22	10	85-139	N.S.
Possible Carriers: Sisters of DMD Patients	8	96	99	11	4	82-111	N.S.

Fluidity studies were therefore undertaken on DMD clinical blood specimens using the approach described by Bloj et al. [77]. These investigations demonstrated, using rats fed with different fat-supplemented diets, that the degree of NaF-induced allosteric inhibition of erythrocyte AChE (as assessed by the magnitude of the slope of the Hill plot) is indicative of the state of membrane fluidity. Bloj et al. have found strong correlation between the estimates of membrane fluidity made by this biochemical method and those made by physical techniques, such as electron spin resonance spectroscopy [77]. Figure 5 shows the effect of increasing concentrations of NaF on the maximal rate of hydrolysis of ATCh by DMD and control erythrocyte membranes, with an inset of the same data in the form of Hill plots. The $K_{.5}$ for F^- inhibition of AChE and the Hill coefficient (the slope of the Hill plot) are summarized for the various clinical and control groups in Tables XI (A and B). It will be noted that no significant differences have been observed in any of the groups studied.

Gel Electrophoresis

Margareth et al. were able to demonstrate that the gel electrophoretic pattern of microsomal membrane proteins obtained from rat skeletal muscle underwent marked alterations following muscle denervation [108]. Boegman's work on dystrophic mouse has revealed appreciable modification of the protein fraction of skeletal muscle sarcolemma; the affected murine proteins were found to differ from control proteins in quantity and gel migration rate [10]. However, when the gel electrophoretic patterns of erythrocyte membrane proteins obtained from DMD patients and carriers were studied by Roses et al.,

FIGURE 5: Fluoride Inhibition of Erythrocyte Membrane Acetylcholinesterase in Normal and DMD Erythrocytes. Inset Hill Plot Analysis of the Same Data.

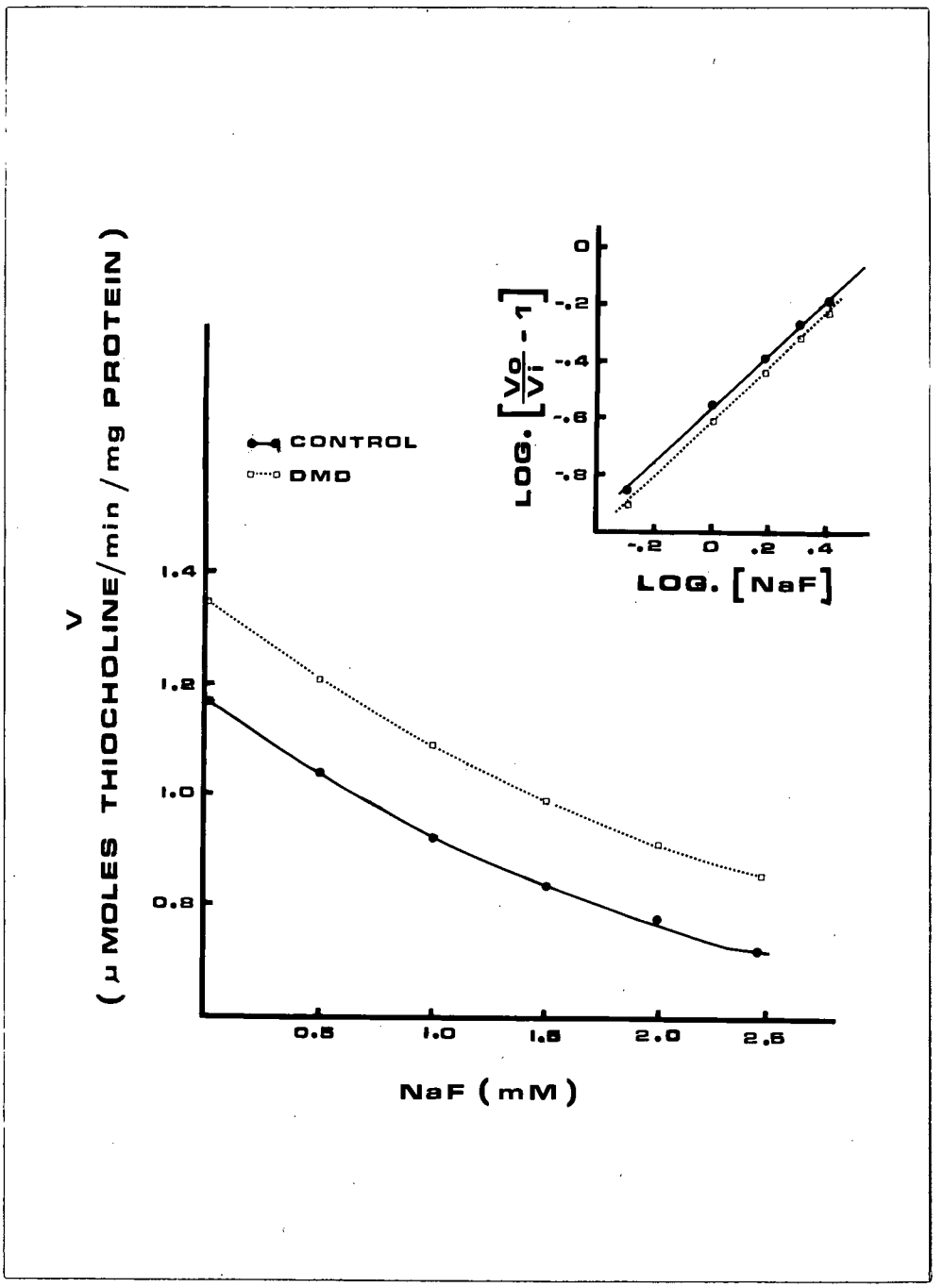

V
(μ MOLES THIOCHOLINE/min /mg PROTEIN)
CONTROL
DMD
1.4
1.2
1.0
0.8
0.5
1.0
1.5
2.0
2.5
NaF (mM)
LOG. [Vo/Vi - 1]
0
-.2
-.4
-.6
-.8
-.2
0
.2
.4
LOG. [NaF]

TABLE Xl (A)

Flouride Inhibition of Erythrocyte Membrane Acetylcholinesterase: Hill Coefficient

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	0.94	0.90	0.12	0.05	0.84-1.14	-
Children with DMD	6	0.97	0.97	0.09	0.04	0.86-1.07	N.S.
Normal Female Adults	6	0.96	0.91	0.20	0.08	0.76-1.36	-
Definite and Probable DMD Carriers	2	-	-	-	-	0.86 1.16	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	0.92 1.09	?
Possible Carriers: Sisters of DMD Patients	8	0.95	0.96	0.22	0.08	0.60-1.28	N.S.

TABLE Xl (B)

Flouride Inhibition of Erythrocyte Membrane Acetylcholinesterase: $K_{.5}$ for F^-

(units: millimolar)

Group	n	Mean	Median	SD	SE	Range or Individual Values (where "n" is small)	Significance at 0.05 level
Age-matched Normal Children	5	4.07	4.02	0.93	0.41	2.98-5.38	-
Children with DMD	6	4.16	4.20	0.43	0.18	3.67-4.73	N.S.
Normal Female Adults	6	4.54	4.50	0.64	0.30	3.82-5.43	-
Definite and Probable DMD Carriers	2	-	-	-	-	3.98 4.05	?
Possible Carriers Mothers of DMD Patients	2	-	-	-	-	3.37 4.22	?
Possible Carriers: Sisters of DMD Patients	8	4.06	4.19	1.01	0.36	2.45-5.48	N.S.

no reproducible departures from normality were observed [57].

Despite this negative report by Roses et al., gel electrophoretic analysis of red cell membrane proteins was performed upon a small but representative sample of clinical and control blood specimens (see Table XII). The erythrocyte membranes of the six subjects tested were analysed as previously described (see Materials and Methods); the stained gel was optically scanned with a densitometer. No gross differences in the protein fractional migration patterns could be discerned for these six subjects (not shown in Table XII).

Table XII provides a comparison of the relative protein contents of several major bands observed when the erythrocyte membrane material of the six subjects under consideration was analysed by gel electrophoresis. The protein content of gel bands I, II, III, V and VI was estimated for each subject by measuring the densitometer scan peak height for each band and expressing this as a percentage of the total height of all five protein bands. Examination of these tabulated data will reveal that no gross differences were observed in the quantities of these protein sub-fractions for the clinical and control specimens. More subtle differences, if such exist, would require extensive investigation to ascertain the limits of normality and the variability of clinical material.

Erythrocyte Fragility

Hereditary spherocytosis and hereditary stomatocytosis are hematological disorders both of which are characterized by increased fragility of erythrocytes to hypotonic stress [109, 110]. Furthermore, Kuiper and Livne have shown that in hereditary spherocytosis there is a marked decrease in

TABLE XII

Polyacrylamide Gel Electrophoretic Analysis of Erythrocyte Membrane Proteins: Relative Peak Heights Derived from Densitometry Scan

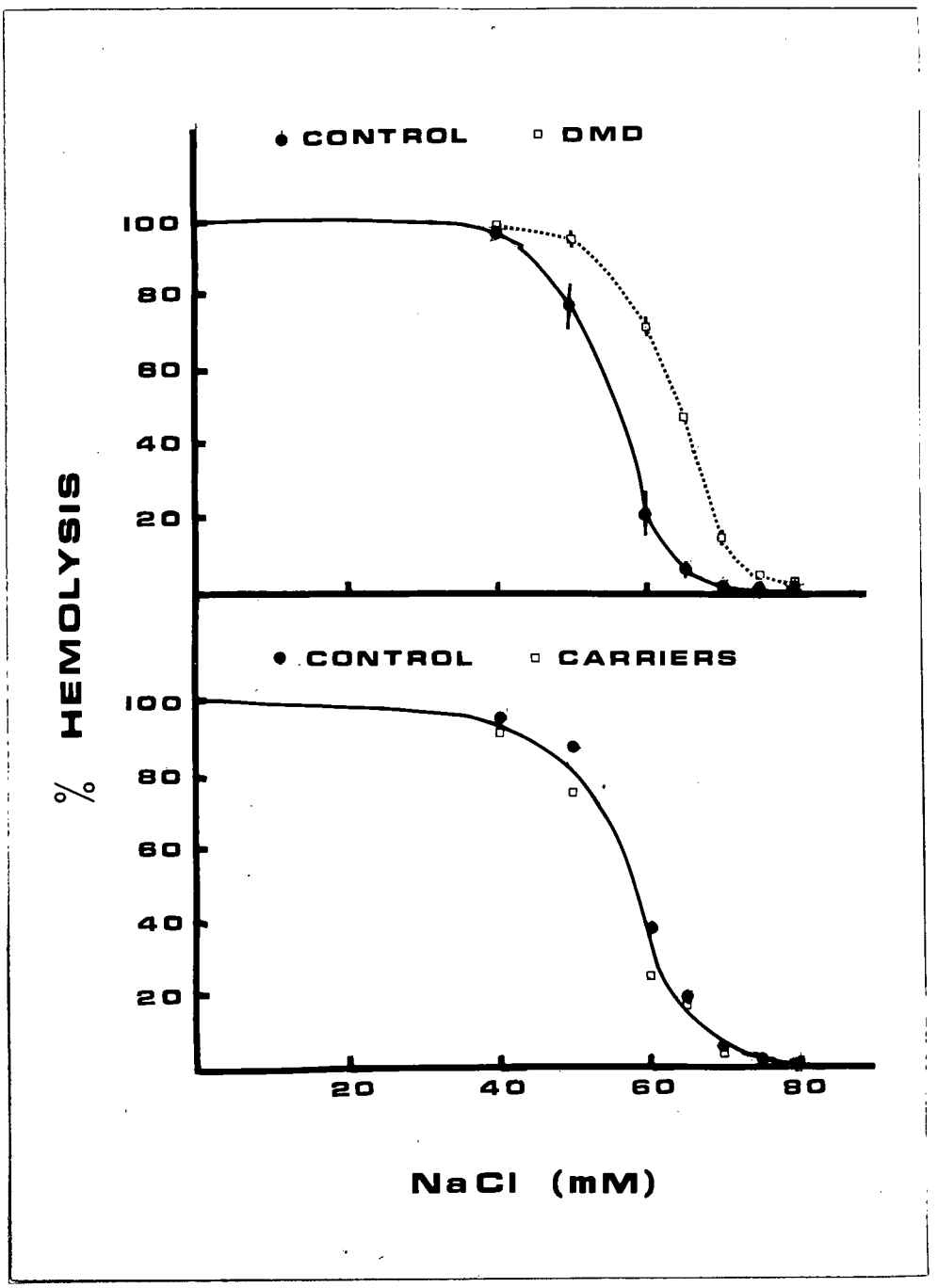
(Each peak height is expressed as the percentage of the total for each individual. Band designation is as described by Fairbanks et al.[78])

Subject	Band I	Band II	Band III	Band V	Band VI
Age-matched Normal Child	20	30	30	10	10
Child with Hereditary DMD	21	32	29	9	10
Child with sporadic DMD	24	29	28	9	10
Normal Female Adult	21	35	23	9	11
Possible Carrier: Mother of DMD Patient	23	32	24	9	11
Possible Carrier: Sister of DMD Patient	18	29	31	10	12

the percentage of long chain fatty acid components of red cell membrane phospholipids [109]. Mentzer et al. have observed an abnormally high resting membrane permeability to potassium and sodium in hereditary stomatocytosis [111]. Similar changes with respect to potassium fluxes and lipid composition have been reported for DMD erythrocytes [51, 55]. Also DMD, like hereditary spherocytosis and stomatocytosis, is a disorder associated with erythrocyte morphological alterations [52, 53, 54]. Given these findings and despite an apparently cursory study by Miale et al. which produced only negative findings [112], an assessment of erythrocyte osmotic fragility was deemed appropriate. Such a study was, in fact, considered essential, because hitherto all of the experimental methods utilized in these investigations involved erythrocyte membranes (processed to eliminate virtually all erythrocyte cytoplasmic components) rather than intact erythrocytes. It is known that the properties of isolated leaky erythrocyte membranes [113, 114, 115, 116] or even resealed membrane preparations [117] may differ quite substantially from those of the membrane in the intact cell. In this regard, Tanaka and Ohnishi using spin label techniques showed that the asymmetric fluidity pattern manifested by the membrane of intact red cells is lost upon hemolysis [118]. Thus the exclusive use of erythrocyte membrane material in the study of DMD might result in the failure to detect the more subtle molecular features which characterize this disorder.

Figure 6 illustrates the pooled results of the osmotic fragility study of clinical and control erythrocytes. The top panel clearly reveals that the red cells of the three DMD patients tested exhibit a greater susceptibility to hemolysis when exposed to hypotonic stress than is seen in

FIGURE 6: Osmotic Fragility of Intact Erythrocytes. Top Panel: DMD and Age-matched Normals. Bottom Panel: Normal Females and Definite Female Carriers of DMD.


● CONTROL
□ DMD
100
80
60
40
20
% HEMOLYSIS
● CONTROL
□ CARRIERS
100
80
60
40
20
20
40
60
80
NaCl (mM)

the erythrocytes of the four control children tested. Standard error bars are included for the NaCl range of 0.05-0.07 M to illustrate that no overlap exists between these two groups over this range. The lower panel of Figure 6 reveals no significant differences in the osmotic fragility of red cells of definite carriers of DMD (n = 2) and normal female adult controls (n = 3). Standard error bars for these two data pools exhibit considerable overlap and have been omitted from the diagram for purposes of clarity.

DISCUSSION

The foregoing experimental results comprise a screening study wherein erythrocytes obtained from patients with DMD and their female relatives were analysed by a wide variety of analytical techniques to evaluate red cell structural and functional characteristics. Statistical methods were employed in the comparison of erythrocyte parameters in both clinical and control blood samples. These comparisons have revealed promising areas for future in-depth investigation. It is hoped that further studies of DMD erythrocytes will contribute to the elucidation of the pathogenic lesion underlying this disorder and will provide unambiguous red cell markers of the affected and carrier states in DMD.

As indicated in the Results section, a number of red cell abnormalities were found to be present in erythrocytes from patients with DMD or from their female carrier relatives: (a) the content of phosphatidyl serine in the erythrocyte membrane lipid fraction was found to be elevated by 50%; (b) evidence for the possibility of two distinct sphingomyelin populations in erythrocyte membrane lipids was suggested by their different fractional migration characteristics upon thin layer chromatographic analysis; (c) membrane basal (Mg^{2+}-dependent) p-NPPase activity was found to be significantly lowered; (d) the apparent dissociation constant ($K_{.5}$) for potassium stimulation of p-NPPase was also observed to be depressed; (e) the activity of erythrocyte membrane Mg^{2+}-dependent, Na^{+}, K^{+}-stimulated ATPase was likewise lowered; (f) a decrease was also seen in the maximal velocity of ATP hydrolysis by the high-affinity component of erythrocyte Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase; (g) the possiblity of similar lowering of the V_{max} of the

Ca^{2+}-ATPase low-affinity component was also noted ($p < 0.10$); and finally, (h) an increase in the fragility of intact erythrocytes to osmotic stress was observed. No apparent abnormalities were evident in the erythrocyte membrane contents of sialic acid, cholesterol or total phospholipid. Clinical red cell membranes likewise showed normal primary amino and sulfhydryl group surface and latent titers. The TLC fractional mobilities and relative quantities of most membrane phospholipid subfractions were normal in DMD patients and their female relatives, as were the kinetic characteristics of erythrocyte membrane AChE and its inhibition by NaF. Finally, in a small but representative number of clinical blood samples tested, no gross differences were found in the relative quantities and fractional migration properties of red cell membrane proteins subjected to gel electrophoretic analysis. Clearly, erythrocyte membrane phospholipids, the enzyme activities associated with active sodium and potassium transport, as well as those believed to be related to active calcium extrusion, and the osmotic integrity of intact red cells appear to be promising areas for further in-depth investigation of DMD.

The results of several lines of investigation [51, 52, 53, 54, 55, 56, 57, 58, 67], as well as those summarized above, suggest that erythrocytes in DMD are abnormal in several respects. These findings, as well as those for other non-muscular DMD tissues (see Introduction), strongly argue in favour of the hypothesis that the defect in DMD is a generalized one involving a number of membrane systems. It may well be that the essential pathological characteristics of this disease are demonstrable in red blood cell membranes, and unequivocal markers of DMD and the carrier state may likewise be revealed by erythrocyte research.

Study of red cells may also provide answers regarding the homogeneity of DMD and carrier groups. There have been certain indications that DMD is not a single pathological entity, but rather a collection of diseases of similar symptomatology, but different etiology: For example, there are reports that apparent sporadic and hereditary DMD cases may be distinguished by the extent of palmitate oxidation occuring in their skeletal muscle mitochondria [119] or by the levels of diphenoloxydase activity found in their platelets [120]. However, the work of Roses and Appel [67], discussed earlier (see Introduction), argues against the occurrence of biochemically distinct subclasses of DMD carriers, since most subjects classified as "possible carriers" [1] revealed their abnormal status in exactly the same fashion as did known and probable carriers, the erythrocytes of subjects from all three groups possessing apparently identical morphological and functional alterations [67].

The suggestion by Roses and Appel that many possible carriers are in fact true carriers of the DMD gene(s), albeit "silent carriers" [65], is further strengthened by the findings of the present investigation. The possible carriers who are sisters of DMD patients exhibited certain compositional [see Table VII (A)] and functional [see Tables VIII (D) and IX (B)] abnormalities. However, these particular alterations were not observed in the DMD patients or in other carrier groups. An entirely different, though probably related, group of changes were to be found in DMD erythrocyte membranes [see the "twin sphingomyelin-like" TLC spot discussion in Results, also Tables VIII (A) and IX (C) and Figure 6]. These particular alterations likewise were not found in any of the DMD "carrier" groups. It should be noted that any female relative of a child afflicted by DMD may, on the bases

described in the Introduction, be designated a "carrier"; thus one would expect to find a certain proportion of the possible carrier group to be perfectly normal. The above observations may well argue for the existence of pathologically distinct sub-varieties of the affected and carrier states in DMD. However, the data on these altered parameters, particularly in the definite/probable carriers and possible carrier mothers of DMD patients, are as yet insufficient to adequately assess the homogeneity of this disease in affected children and their female relatives.

It was previously stated (see Results) that the present data could be considered sufficient to rule out the possibility that the erythrocytes of definite/probable carriers and possible carrier mothers are abnormal with respect to the properties examined here. In nearly every case these two data-poor groups show at least one observation which falls within the normal range of values [see Tables VI (A-D), VII (A-D), VIII (C and D), IX (A-F) and XI (A and B)]. The only exceptions to this rule are seen in certain single observations of Ca^{2+}-ATPases [see Tables IX (B-F)]. These two carrier groups may well be normal with respect to parameters where data overlap the range of normal values. This "overlap argument" is only valid for small sample sizes and may be stated as follows: Assume for the moment that the population from which a given data-poor group of individuals is taken does differ substantially from normal with respect to a given parameter; then the probability is exceedingly small ($p \ll 0.05$) that random sampling of this abnormal population would by chance alone produce a sample of size $n = 1$ or $n = 2$ where one or both observations fall within the range of normal values as estimated by a relatively small sampling of the normal population, $n = 6$. Thus, if such an

overlap of the normal range does occur, one could argue that it is highly likely that the data-poor group is normal with respect to the parameter under consideration. Underlying this argument are two assumptions: the sampling groups are assumed to reflect homogeneous populations, and untoward experimental errors are assumed to have negligible influence on parameter evaluation. In Table VIII (C), the two divergent values of K^+-NPPase V_{max} for the definite/probable carrier group could suggest that perhaps one or both of these conditions are not fulfilled in every case. Clearly, further study of these parameters is indicated if the normality of data-poor carrier groups is to be assessed. If abnormalities are found, then the homogeneity of the various carrier groups and of the DMD patient subclasses (e.g., sporadic and hereditary) may be evaluated.

Having identified several possible alterations in erythrocytes of DMD patients and carriers, one must now consider how these abnormalities might be related to the overall disease process. Since the present findings require extensive verification and elucidation, any such discussion must involve a good deal of speculation. Therefore in the discussion which follows, certain assumptions have been made for the sake of simplicity. It is assumed that (a) all cases of DMD reflect the same underlying pathology (i.e., DMD is a homogeneous disorder that is not composed of biochemically distinct subvarieties) and (b) the abnormalities exhibited by known and silent carriers of DMD represent an attenuated form of the pathology underlying the disorder of afflicted children. This latter assumption is made, because female carriers, apparently even silent ones [67], often exhibit some degree of demonstrable myopathy, although rarely are they afflicted to the degree

observed in DMD patients [1]. The discussion which follows attempts to rationalize the various experimental data from this work and from the investigations of others in terms of recognized mechanisms of cellular injury. Numerous parallels are drawn between the alterations observed in DMD red cells and those exhibited by erythrocytes in various other disorders and experimental models.

Many of the changes described for erythrocytes in DMD (e.g., alterations in membrane ionic permeability and cellular ionic homeostasis [55, 56, 121], transformation of a significant proportion of cells into echinocytes [52, 53, 54, 67], modification of membrane phospholipid subfractions [see sphingomyelin commentary in Results, also Table VII (A)] [51], enhanced osmotic fragility [see Figure 6] and reduction of membrane deformability [121]) are similar to those observed when red cells are exposed to oxidant injury with subsequent peroxidation of plasma membrane lipids. Oxidant injury may occur in erythrocyte aging and may trigger removal of the red cell from the circulation and its destruction in the spleen [122]. Aged erythrocytes exhibit a diminution in the in vivo lipoperoxidation protective system (i.e., glutathione, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase), resulting in peroxidative injury to membrane lipids [122], as well as abnormally high levels of potassium conductance, decreased intracellular potassium, increased intracellular sodium, decreased membrane deformability, enhanced osmotic fragility and hemolysis [122, 123, 124]. Lipoperoxidation is known to be the result of free radical attack on unsaturated bonds found within the fatty acid residues of membrane lipids; once

begun the process is autocatalytic [124]. Oxidant injury resulting from the deficiency of natural antioxidant vitamin E is believed to underlie the production of a number of nutritional muscular dystrophies in animals [125]. It is of interest that the echinocyte morphology observed in human DMD erythrocytes [52, 53, 54, 67] is also to be found in red cells of rats rendered dystrophic by dietary deficiency of vitamin E [126]. Furthermore, the elevated potassium conductances seen in human DMD skeletal muscle and red cells [127, 55] also occur in brain and liver mitochondria of vitamin E-deficient rats [55].

That oxidant injury and lipoperoxidation may underlie or at least contribute to many of the abnormalities observed in DMD erythrocytes is suggested by the findings of Kunze et al., who detected alterations in the fatty acid patterns of phosphatidyl ethanolamine and sphingomyelin [51]. These investigators found that the content of saturated fatty acid residues in these phospholipid subfractions were increased at the expense of unsaturated residues. It is known that moderate oxidant injury triggers a pre-lytic redistribution of membrane phospholipid fatty acid residues, the rate of acylation of both saturated and unsaturated fatty acids into these phospholipids being accelerated; however, since lipoperoxidative attack on unsaturated residues is ongoing, these fatty acids continue to be rapidly lost, allowing the accumulation of saturated residues [124]. This increase in saturated fatty acid content in membrane lipids may be responsible for the reduction in deformability observed in erythrocytes subjected to lipoperoxidation. Furthermore, the lysophosphatides produced during membrane lipid oxidant injury may well be involved in enhancing osmotic fragility and precipitating hemolysis in red cells, since even low concentrations of these detergent-like

molecules (2×10^{-4}M) are known to be capable of lysing erythrocytes [128]. Conversion from the discocytic to the echinocytic morphology has also been observed when normal red cells are incubated with lysophosphatides in vitro [52].

In addition to producing lipid damage, oxidant injury is also capable of destroying the sulfhydryl residues of erythrocyte membrane proteins [124]. It is well known that the integrity of these membrane sulfhydryl groups is required for the maintenance of normal membrane ionic permeability [129]; therefore, the destruction of these residues could have grave consequences on erythrocyte homeostasis. Experiments employing treatment of erythrocytes with sulfydryl-modifying agents have revealed that cells so treated exhibit membranes leaky to sodium and potassium [129], decreased deformability and enhanced osmotic fragility [130].

Contrary to the findings of Omaye and Tappel which suggest that lipoperoxidation injury is associated with genetic muscular dystrophy in some species of chickens and mice [131], the DTNB labelling studies of erythrocytes of DMD patients and their female "carrier" relatives failed to show any evidence of loss of membrane sulfhydryl groups [see Tables IV (A and B)]. However, it might be suggested that the twin "sphingomyelin-like" spots observed upon TLC analysis of some clinical blood samples may reflect the occurrence of lipoperoxidative injury in the membranes of these cells, the second spot arising from newly acylated phospholipids rich in saturated fatty acid residues. If lipoperoxidation is occurring in DMD erythrocytes, it seems unlikely that it represents the mechanism of primary importance in the pathogenesis of this disease, since these twin "sphingomyelin-like" spots were only observed in half

of all patients analysed. Furthermore, phosphatidyl ethanolamine, which is particularly abundant in poly-unsaturated fatty acids, should have exhibited detectable thin layer chromatographic alterations as a result of re-acylation with saturated fatty acid residues [124], but in fact, no such alterations were seen in any of the clinical samples. These facts might suggest that a more specific mechanism of lipid modification than lipoperoxidation is operating in DMD erythrocytes. Should analysis prove these twin "sphingomyelin-like" TLC spots to be sphingolipids, then one might suspect the occurrence of some abnormality in sphingomyelin synthesis or renewal or perhaps the activation of lipases which selectively attack this phospholipid subfraction. A recent report that short and long-chain triglyceride lipase activities are markedly elevated in skeletal muscle of hereditarily dystrophic mice [132] may have a bearing on this point.

Even if lipoperoxidation does not represent a major cause of lipid modification in DMD erythrocytes, a low level of oxidative stress may be occurring in these cells, compensated for by stimulation of the in vivo lipoperoxidation protection system [131]. Low levels of oxidant injury might not be discernible as a reduction in membrane sulfhydryl residues [124]; therefore, identification of the occurrence of this subtle, albeit injurious process within DMD red cells might not be possible using techniques which quantify membrane sulfhydryl groups (e.g., the DTNB labelling technique used in the present investigation). However, analysis of erythrocyte contents of glutathione and the various enzymatic components of the in vivo lipoperoxidation protective system could provide a sensitive index of membrane peroxidative damage.

If low level peroxidative damage is occurring within DMD erythrocytes, it conceivably could continue to the enhancement of osmotic fragility observed in these cells (see Figure 6). If this is so, and despite the fact that the erythrocytes of DMD carriers failed to exhibit any abnormality in their response to osmotic stress (see Figure 6), examination of the fragility characteristics of DMD carrier red cells in the presence of thyroxine, a hormone reported to potentiate the hemolytic effects of peroxides on erythrocytes [122], might unmask the presence of an osmotic defect similar to that seen in the cells of DMD patients and might afford a convenient marker of the carrier state.

Another disorder of erythrocytes which produces many alterations similar to those observed in DMD red cells is hereditary spherocytosis (HS). Consideration of this hematological disorder may shed some light upon the involvement of erythrocytes in DMD pathology. HS is a genetically transmitted disorder in which erythrocytes exhibit alterations of cellular morphology and membrane lipid composition, as well as showing reduced deformability, enhanced osmotic fragility and abnormalities of transmembrane ionic fluxes [109, 110, 129, 133]. The passive permeability of sodium ions in HS has been found to be twice normal [134], but without concomitant membrane leakiness to potassium ions [130]. This high sodium influx is compensated by an elevated level of active sodium extrusion, energized by abnormally high rates of glycolysis in erythrocytes [135]. DMD erythrocytes, on the other hand, show a different pattern of ionic fluxes; these cells have a predilection to leak potassium (instead of sodium), while maintaining apparently normal intracellular levels of potassium [55, 122]. Normal potassium homeostasis in the face

of this five-fold increase in conductance [55] may be maintained by a compensatory increase in active potassium influx, since a significant elevation in active potassium pumping has been reported to occur in the erythrocytes of DMD patients and carriers; however, this alteration is apparently associated with normal levels of sodium extrusion [56]. This might suggest a departure from the normal 3:2 sodium-potassium exchange ratio in the sodium-potassium pump of these cells. A stoichiometric alteration in the operation of the sodium-potassium exchange pump has been observed by Hull and Roses to occur in erythrocytes obtained from patients suffering from myotonic muscular dystrophy [136]. These investigators have evaluated the exchange ratio in myotonic erythrocytes, finding two sodium ions exchanged for an equal number of potassium ions, and they suggest that this modification may arise from a reduction in the affinity of one of the three binding sites for sodium at the level of the membrane cation pump [136]. Perhaps the increased active potassium uptake, but normal sodium extrusion, reported in DMD red cells [56] reflects an alteration, not in the affinity of the pump for sodium as seen in HS, but rather in its affinity for potassium. The membrane sodium-potassium exchange pump in DMD erythrocytes may have undergone some modification which increases its affinity for potassium without affecting its affinity for sodium. Tentative evidence for such a modification might be seen in the increased affinity (decreased $K_{.5}$) of membrane p-NPPase for potassium ion observed in certain clinical subjects [see Table VIII (D)]. If further investigation bears out this observation in DMD erythrocytes, then stoichiometric studies of active sodium-potassium exchange in these cells would be warranted.

Apparently ionic homeostasis is not precisely maintained in DMD erythrocytes, since a small but significant increase in intracellular sodium is reported to occur in these cells [122]. Secondary to this increase in intracellular sodium, the red cell may imbibe some water, and this may explain the tendency of DMD erythrocytes to lyse in hypo-osmolar media before normal erythrocytes (see Figure 6). The decreased activity of membrane Na^+, K^+-ATPase noted in the present studies for some clinical subjects [see Table IX (B)] may produce this increase in intracellular sodium levels, but this explanation is not entirely compatible with an alteration in the mechanism of cation pumping just postulated to explain the normal potassium levels found within DMD red cells. Perhaps the observed decreases in enzymatic activities associated with the sodium-potassium pumping mechanism [see Tables VIII (A) and IX (B)] are indicative of the operation of complex homeostatic compensatory mechanisms triggered by cation imbalances within the intact erythrocyte. The situation is further complicated by uncertainty as to the extent to which the functional characteristics of the active sodium-potassium transporting mechanism in intact erythrocytes are reflected by the properties of the Na^+, K^+-stimulated ATPase activity in isolated membranes. One cannot eliminate the possibility, for example, that a decrease in Na^+, K^+-ATPase activity in isolated membranes may be a reflection of the decreased stability of the enzyme in a pathologically altered membrane under the conditions of membrane isolation. It is interesting, however, that a depression of Na^+, K^+-ATPase activity has been observed in sarcolemmal membrane preparations derived from DMD skeletal muscle [9]. There is evidence that ionic homeostatic mechanisms are perturbed in DMD skeletal muscle, since abnormally high levels of potassium

efflux have been reported to occur in these tissues [127]. Furthermore, whole body studies of DMD carriers have revealed a reduction in intracellular potassium contents and an elevation in cellular water [92]. Such imbalances might give rise to sublethal injuries in erythrocytes and other non-excitable tissues, but these same defects in ionic homeostasis would be devastating to excitable tissues, perhaps producing many of the myriad pathological changes of DMD muscle and nerve discussed earlier (see Introduction) and ultimately culminating in necrosis [55]. The importance of ionic flux imbalances in the process of muscle cell death is emphasized by the report that treatment of DMD patients with lithium gluconate, purported to be a non-competitive inhibitor of potassium translocation in a variety of biological membranes [137], decreases serum CPK activity [138], one of the indices of muscle deterioration in the early stages of this disease [1]. Lithium alone or in combination with other drugs may prove useful in ameliorating the rapid progress of DMD [139].

While alterations in sodium-potassium homeostasis may be invoked to explain deleterious changes which occur in excitable tissues in DMD, as well as some of the functional disturbances observed in DMD and HS erythrocytes (e.g., increased osmotic fragility), other abnormalities in the red cells of patients with DMD and HS (e.g., decreased deformability, echinocytic morphology, etc.) are not so readily explained on this basis. However, there is a good deal of evidence that alterations in cellular membrane lipids may represent a more fundamental change in the production of abnormal erythrocytes in DMD and HS, and may underlie skeletal muscle myopathy in DMD. Such a basic modification of membrane integrity could well have profound effects upon the functioning

of membrane enzymes by altering membrane lipid-protein interactions. For example, Kuiper and Livne have correlated their observation of a reduction in the quantity of long chain fatty acid conjugates of membrane phospholipids in HS erythrocytes with other features of this hematological disorder (e.g., reduction in cell surface area, increased sodium conductance, enhanced osmotic fragility, etc.) [109]. Despite the difference in ionic permeability characteristics between DMD and HS erythrocytes (discussed in the preceeding paragraphs), basic modifications in the phospholipid subfractions of erythrocyte membranes are also observed in DMD [51] [see Table VII (A) and sphingomyelin commentary in Results]. These modifications of red cell membrane lipids in DMD may have profound effects on cation permeability, as well as on membrane morphology, fluidity, deformability and osmotic fragility [124, 128]. Possible mechanisms whereby these changes may occur will be discussed more fully in the pages which follow. Emphasizing the potential importance of lipid modification in the underlying pathology in DMD tissues are the reports of Hughes and Kunze et al. [5, 51]. These investigators have found significant alterations of lipid fractions in DMD skeletal muscle similar to those seen in DMD erythrocytes. However, these findings are somewhat ambiguous since skeletal muscle necrosis is usually associated with lipogenesis. For these reasons, it appears that a thorough biochemical characterization of erythrocyte lipids obtained from DMD patients and their female relatives would be warranted. In addition to utilizing TLC analysis of membrane lipids to detect gross abnormalities in the various phospholipid subfractions, hydrolysis of these subfractions followed by gas-liquid chromatography would allow accurate identification of phospholipid fatty acid conjugates in clinical blood specimens.

Once the composition of erythrocyte membrane lipid components is established for DMD patients and carriers, utilization of techniques which probe the lipid-protein interactions within intact red cells might prove valuable. Perhaps the sulfhydryl and primary amino group labelling techniques, as well as the biochemical method of assessing membrane fluidity advanced by Bloj et al. [77] (see NaF inhibition of AChE in Materials and Methods and Results), may prove more useful in the study of intact erythrocytes than these methods proved to be in the study of isolated red cell membranes. The fact that these techniques failed to detect any abnormalities in the fine structure of isolated DMD erythrocyte membranes [see Tables IV (A and B), V (A and B) and XI (A and B)] in no way eliminates the possibility that such abnormalities do occur in the membranes of intact cells. As has been previously discussed (see Results), when isolated erythrocyte membranes ("ghosts") have been rigorously compared to the membranes of intact cells utilizing various probes of membrane integrity, red cell ghosts were found to deviate considerably from the native structure exhibited by the membranes of intact erythrocytes [113, 114, 115, 116, 117, 118]. Preliminary studies from this laboratory indicate that the kinetic characteristics of substrate hydrolysis by erythrocyte membrane AChE and its inhibition by NaF differ markedly between normal intact eythrocytes and ghosts prepared from normal cells (data not presented in Results). While such findings do not negate the value of utilizing red cell ghosts in the study of DMD, they do suggest caution in the interpretation of data obtained from isolated membranes and emphasize the value of using intact erythrocytes wherever possible in these investigations.

It is not clear to what extent alterations in membrane lipid-protein interactions in DMD erythrocytes influence calcium homeostasis in these cells, but the results of the present investigations and the work of others indicate that cellular calcium handling may be impaired in DMD tissues. For example, the maximal velocity of the high-affinity component of erythrocyte membrane Ca^{2+}-ATPase, believed by Schatzmann and his colleagues [98, 101] to represent the red cell's calcium extrusion mechanism, is significantly depressed in DMD subjects [see Table IX (C)]. A similar alteration may also be present in the low-affinity component of this enzyme [see Table IX (E)], which Schatzmann et al. have tentatively identified as a slightly denatured form of the high-affinity Ca^{2+}-ATPase produced during the preparation of red cell ghosts [98, 101]. Thus the calcium pump may be defective in DMD erythrocytes. A similar disturbance in Ca^{2+}-ATPase activity with impairment of active calcium uptake has been reported to occur in sarcoplasmic reticular vesicles derived from DMD skeletal muscle [12].

It is not clear whether these apparent abnormalities in the calcium-pumping mechanism identified in DMD erythrocytes and skeletal muscle organelles arise from membrane lipid alterations with subsequent modification of membrane lipid-protein interactions, or whether they may represent some basic, inheritable change in a protein component of this cationic transport mechanism. It is clear, however, that such a change in calcium homeostatic machinery could be potentially deleterious to the cellular integrity of skeletal muscle [140]. Furthermore, this defect could produce many of the abnormalities in cellular morphology and function described for DMD erythrocytes. For example, the

intracellular accumulation of calcium ions in the DMD red cell, secondary to defective calcium extrusion, may predispose the cell to leak potassium at an abnormally high rate; the ability of calcium to modify potassium permeability in erythrocytes has been well documented by investigators who studied potassium efflux from metabolically depleted red cells incubated in calcium Ringers solution [141, 142]. Similarly, elevation of intracellular calcium concentrations in erythrocytes by introduction of the cation via the antibiotic ionophore A23187 produces a marked increase in potassium efflux, as well as demonstrable reduction in red cell deformability [143]. Furthermore, metabolic energy depletion and elevation of intracellular calcium ion concentration with A23187 in red cells has been shown to initiate the transformation from the normal discocytic morphology to the spiculated echinocytic form; this change is apparently related to the accumulation of 1,2-diacylglycerol within the inner leaflet of the erythrocyte membrane, producing outward evaginations of the membrane ("spicules"); 1,2-diacylglycerol is believed to result from the calcium-stimulated breakdown of membrane phosphatidyl choline by endogeneous phospholipase C [102, 103, 145, 146]. Allan et al. suggest that the mechanism just described may explain the loss of membrane components and the enhanced osmotic fragility observed to occur in aging erythrocytes, as well as in HS red cells [146]. As described in detail earlier, many interesting parallels exist between the alterations seen in HS erythrocytes and those noted for DMD erythrocytes. It is hardly surprising therefore that Feig and Guidotti have observed a deficiency of Ca^{2+}-ATPase activity in HS red cells, which these investigators believe may reflect a basic alteration in the calcium extrusion mechanism

[147]. It is conceivable that these parallel alterations in DMD and HS red cells may arise from a similar defect in calcium homeostasis. The close correlation between the data reported in this thesis [see Tables IX (C and E)], the other published observations of erythrocyte abnormalities in DMD, and the known sequelae of high levels of intracellular calcium established by ionophore studies suggests that potentially valuable insights into DMD pathogenesis may be obtained by a rigorous study of calcium extrusion in these erythrocytes.

Another possible locus for the primary defect in DMD erythrocytes might be the microfibrillar elements, which form an anastomosing network attached to the membrane's inner surface [64]. The function of this network has not been established, but some investigators believe that this microfibrillar system in red cells represents an actomyosin-like contractile mechanism analogous to that found in the skeletal muscle cell [65]. If this is the case, then characterization of this system in DMD erythrocytes should receive high priority in future investigations, especially since there is evidence that the contractile elements of DMD skeletal muscle may be defective. Furukawa and Peter have observed decreased actomyosin-ATPase activity and impaired superprecipitation of myosin B derived from skeletal muscle of patients with DMD [22].

The speculation that an actomyosin-like system exists in erythrocytes was initiated in 1962 by Ohnishi who demonstrated with viscometry that skeletal muscle myosin interacts with a protein in the water-soluble extracts of acetone powder preparations of red cell ghosts; Ohnishi also observed that this interaction is inhibited by addition of ATP to the extract [148]. This protein has subsequently been identified as actin by its molecular weight, net charge,

ability to polymerize into filaments with the double helical morphology and its decoration with heavy meromyosin; in situ erythrocyte actin is associated with a high molecular weight protein loosely bound to the inner membrane surface, called "spectrin" (gel electrophoretic bands I and II [78]), forming the fibrillar network previously described [64]. The question of whether or not this spectrin-actin system possesses a Mg^{2+}-dependent ATPase activity, like skeletal muscle actomyosin-ATPase, is greatly disputed, some even contending that spectrin may be related to one component of the membrane's Ca^{2+}-ATPase activity [63, 64, 121, 149, 150, 151, 152].

A number of lines of evidence suggest that the spectrin-actin network, together with attached glycophorin, a membrane-traversing protein, constitutes the cytoskeleton of erythrocytes and is the chief determinant of erythrocyte shape and deformability [110] -- two parameters known to be altered in DMD red cells. For example, when erythrocytes are heated, a discocyte-spherocyte transition is observed to occur at the precise temperature at which a membrane protein possessing ATPase activity denatures [153]; Jacob has strongly suggested that this protein is spectrin [110]. A similar morphological alteration is observed when erythrocytes are subjected to treatment with vinblastine, a drug which interacts with microfibrillar proteins and is known to denature erythrocyte spectrin with some specificity [154, 155]. Finally, Jacob et al. have found indications that spectrin polymerization may be defective in HS erythrocytes, cells known to exhibit echinocytic morphology [154]. That the anastomosing spectrin-actin network attached to the inner surface of erythrocyte membranes may also be capable of influencing red cell cationic permeability is suggested by the work of Lubin et al. [156]. These investigators found that when erythrocytes obtained from patients suffering from sickle

cell disease are treated with dimethyl adipimidate, a protein cross-linking drug with special affinity for glycophorin, the excessively high potassium conductance usually observed in these cells is reduced to normal levels. Lubin and his colleagues further showed that this same drug is capable of normalizing cation permeability, morphology, and osmotic fragility in erythrocytes from patients with hereditary stomatocytosis [157]. The above evidence suggests that erythrocyte cationic permeability, morphology, deformability and osmotic fragility may all be influenced by the membrane's spectrin-actin microfibrillar network. A defect in this system might underlie the similar alterations observed in red cells in HS [154, 155], hereditary stomatocytosis [156, 157] and DMD. Whether this defect represents the primary lesion in these disorders or is merely one of the many alterations in membrane structure and function, possibly resulting directly from changes in membrane lipid-protein interactions, remains to be determined. In terms of ongoing investigation of DMD erythrocytes, isolation and biochemical characterization of DMD red cell spectrin and actin components might be highly informative. It would also be of interest to determine if in vitro treatment of DMD erythrocytes with dimethyl adipimidate results in a normalization of membrane functional alterations associated with DMD.

Finally, the work of Strickland and Ellis [24] will be briefly considered with a view to suggesting how a generalized metabolic defect in glycolysis might be deleterious to skeletal muscle cells and erythrocytes, should such a defect prove to be even more primary than the abnormalities observed in lipids and some proteins in affected tissues. These investigators demonstrated the occurrence of a compositionally and functionally abnormal isoenzyme II of

hexokinase in DMD skeletal muscle, resulting in a high conversion of glucose to fructose with a concomitant drop in glucose-6-phosphate production [23, 24]. Strickland and Ellis have also found evidence of the same enzymatic defect in DMD liver, peripheral nerve and brain, but not in DMD adipose and connective tissue [24]. Such a metabolic abnormality might be expected to produce fatty change in skeletal muscle fibers via the conversion of the large quantities of fructose into triglycerides. Furthermore, this defect might be expected to produce impaired fatty acid synthesis in liver by blocking NADPH generation by the hexose monophosphate pathway [160]. Since mature erythrocytes are incapable of de novo synthesis of fatty acids, but are continuously renewing their lipid components via various lipid exchange mechanisms, many of which draw upon circulating lipid stores in the plasma [128], and since the liver is a prime contributor to plasma lipid stores [160], an alteration in liver fatty acid synthesis in DMD would be expected to produce alterations in lipid constituents of erythrocytes. Furthermore, if this glycolytic defect should extend to the erythrocyte itself in DMD, and since the energy needs of mature red cells are supplied primarily by anaerobic glycolysis and from products of the hexose monophosphate pathway [160], one would expect to observe ATP depletion in the DMD erythrocyte; this in turn could seriously affect the ATP-requiring calcium and sodium-potassium transport pumps and pump-linked transport of non-ionic substances, as well as decreasing the supply of energy required for the various membrane lipid renewal pathways. Therefore, a generalized metabolic defect such as the one observed by Strickland and Ellis might produce sublethal or lethal injuries to erythrocytes, as well as to other body tissues in this disease. It is interesting to note in this regard that erythrocyte pyruvic

kinase deficiency, a hemolytic anemia arising from a congenitally defective enzyme of the red cell's glycolytic pathway and thereby resulting in a deficiency of erythrocyte ATP stores, produces a picture in erythrocytes strongly resembling that seen in DMD (i.e., echinocytic morphology, an accelerated rate of potassium efflux, compensatory Na^+, K^+-ATPase pumping, decreased deformability and osmotic fragility [129, 161]). The foregoing discussion suggests that a thorough study of glycolytic metabolism of erythrocytes, liver and other tissues might be of real value in the investigation of the molecular basis of DMD pathogenesis.

In addition to discussing a variety of interesting methodological approaches not already employed in the present investigation, consideration has been given in the preceeding pages to suggesting which of the experimental techniques already employed may prove useful in further characterization of DMD erythrocytes. Therefore, a brief examination of possible refinements of the present techniques which might improve their research value would be appropriate here.

A report by Cohen et al. [123] on the biochemical characterization of density -separated normal human erythrocytes demonstrated that the age of the various red cell populations comprising whole blood has a profound effect upon the magnitude of erythrocyte compositional and functional parameters. These investigators found that increasing red cell age correlates with elevation of hemoglobin and intracellular sodium, but with reduction of intracellular potassium, as well as with reduction in membrane protein, sialic acid, phospholipid, cholesterol, and AChE activity [123]. Perhaps the negative results observed in the present studies of membrane sialic acid,

cholesterol, phospholipid and AChE activity in DMD erythrocytes arise from a "swamping out" process, whereby significant alterations in erythrocytes of a given age are obscured by the presence of cells of different ages which display normal parameters or alterations in the opposite direction. Segregation of erythrocytes according to cell age by use of density-separation techniques may allow greater sensitivity in the characterization of abnormalities in DMD red cells.

Another important modification of the present methodology employed in these DMD studies might be to utilize intact erythrocytes instead of isolated red cell membrances in the analysis of the membrane fluidity characteristics of clinical and control blood cells. Tanaka and Ohnishi have demonstrated that the fluidity patterns exhibited by the various regions of the erythrocyte membrane, based upon the asymmetrical distribution of membrane phospholipid components, are lost when red cells undergo hemolysis [118]. Since the preparation of isolated plasma membranes of clinical and control erythrocytes involves step-wise hemolysis, possible alterations in membrane fluidity characteristics of DMD erythrocytes may be obscured by these preparative operations. Since Bloj et al. [77] have suggested that the Hill coefficient for the inhibition by NaF of membrane-bound AChE is a useful probe of membrane fluidity, and since AChE is an enzyme located on the outer surface of the erythrocyte membrane, analysis of the membrane fluidity characteristics of intact erythrocytes from DMD and normal subjects should be possible. Preliminary studies on intact red cells conducted in this laboratory have demonstrated that the kinetic characteristics of substrate hydrolysis by membrane AChE and its inhibition by NaF differ markedly in these cells

from those observed for isolated erythrocyte membrane preparations. The results of other preliminary studies, if confirmed by further investigation, may cast some doubt upon the sensitivity of this biochemical technique, purported to be a reliable means of assessing membrane fluidity characteristics. When normal erythrocyte membranes are treated with agents known to severely modify membrane protein and lipid components (e.g., trypsin and phospholipase A), these membranes exhibited Hill parameters in NaF-AChE studies similar to those obtained for untreated control membranes. Bloj et al. conducted their experiments on isolated membranes prepared from erythrocytes of rats fed various fat-modified diets [77] and not on human erythrocyte membranes. Therefore, a thorough assessment of the usefulness of this technique in the characterization of fluidity properties of human control erythrocytes should be conducted prior to undertaking a full-scale study of membrane fluidity in DMD erythrocytes.

Another potentially valuable modification of the present methodology employed in the investigation of DMD erythrocytes would entail analysis of the osmotic fragility characteristics of DMD patient and carrier red cells in the presence of membrane stabilizers of destabilizers. For example, it might be of considerable clinical interest if non-steroidal anti-inflammatory drugs possessing membrane-stabilizing properties, e.g. indomethacin [162], could be shown to correct the abnormal osmotic fragility of DMD erythrocytes. That indomethacin may be of therapeutic value in DMD is suggested by a recent report by Bulien and Hughes where treatment of dystrophic hamsters with this drug was shown to reduce CPK levels in the serum of these animals, as well as normalize skeletal muscle levels of this enzyme [163]. These findings suggest

the possibility that the defect in skeletal muscle membrane integrity in muscular dystrophy, resulting in the release of cytoplasmic enzymes, may have some bearing on the fragility of erythrocyte membranes in DMD. Also, the ability of drugs to correct the defect in the hypotonic stability of erythrocytes in vitro may prove to be a useful screening procedure for the evaluation of drugs to be tested for possible beneficial effects in vivo.

With regard to DMD carrier erythrocytes, no abnormalities in gross osmotic stability were detected in these cells (see Figure 6). However, it may be possible to unmask more subtle alterations in the hypotonic stability of carrier erythrocytes by examining their stabilization and destabilization properties in the presence of membrane-active drugs (e.g., propranolol), since it has recently been shown that the effects of these drugs upon the osmotic stability of erythrocytes is critically dependent upon lipid-protein interactions within the membrane [164]. Comparison of the osmotic behavior of DMD carrier and normal erythrocytes following treatment with membrane-active drugs may reveal subtle differences in the fine structures of the membranes of these cells and may even provide a convenient means of identifying the carrier state in the female relatives of DMD patients.

In summary, the findings of the present investigation, albeit tentative, and those of other research workers suggest that DMD is a generalized disorder affecting many body tissues in affected children, even erythrocytes. The cellular locus of the defect is still uncertain, although a strong case may be made for localization of the lesion in the plasma membranes of affected cells. The abnormalities in red cell membranes discussed previously lend strong credibility to this hypothesis that DMD is a generalized plasma membrane defect,

although abnormalities have also been noted in organelle membranes of certain other tissues. A variety of alterations of red cell composition and function have been discussed with a view to discerning which alteration might reflect the primary lesion in this disorder and thus gives rise to the other abnormalities observed. Lipoperoxidative mechanisms of membrane injury, alterations in ionic homeostasis, abnormalities in erythrocyte microfibrillar components and modifications of membrane lipid components with subsequent alteration of lipid-protein interactions vital to the maintenance of cellular integrity have all been discussed as possible candidates in the search for the primary DMD lesion. Although at present, there is no conclusive means of choosing between these possibilities, the author favors the hypothesis that DMD arises from a fundamental alteration in membrane lipids, producing various perturbations of membrane structure and function which are ultimately lethal in affected cells. The possibility that this alteration in membrane lipids might be secondary to a genetic defect in cellular glycolytic machinery has been discussed and proposals made as to the mechanics of erythrocyte involvement in such a disorder. Finally, throughout the discussion suggestions have been made regarding potentially promising avenues for the further investigation of erythrocytes in DMD.

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APPENDIX

This section illustrates the use of the Wilcoxon rank sum method of statistical analysis. This method may be employed in the comparison of groups with a minimum sample size of four observations. In the present example, membrane cholesterol contents of erythrocytes drawn from Duchenne dystrophic and normal subjects are compared. The experimental observations (expressed as micrograms cholesterol per milligram membrane protein) are as follows:

Controls ($n_1 = 6$)	DMDs ($n_2 = 9$)
168.1	275.0
167.8	195.3
186.7	177.7
209.1	209.3
203.9	217.4
221.3	219.3
	215.9
	216.4
	220.4

The Wilcoxon rank sum test proceeds in the following manner (79): both samples are combined, ordering the observations from low to high, then a rank is assigned to each observation. For tied observations (i.e., those having the same numerical value), one assigns the corresponding average rank to each tie. (Observations from the group with the smaller sample size are tagged with an asterisk to permit their identification in the pooled data array.)

Ordered Array	Rank
167.8*	1
168.1*	2
177.7	3
186.7*	4
195.3	5
203.9*	6
209.1*	7
209.3	8
215.9	9
216.4	10
217.4	11
219.3	12
220.4	13
221.3*	14
275.0	15

The test consists of summing the ranks of the group having the smaller sample size. For comparison of samples of equal size, one may sum the ranks of either group.

$$\text{Rank Sum} = 1 + 2 + 4 + 6 + 7 + 14 = 34$$

The rank sum is next compared with the tabulated limits, which have been compiled for the desired level of statistical significance. In the present example where $n_1 = 6$ and $n_2 = 9$, the significance limits for the Wilcoxon test at $2\alpha = 0.05$ are 31-65 [165]. If the rank sum attains these levels or exceeds them in the outward direction, the null hypothesis (i.e., that the population distributions of cholesterol content values in each group are equal) must be rejected. However, in the present example, the rank sum falls within the significance limits range, and therefore, the null hypothesis is accepted: i.e., at the 5% level of statistical significance, no discernible difference in erythrocyte membrane cholesterol content has been found between samples drawn from Duchenne dystrophic and normal control populations.