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Erythrocyte-ghost Ca^{2+}-stimulated Mg^{2+}-dependent adenosine triphosphatase in Duchenne muscular dystrophy

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The Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activities (Ca^{2+}-ATPase) of erythrocyte-ghost membranes from patients with Duchenne muscular dystrophy (DMD) and carriers of DMD were compared with activities of normal controls. The Ca^{2+}-ATPase activity of DMD-patient ghost preparations was found to follow the same pattern of activation by Ca^{2+} as the control membranes. However, the Ca^{2+}-ATPase activity in DMD and some DMD-carrier preparations was substantially elevated compared with controls. To characterize further the elevated Ca^{2+}-ATPase activity found in DMD-patient ghost membrane preparations, we estimated kinetic parameters using both fine adjustment and weighting methods to analyse our experimental data. It was established that in both DMD and DMD-carrier preparations the increase in Ca^{2+}-ATPase activity was reflected by a significant increase in $V_{max.}$ rather than by any change in K_m. The response of the membrane Ca^{2+}-ATPase activity to changes in temperature was also investigated. In all preparations a break in the Arrhenius plot occurred at 20°C, and in DMD and DMD-carrier preparations an elevated Ca^{2+}-ATPase activity was detected at all temperatures. Above 20°C the activation energy for all types of preparation was the same, whereas below this temperature there appeared to be an elevated activation in DMD and DMD-carrier preparations compared with normal controls. The concept that a generalized alteration in the physicochemical nature of the membrane lipid domain may be responsible for the many abnormal membrane properties reported in DMD is discussed.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease characterized by progressive degeneration of skeletal muscle. Despite the intense investigation to which DMD has been subjected, the underlying molecular abnormality remains unidentified. The concept that is currently enjoying considerable popularity is that the functional genetic fault of DMD results in abnormal composition and altered function of muscle cell-surface membrane (reviewed by Rowland, 1980). Unfortunately it has proved difficult to isolate sarcolemma from muscle in sufficient quantity and in a pure enough state to facilitate biochemical analysis. As biochemical defects may be expressed in cells other than the symptomatic tissue, much attention has been devoted to the study in DMD of other membrane systems, particularly that of the erythrocyte (reviewed by Roses *et al.*, 1980).

Abbreviations used: Ca^{2+}-ATPase, Ca^{2+}-stimulated Mg^{2+}-dependent ATPase; DMD, Duchenne muscular dystrophy; SDS, sodium dodecyl sulphate.

ATPase enzymes of the erythrocyte membrane have received considerable attention since Brown *et al.* (1967) reported a stimulation of Na^+- and K^+-stimulated Mg^{2+}-dependent ATPase (Na^+,K^+-ATPase) by ouabain in ghost preparations from patients with DMD. However, we (Dunn *et al.*, 1980*a*), using various methods for ghost preparation and enzyme assay, were unable to reproduce the spectacular abnormality described by Brown *et al.* (1967). Instead we found a decreased susceptibility of the enzyme to ouabain specifically in membrane preparations from patients with DMD.

The Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity (Ca^{2+}-ATPase) of DMD-patient erythrocyte membranes has received less attention, but several reports have indicated that this enzyme activity is increased in ghost preparations from patients with DMD (Hodson & Pleasure, 1977; Luthra *et al.*, 1979; Ruitenbeek, 1979). In the present paper we have reinvestigated the Ca^{2+}-ATPase activity, some of its kinetic parameters and its response to changes

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in temperature in erythrocyte membrane preparations from normal individuals, patients with DMD and carriers of DMD.

Materials and methods

Patients

Samples of blood were collected into heparin by venipuncture with informed consent from 21 patients with DMD (4–13 years), 18 carriers of DMD (17–42 years), six patients with a variety of other neuromuscular diseases (3–16 years) and 16 normal individuals (7–36 years). The diagnosis of the patients was assessed by clinical, histochemical, electron-microscopic, serum enzyme and electromyographic criteria (Dubowitz, 1978).

Preparation of erythrocyte ghosts

Ghost membranes were prepared at 4°C by a method similar to that of Fairbanks *et al.* (1971), but phosphate was replaced with an imidazole buffer to facilitate the ATPase assay. The blood specimen was centrifuged for 10min at 100**g**, the plasma and buffy coat were removed and the erythrocytes washed in 3 × 5 vol. of 150mM-NaCl/10mM-imidazole, pH 7.4. The cells were haemolysed in 10vol. of 10mM-imidazole, pH 7.4, centrifuged for 30min at 39000**g** and washed repeatedly in the same buffer until the pellet of erythrocyte ghosts was creamy white. In initial studies of the effect of Ca^{2+} concentration on Ca^{2+}-ATPase activity, blood samples were stored for 72h at 4°C before preparation of ghosts. In all subsequent studies the ghosts were prepared the same day as the blood was collected, stored at −40°C and assayed within 4 days.

Gel electrophoresis

Ghost preparations (50μg) were solubilized in SDS sample buffer [3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.125M-Tris/HCl (pH6.8), 0.001% Bromophenol Blue] and the samples heated at 100°C for 5min. Discontinuous SDS/polyacrylamide gradient gels were prepared by the method of Laemmli (1970), except that a 4–18% acrylamide gradient combined with a gradient of bisacrylamide (0.11–0.08%) was used. The stacking gel contained 3% acrylamide and 0.11% bisacrylamide. This modified gel composition was used as it was found to give improved separation of the high-molecular-weight components. The gels were run at 4mA/tube until the Bromophenol Blue reached the bottom of the gels. The gels were fixed in 20% trichloroacetic acid, stained in 0.2% Coomassie Blue R250 in destain solution [45% (v/v) methanol, 10% (v/v) acetic acid] and destained until a clear background was obtained. Stained gels were scanned at 560nm with a Gelman DCD16 scanner and the scans quantified by using a Reichert MOP digitizer linked to a Commodore microcomputer.

ATPase assay

Protein content of the erythrocyte ghost membrane preparations was measured by the method of Lowry *et al.* (1951), modified by the prior solubilization of the ghosts in 0.05% SDS. Release of P_i in the ATPase assay was assessed by the spectrophotometric measurement of the soluble complex of phosphomolybdic acid and the non-ionic detergent Cirrasol ALN-WF (Atkinson *et al.*, 1973). The addition of this mixture served to terminate the ATPase reaction. [The Cirrasol ALN-WF was generously given by Atlas Chemical Industries (U.K.) Ltd., Leatherhead, Surrey, U.K.]

Ca^{2+}-ATPase activities of erythrocyte plasma membranes were assayed by incubating ghosts (0.3–0.5mg of protein) for 30min at 37°C in a solution containing 3mM-ATP, 3mM-$MgCl_2$, 80mM-NaCl, 80mM-imidazole, pH 7.2. The total volume of the assay was 1ml. The enzyme reaction was linear for at least 75min under these conditions. EGTA was included in the incubation mixture at a concentration of 0.5mM. Various concentrations of $CaCl_2$ were added and the free Ca^{2+} concentration was calculated (Pershadsingh & McDonald, 1980). Ca^{2+}-independent basal Mg^{2+}-ATPase activity was measured in the absence of added $CaCl_2$, and Ca^{2+}-ATPase activity was determined as the difference between the ATPase activity measured in the presence of $CaCl_2$ and that measured in its absence. Vanadium-free ATP (Sigma Chemical Co., Poole, Dorset, U.K.) was used, as the presence of vanadium in certain ATP preparations can inhibit erythrocyte membrane Ca^{2+}-ATPase (Bond & Hudgins, 1978).

Response of Ca^{2+}-ATPase to changes in temperature

The enzyme assay was carried out as described above except that ghosts were incubated for either 60min at temperatures between 15°C and 25°C, or 30min at 30°C and 37°C.

Kinetic analysis

The Ca^{2+}-ATPase activities of erythrocyte-ghost membranes as a function of concentration of ATP were assayed by incubating ghosts (0.2–0.3mg of protein) for 60min at 25, 30 or 37°C in the assay mixture described above. A constant added $CaCl_2$ concentration of 0.55mM (free Ca^{2+} concentration 51.3μM) was used and the ATP and the Mg^{2+} concentrations were varied co-ordinately between 0.1 and 3mM. The kinetic parameters K_m and $V_{max.}$ for the reaction were calculated by a number of graphic methods both with and without fine adjustment of the provisional values (Wilkinson, 1961).

Results

Gel electrophoresis

A typical densitometric scan of erythrocyte membrane proteins separated on a polyacrylamide gradient gel is shown in Fig. 1. Densitometric scans of the gels were divided up into various regions for quantification (Fig. 1) and the percentage of the total protein associated with each region was calculated. When quantitative data for a series of normal, DMD-patient and DMD-carrier membrane profiles were compared, no significant differences were observed (Table 1).

Effect of Ca^{2+} concentration on Ca^{2+}-ATPase activity

Plasma-membrane Ca^{2+}-ATPase activities are known to be dependent on free Ca^{2+} concentration. In order to investigate the Ca^{2+}-sensitivity of the normal erythrocyte membrane enzyme, Ca^{2+}-ATPase was assayed in ghost preparations in the presence of various amounts of added $CaCl_2$. At very low free Ca^{2+} concentrations little Ca^{2+}-ATPase activity was apparent (Table 2), but as the Ca^{2+} concentration was increased substantial Ca^{2+}-ATPase activity was elicited. However, high free Ca^{2+} concentrations ($>100\,\mu$M) were inhibitory to enzyme activity. The enzyme therefore appeared to be maximally activated at free Ca^{2+} concentrations in the range 10–100 μM. Such behaviour is consistent with previous reports of the Ca^{2+}-sensitivity of human erythrocyte membrane Ca^{2+}-ATPase (Schatzmann & Rossi, 1971). It should be noted that these experiments were carried out on blood samples that had been stored at 4°C before preparation of the ghosts. Subsequent experiments using ghosts prepared the same day as the blood was collected have demonstrated a similar response to Ca^{2+} concentration, but the actual activities observed were substantially increased (0.74 μmol/h per mg of protein at 0.55 mM added Ca^{2+} for a normal ghost preparation).

Ca^{2+}-ATPase activity of erythrocyte membranes from DMD patients

When Ca^{2+}-ATPase activity of erythrocyte-ghost preparations from patients with DMD was investigated, it was found to follow the same pattern of activation by Ca^{2+} as the control membranes (Table 2), but the mean Ca^{2+}-ATPase activity that was elicited was substantially elevated when compared with controls. However, there was overlap between the range of activities detected in the normal and DMD populations at each Ca^{2+} concentration used, and this was due to a considerable variation in enzyme activity between preparations from different individuals. The reason for this individual variability

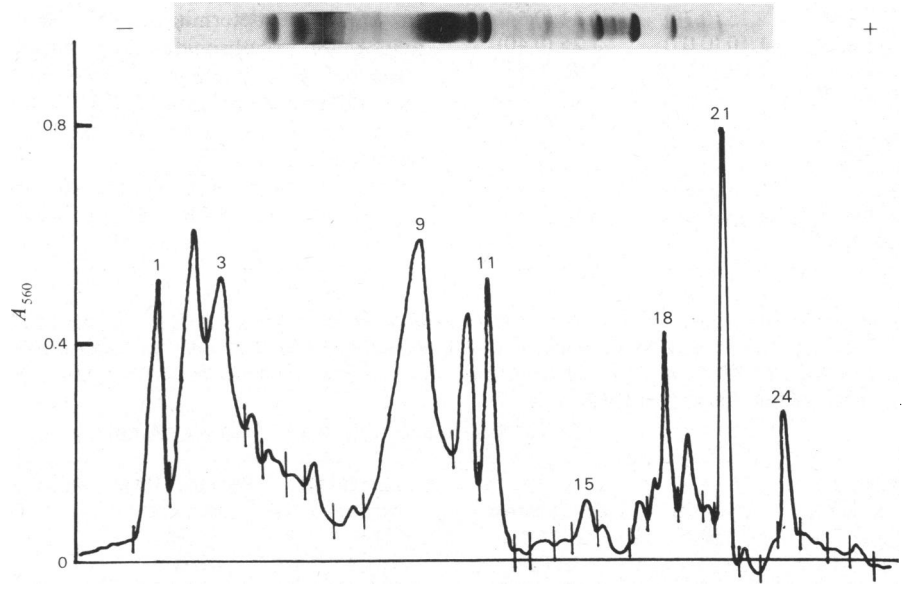

Fig. 1. *Densitometric scan of Coomassie Blue-stained gradient SDS/polyacrylamide gel of erythrocyte membrane proteins*

SDS/4–18%-polyacylamide gradient gels of erythrocyte membrane proteins stained with Coomassie Blue R250 were scanned at 560nm with a Gelman DCD16 scanner and each region was quantified by using a Reichert digitizer.

in Ca^{2+}-ATPase activity is not known, but similar behaviour has been noted by other workers (Hodson & Pleasure, 1977; Luthra *et al.*, 1979). Interestingly, the Ca^{2+}-ATPase activity of definite carriers of DMD also appeared to be elevated compared with controls, as did some preparations from possible carriers of DMD. Erythrocyte membrane preparations from patients with a variety of other neuromuscular diseases, however, exhibited similar Ca^{2+}-ATPase activities to those of controls.

Table 1. *Quantification of erythrocyte membrane proteins separated on SDS/4–18%-polyacrylamide stained with Coomassie Blue*

Gels were scanned at 560nm with a Gelman DCD16 scanner, and the proportion of each band shown in Fig. 1, expressed as a percentage of the total, was calculated. Values are means (±S.D. in parentheses) for *n* subjects.

	Relative intensity (%)		
Peak	Normal ($n = 6$)	DMD patients ($n = 2$)	DMD carriers ($n = 4$)
1	8.16 (3.24)	5.92 (1.34)	5.40 (1.02)
2	12.89 (1.56)	12.85 (2.58)	11.33 (0.21)
3	7.68 (1.93)	10.70 (0.25)	8.80 (1.99)
4	2.56 (0.69)	2.18 (0.10)	2.78 (0.26)
5	2.11 (0.47)	2.37 (0.56)	2.37 (0.69)
6	1.98 (0.21)	2.26 (0.08)	2.20 (0.18)
7	2.06 (0.44)	1.68 (0.53)	2.04 (0.36)
8	2.28 (0.61)	2.12 (0.63)	2.38 (0.55)
9	23.11 (2.21)	24.98 (0.88)	24.27 (1.85)
10	5.70 (0.96)	5.85 (0.78)	5.67 (0.93)
11	4.72 (1.54)	4.72 (0.08)	4.86 (0.52)
12	0.67 (0.20)	0.47 (0.01)	0.80 (0.28)
13	0.99 (0.17)	0.47 (0.12)	1.13 (0.12)
14	1.00 (0.18)	0.89 (0.57)	1.14 (0.30)
15	1.72 (0.14)	1.68 (0.78)	2.16 (0.06)
16	0.90 (0.27)	0.67 (0.47)	1.02 (0.30)
17	1.42 (0.36)	1.50 (0.41)	1.79 (0.21)
18	4.48 (0.51)	4.60 (0.23)	4.61 (1.18)
19	1.50 (0.38)	2.15 (0.79)	2.34 (0.64)
20	1.56 (0.50)	1.10 (0.07)	1.25 (0.40)
21	6.06 (1.26)	6.07 (0.82)	5.08 (0.62)
22	0.46 (0.27)	0.11 (0.13)	0.27 (0.05)
23	0.53 (0.30)	0.33 (0.10)	0.50 (0.12)
24	2.24 (1.25)	1.50 (0.12)	1.79 (0.40)
25	1.13 (0.62)	0.76 (0.30)	1.08 (0.12)
26	0.72 (0.21)	0.67 (0.19)	0.69 (0.15)
27	0.89 (0.54)	0.90 (0.38)	0.70 (0.21)

Response of Ca^{2+}-ATPase activity to changes in temperature

The response to changes in temperature over the range 15–37°C of the Ca^{2+}-ATPase activity of erythrocyte plasma membranes was examined. Progress curves of Ca^{2+}-ATPase activity were linear for at least 75 min under the assay conditions used at both 18 and 37°C. Arrhenius plots (Fig. 2) clearly revealed an elevated enzyme activity at all temperatures (15–37°C) in preparations from both DMD patients and DMD carriers. A discontinuity in the Arrhenius plot occurred at 20°C in all preparations examined. Above 20°C the activation energy for all types of preparation was 74.9kJ/mol (17.89kcal/mol). Below 20°C, however, there appeared to be an elevated activation energy in preparations from DMD patients (130kJ/mol; 31.05kcal/mol) and DMD carriers (133kJ/mol; 31.77kcal/mol) compared with normal controls (114kJ/mol; 27.23kcal/mol).

Kinetic studies of Ca^{2+}-ATPase

To characterize further the elevated Ca^{2+}-ATPase activity found in erythrocyte-ghost membranes from patients with DMD, some kinetic parameters of the enzyme were determined. The Ca^{2+}-ATPase activities of erythrocyte-ghost membranes were measured as a function of ATP concentration at three different temperatures (25, 30 and 37°C), and these data were used to calculate the kinetic parameters K_m and $V_{max.}$.

The double-reciprocal transformation (Lineweaver & Burk, 1934) of the Michaelis–Menten

Table 2. *Effect of free Ca^{2+} concentration on Ca^{2+}-ATPase activity in erythrocyte membrane preparations from normal individuals, patients with DMD, carriers of DMD and patients with other neuromuscular diseases*

For full details see the text. *$P < 0.05$ for differences from normals. Results are means ± S.E.M.; values in parentheses indicate the numbers of individuals per group.

		Ca^{2+}-ATPase activity (μmol of P_i/h per mg of protein)				
$CaCl_2$ added (mM)	Calculated free Ca^{2+} (M)	Normal (7)	DMD patients (14)	Definite DMD carriers (3)	Possible DMD carriers (7)	Other neuromuscular diseases (6)
0.2	8.74×10^{-8}	0.03	0.06	0.04	0.04	0.04
0.4	5.21×10^{-7}	0.22 ± 0.048	0.35 ± 0.043	0.22 ± 0.06	0.23 ± 0.05	0.17 ± 0.04
0.5	8.03×10^{-6}	0.29 ± 0.055	0.45 ± 0.064	0.31 ± 0.08	0.33 ± 0.03	0.24 ± 0.05
0.55	5.13×10^{-5}	0.29 ± 0.055	0.47 ± 0.055*	0.39 ± 0.05	0.34 ± 0.04	0.26 ± 0.06
0.6	1.10×10^{-4}	0.28 ± 0.059	0.45 ± 0.046*	0.38 ± 0.06	0.35 ± 0.03	0.27 ± 0.05
1.0	5.00×10^{-4}	0.25 ± 0.058	0.37 ± 0.036*	0.35 ± 0.03	0.33 ± 0.04	0.26 ± 0.06

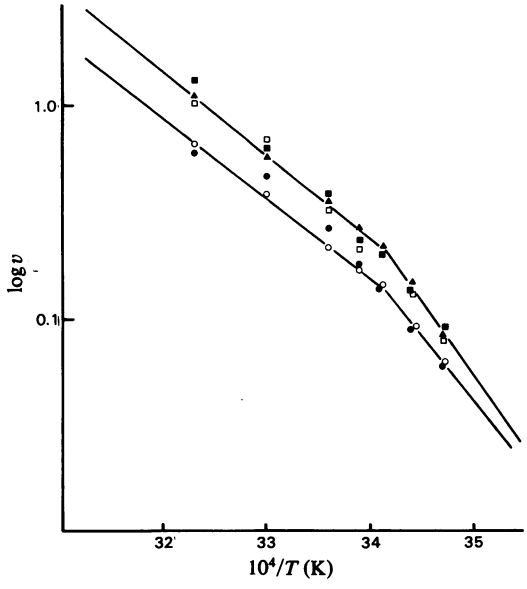

Fig. 2. *Response of Ca^{2+}-ATPase to changes in temperature*

Arrhenius plots of the response to temperature of Ca^{2+}-ATPase activities of erythrocyte ghosts from normal individuals (●; $n = 9$), patients with DMD (▲; $n = 12$), definite (■; $n = 8$) and possible (□; $n = 10$) carriers of DMD, and patients with other neuromuscular diseases (○; $n = 11$). The unit of v is μmol of P_i/h per mg of protein.

relation is the most widely used graphical method for the determination of K_m and $V_{max.}$. However, this method can be seriously criticized on the grounds that small errors in the determination of v are magnified when reciprocals are taken (Cornish-Bowden, 1979). The linear form discussed by Hofstee (1952) of v versus v/[S] suffers to a lesser extent from the same statistical disadvantage as the Lineweaver–Burk plot, with the added complication that both the variables are affected by experimental variability in v (Wilkinson, 1961). We have therefore chosen to analyse our experimental data using the Hanes–Woolf plot of [S]/v versus [S] (Hanes, 1932), as statistical analysis has shown this transformation to give the best estimates for kinetic parameters when there is variability in v (Dowd & Riggs, 1965).

We have used the least-squares method to fit our experimental data to the Hanes–Woolf transformation to yield preliminary estimates of $V_{max.}$ and K_m (Table 3). As the temperature was raised from 25°C to 37°C the values of both $V_{max.}$ and K_m increased in all types of preparation. However, at all temperatures the value of $V_{max.}$ was consistently

Table 3. *Kinetic parameters for erythrocyte membrane Ca^{2+}-ATPase as a function of ATP concentration*

Data were analysed by using the Hanes–Woolf plot (for details see the text). Units: $V_{max.}$, μmol/h per mg of protein; K_m, mM. 'Weighted' and 'refined' values are means (±S.E.M. in parentheses). Correlation coefficients are for the regression lines calculated from the experimental data. Significance of differences from normals: $^{*}P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.1$, $^{****}P < 0.2$. Numbers of subjects are shown in parentheses.

Temp. ...	25°C			30°C			37°C		
	Normal (8)	DMD patients (12)	DMD carriers (6)	Normal (6)	DMD patients (12)	DMD carriers (6)	Normal (8)	DMD patients (12)	DMD carriers (6)
Correlation coefficient	0.93 (±0.026)	0.92 (±0.05)	0.69 (±0.099)	0.96 (±0.038)	0.89 (±0.087)	0.72 (±0.245)	0.79 (±0.105)	0.93 (±0.097)	0.67 (±0.303)
$V_{max.}$	0.284	0.335	0.450	0.473	0.507	0.498	0.806	1.08	0.653
$V_{max.}$ (weighted)	0.347 (±0.019)	0.494* (±0.027)	0.673* (±0.063)	0.545 (±0.029)	0.807* (±0.044)	1.083** (±0.13)	1.100 (±0.077)	1.306*** (±0.065)	1.628*** (±0.181)
$V_{max.}$ (refined)	0.312 (±0.019)	0.363**** (±0.027)	0.530* (±0.063)	0.507 (±0.029)	0.571**** (±0.044)	0.694**** (±0.13)	0.912 (±0.077)	1.099**** (±0.065)	1.085**** (±0.181)
K_m	0.193	0.202	0.332	0.309	0.238	0.364	0.407	0.440	0.254
K_m (weighted)	0.172 (±0.043)	0.196 (±0.07)	0.322 (±0.142)	0.206 (±0.052)	0.223 (±0.08)	0.426 (±0.261)	0.402 (±0.105)	0.357 (±0.073)	0.480 (±0.229)
K_m (refined)	0.205 (±0.038)	0.193 (±0.051)	0.366 (±0.113)	0.272 (±0.037)	0.224 (±0.058)	0.375 (±0.22)	0.384 (±0.077)	0.384 (±0.058)	0.392 (±0.211)

elevated in DMD-patient preparations compared with normal controls. There appeared to be no consistent difference in the value of K_m for the three types of preparation. Examples of Hanes–Woolf plots for the data obtained at 37°C are shown in Fig. 3. This plot confirms the similarity in the value of K_m between control, DMD-patient and DMD-carrier preparations, and indicates an elevation in the value of $V_{max.}$ for the DMD-patient and DMD-carrier preparations compared with the control. It should be noted that the experimental points conform well to the regression lines and show no evidence of kinetic co-operativity.

However, such a series of experimental observations may not be homogeneous in variance, and it is therefore necessary to allow for the differing accuracies in fitting the regression function. The appropriate method is to fit the function so that the weighted sum of squares of deviations is a minimum, the relative weights being inversely proportional to the variances of the v values. We have carried out such a weighted analysis as described by Wilkinson (1961) in which the variance of the velocities is assumed to be constant, and therefore applying weights of $v^4/[S]^2$. The weighted values of $V_{max.}$ and K_m together with their standard errors calculated as described by Wilkinson (1961) are shown in Table 3. The values for $V_{max.}$ and K_m for the different preparations were compared by using a t test as described by Cleland (1967, 1979). The value of $V_{max.}$ is significantly elevated in both DMD-patient and DMD-carrier preparations at all temperatures tested. Although there appeared to be variation in the values of K_m in the different preparations, these changes were not, however, statistically significant.

We have also performed fine adjustment of the provisional estimates of $V_{max.}$ and K_m by a process based on fitting a bilinear regression of v on the corresponding values of the provisionally fitted Michaelis–Menten function and its first derivative, as described by Wilkinson (1961). This analysis (Table 3) confirms the significantly elevated $V_{max.}$ value in both DMD-patient and DMD-carrier preparations at the three temperatures employed. Again there was no significant difference in K_m values between DMD-patient and DMD-carrier preparations compared with normal controls.

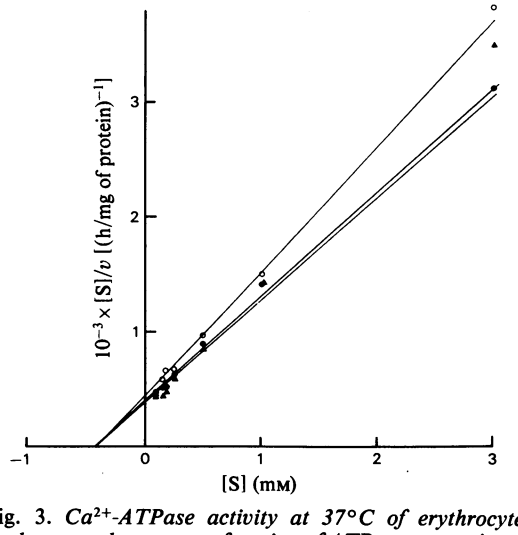

Fig. 3. *Ca^{2+}-ATPase activity at 37°C of erythrocyte-ghost membranes as a function of ATP concentration*
The data are plotted as a Hanes–Woolf plot of $[S]/v$ against [S] (for details see the text). The lines are the refined regression lines and the points are the mean values of the experimental data. The intercept on the ordinate is equal to $-K_m$ and the intercept on the abscissa is equivalent to $K_m/V_{max.}$. O, Normal individuals; ●, patients with DMD; ▲, carriers of DMD.

Discussion

The ATPase activities of the erythrocyte membrane have been the subject of intense investigation since Brown *et al.* (1967) reported an abnormal response to ouabain of Na^+,K^+-ATPase in ghost preparations from patients with DMD. In the present paper we have investigated the properties of the erythrocyte membrane Ca^{2+}-ATPase and have unequivocally demonstrated a marked increase in Ca^{2+}-ATPase activity in membrane preparations from both DMD patients and DMD carriers.

Kinetic studies of erythrocyte membrane Ca^{2+}-ATPase can be complicated by the appearance of a form of the enzyme with low affinity for Ca^{2+} when certain procedures are used for the isolation of ghost membranes. It has been proposed that there is a reversible shift between low- and high-affinity states of Ca^{2+}-ATPase induced by the Ca^{2+}-dependent binding of the low-molecular-weight cytoplasmic activator protein, calmodulin, to the erythrocyte membrane (Sarkadi, 1980). The method of ghost preparation in the absence of chelating agents that we have adopted, however, is known to yield only a high-affinity Ca^{2+}-ATPase activity (Schatzmann, 1973). Our results on the activation of ATPase by Ca^{2+} have not revealed any evidence for a dual nature of the enzyme, although we have not carried out a thorough kinetic analysis of these data. Moreover, the Ca^{2+}-ATPase activity of ghost membranes from patients with DMD was found to follow the same pattern of activation by Ca^{2+} as the control membranes (Table 2), despite the elevation of Ca^{2+}-ATPase activity observed in DMD-patient preparations. An increase in the specific activity of erythrocyte membrane Ca^{2+}-ATPase, such as we

have observed, could be due to an alteration in the relative amounts of other membrane proteins in preparations from DMD patients. However, this possibility can be excluded, as we have demonstrated no significant quantitative differences in the polypeptide profiles of normal, DMD-patient and DMD-carrier membranes (Table 1).

An elevation in Ca^{2+}-ATPase activity in erythrocyte membranes from DMD patients has been observed by other workers (Hodson & Pleasure, 1977; Luthra *et al.*, 1979; Ruitenbeek, 1979), but studies on the kinetic basis for this difference have produced conflicting results. A greater affinity of the DMD-patient enzyme for its substrate (ATP) has been claimed on the basis of a decrease in K_m (Hodson & Pleasure, 1977). In contrast, Ruitenbeek (1979) reported that it was the $V_{max.}$ for the reaction that was elevated in DMD-patient preparations, while the K_m remained unchanged. However, the kinetic data in both of these studies were analysed by using simple graphical methods, which suffer from the disadvantage that no estimate of the reliability of the kinetic constants can be derived (Cleland, 1967, 1979). We have therefore reinvestigated the kinetic parameters of Ca^{2+}-ATPase of DMD-patient erythrocyte-ghost preparations using plots of $[S]/v$ against $[S]$ (Hanes, 1932) in conjunction with a procedure for fine adjustment of the preliminary estimates and a weighted sum-of-squares method to improve our estimates of the kinetic parameters (Wilkinson, 1961). These latter two methods have the advantage that the significance of differences in the kinetic parameters can be tested (Cleland, 1967, 1979). Analysis of the experimental data in this way clearly demonstrated that in both DMD-patient and DMD-carrier preparations the increase in Ca^{2+}-ATPase activity was reflected by a significant increase in $V_{max.}$ rather than any change in K_m. It has been reported (Richards *et al.*, 1978; Muallem & Karlish, 1980) that erythrocyte membrane Ca^{2+}-ATPase exhibits a biphasic activation by ATP, resulting in two K_m values (1–3 μM and 200–300 μM respectively). This suggests that the enzyme has two ATP-binding sites with different affinities. The range of ATP concentrations that we have used should result in the occupation of both binding sites by ATP, which is compatible with the high K_m values that we have observed. It should be noted that, although Hodson & Pleasure (1977) reported possible negative co-operativity for Mg–ATP, our more sophisticated kinetic analysis has failed to provide any evidence for either negative or positive co-operativity in either control or DMD-patient preparations, although differences in methods of ghost preparation and the absence of EGTA from their kinetic assay medium could be responsible for this difference.

The low-molecular-weight activator protein, calmodulin (for review see Klee *et al.*, 1980), present in the cytoplasm of erythrocytes, is known to increase both the activity of the Ca^{2+}-ATPase (Bond & Clough, 1973; Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977) and its affinity for Ca^{2+} (Scharff & Foder, 1978; Roufogalis, 1979). It is therefore possible that the elevated Ca^{2+}-ATPase activity that we have observed in ghost preparations from patients with DMD could be related to either changes in the response of the enzyme to calmodulin or alterations in the properties of calmodulin itself. As the method of ghost preparation that we have used will not yield calmodulin-free ghosts (Sarkadi, 1980), the elevated Ca^{2+}-ATPase activity of ghost membranes from DMD patients could be due to differential retention of calmodulin in such preparations. Luthra *et al.* (1979) have examined the effects of calmodulin on membrane Ca^{2+}-ATPase in normal and DMD-patient erythrocytes. The addition of saturating amounts of haemolysate was found to stimulate activities to the same extent in both preparations. Moreover, the difference in Ca^{2+}-ATPase activity between normal and DMD-patient membrane preparations remained as great under these conditions. These results were the same regardless of whether the haemolysate was derived from normal or DMD-patient erythrocytes. In addition, the presence of calmodulin would be expected to modify the response of the enzyme to Ca^{2+} (Luthra & Kim, 1980), but we have found this response to be the same in normal and DMD-patient preparations (Table 2). These results suggest that the difference observed in Ca^{2+}-ATPase activity of patients with DMD is unlikely to be due to differential retention of calmodulin by normal and DMD-patient ghosts or to alterations in the activator protein itself.

Ca^{2+}-ATPase can be purified from erythrocyte membranes with a calmodulin affinity column, and the purified enzyme has been reconstituted into liposome systems (Niggli *et al.*, 1981). When neutral phospholipids were used the enzyme still responded to calmodulin, but if acidic phospholipids (e.g. phosphatidylserine) were used the enzyme was fully activated and could not be further stimulated by the addition of the activator. The intact erythrocyte membrane contains about 12% phosphatidylserine, which is insufficient to activate Ca^{2+}-ATPase activity maximally. Although changes have been reported to occur in the lipid composition of DMD-patient erythrocyte membranes (Kunze *et al.*, 1973; Howland & Iyer, 1977; Kalafoutis *et al.*, 1977), there is no evidence for an increase in the total content of acidic phospholipids. More subtle changes in the physicochemical properties of the lipid domain in membranes from DMD-patient erythrocytes could, however, result in the exposure of Ca^{2+}-ATPase integral membrane protein to a

modified lipid environment, resulting in an elevation of enzyme activity. Indeed, results from our laboratory (Dunn *et al.*, 1980*b*) have indicated a decreased membrane fluidity in erythrocytes from patients with DMD, as measured by the fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene.

Changes in the membrane lipid domain are known to be able to modify the response of membrane-associated enzymes to changes in temperature (Kumamoto *et al.*, 1971; Kimelberg, 1975; De Kruyff *et al.*, 1973). We have therefore investigated the response of erythrocyte membrane Ca^{2+}-ATPase activity to temperature over the range 15–37°C. In all preparations a break in the Arrhenius plot (Fig. 2) occurred at 20°C, and in DMD-patient and DMD-carrier preparations an elevated Ca^{2+}-ATPase activity was detected at all temperatures. Above 20°C the activation energy for all types of preparation was the same, whereas below this temperature there appeared to be an elevated activation energy in DMD-patient and DMD-carrier preparations compared with normal controls. It should, however, be noted that the Arrhenius plots (Fig. 2) are based on observations made at a limited number of temperatures. This was due to the small samples of material available from children with neuromuscular diseases. In addition it is known that variations in substrate-binding affinity with changes in temperature can strongly influence the behaviour of Arrhenius plots (Silvius *et al.*, 1978). Arrhenius plots of $V_{max.}$ and K_m values obtained from our kinetic analysis (Table 3) are linear above the break point, yielding a value for E_a (activation energy) of 68.2 kJ/mol (16.3 kcal/mol), in good agreement with the value derived from the plot of our experimental data (Fig. 2), and a 'ΔH' value of −39.8 kJ/mol (−9.5 kcal/mol). Reliable measurement of kinetic constants below the break point was not possible, owing to the limited amount of each ghost sample combined with the low Ca^{2+}-ATPase activities observed at low temperatures. If large changes in K_m occur with temperature, then anomalous breaks in Arrhenius plots can be observed, owing to the measured velocity (v_0) being an underestimate of $V_{max.}$. Our data, however, suggest that K_m does not change markedly with temperature. In addition the progress curve for the enzyme was linear for at least 75 min at 18°C, implying that excess substrate was present at this temperature and that the measured v_0 reflected true $V_{max.}$.

Several explanations can be put forward to explain discontinuities in Arrhenius plots (Dixon & Webb, 1979; Raison, 1973). The Ca^{2+}-ATPase reaction is thought to involve more than one step subsequent to the formation of the enzyme–substrate complex (Sarkadi, 1980), and it is possible that these reactions have different temperature coefficients. Thus a discontinuity in Arrhenius plots can arise owing to a change in the rate-limiting step. However, Arrhenius-plot analyses of the fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene (Dunn *et al.*, 1980*b*) and of Na^+,K^+-ATPase activity (Dunn *et al.*, 1980*a*) in normal and DMD-patient erythrocyte membranes have also revealed discontinuities at the same temperature. This suggests that the break that we have observed in the Arrhenius plot of membrane Ca^{2+}-ATPase is more likely to be due to a phase change in the membrane lipid (Raison, 1973). Therefore, although our preliminary Arrhenius plots should be interpreted with caution, they nevertheless suggest the possibility of an altered lipid domain in DMD-patient erythrocyte membranes. To elucidate this point further it will be necessary to purify Ca^{2+}-ATPase from normal and DMD-patient erythrocyte ghosts and investigate its properties by using reconstituted liposome systems.

Increased intracellular Ca^{2+} has been proposed to be involved in the pathogenesis of DMD (Duncan, 1979). It is therefore possible that an increased activity of membrane Ca^{2+}-ATPase may compensate for the changes in Ca^{2+} metabolism occurring in DMD-patient erythrocytes. This elevation of Ca^{2+}-ATPase activity is associated with an increased rate of Ca^{2+} transport in erythrocytes from patients with DMD (Mollman *et al.*, 1980). However, although Ca^{2+} accumulation has been reported in muscle fibres from DMD patients (Bodensteiner & Engel, 1978), erythrocyte Ca^{2+} concentrations have been reported to be normal (Dise *et al.*, 1977).

Our results therefore support the concept of an alteration in membrane properties in DMD, and we propose that this may be due to a generalized alteration in the physicochemical nature of the membrane lipid domain. However, the abnormalities that we have described do not unequivocally indicate the presence of a primary defect in the erythrocyte membrane itself, as such abnormalities could be due to factors circulating in the serum of patients with this disease (Peter *et al.*, 1969; Siddiqui & Pennington, 1977). The biochemical basis for changes in membrane properties in DMD therefore awaits clarification.

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