Biochimica et Biophysica Acta, 660 (1981) 227–237
Elsevier/North-Holland Biomedical Press

BBA 69314

AN ABERRANT ADENYLATE KINASE ISOENZYME FROM THE SERUM OF PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY

MINORU HAMADA [a,*], HIROMICHI OKUDA [b], KEIZO OKA [c], TSUTOMU WATANABE [a], KEISHI UEDA [d], MOTOO NOJIMA [e], STEPHEN A KUBY [f–h,*] MICHAEL MANSHIP [f,g], FRANK H TYLER [f,h] and FRED A ZITER [i]

Departments of [a] *Hygiene and* [b] *Biochemistry,* [c] *Central Laboratory Analysis Division,* [d] *Health Administration Center, and* [e] *Orthopedics, Ehime University School of Medicine, Shigenobu, Onsen gun, Ehime 791-02 (Japan)* [f] *Laboratory for Study of Hereditary and Metabolic Disorders, and the Departments of* [g] *Biochemistry,* [h] *Medicine and* [i] *Neurology, University of Utah College of Medicine, Salt Lake City, UT 84132 (U S A)*

(Received November 20th, 1980)
(Revised manuscript received March 26th, 1981)

Key words Adenylate kinase, Aberrant isoenzyme, Muscular dystrophy, (Human serum)

The sera from patients with human Duchenne (X-linked) progressive muscular dystrophy contain elevated adenylate kinase (ATP AMP phosphotransferase, EC 2 7 4 3) activities, in addition to their characteristically high creatine kinase (ATP creatine *N*-phosphotransferase, EC 2 7 3 2) activities By agarose gel electrophoresis of human Duchenne dystrophic serum, the presence of an apparently normal human serum adenylate kinase together with a variant species of adenylate kinase was detected The latter enzyme species appeared, in its mobility, to be similar to that of the normal human liver-type adenylate kinase The presence of this aberrant liver-type adenylate kinase could also be demonstrated by characteristic (for the liver type) inhibition patterns with P^1,P^5-di-(adenosine-5′)pentaphosphate, 5,5′-dithiobis(2-nitrobenzoate) and phospho*enol*pyruvate On the other hand, by inhibition titrations with an anti-muscle-type adenylate kinase, hemolysates from the erythrocytes of several Duchenne and Becker's dystrophics were found to contain approx 96% muscle-type adenylate kinase and their serum approx 97% muscle-type adenylate kinase These same patients contained approx 89% M-M type creatine kinase in their serum (by inhibition against anti-human muscle-type creatine kinase) indicative of the presence also of M-B plus B-B type active isoenzymes All of these data can best be explained by the presence of a variant or mutant adenylate kinase isoenzyme in the dystrophic serum This isoenzyme appears to resemble the liver type in its inhibition patterns with P^1,P^5-di(adenosine-5′)pentaphosphate, 5,5′dithiobis(2-nitrobenzoate) and phospho*enol*pyruvate, and in its heat stability (compare also the agarose gel electrophoresis pattern), but structurally, it is a muscle type, or derived from a muscle type, as shown immunologically by inhibition reactions with anti-muscle-type adenylate kinase Whether this is a fetal-type isoenzyme of adenylate kinase will require further investigation

Introduction

By direct isolation studies, a remarkable observation was made on the tissues from terminal male patients with dystrophy (X-linked, Duchenne) [1] In the atrophied dystrophic muscle tissue, three isoenzymes of creatine kinase were found the muscle, hybrid and brain types The isoenzyme pattern was remarkably similar to that obtained from human fetal muscle On the other hand, in the normal male adult, only one isoenzyme appears to be present in the skeletal muscle (the muscle type) and only one isoenzyme (the brain type) in the brain Kuby et al [2] recently reported that the

* To whom reprint requests should be sent
Abbreviations Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate, DTNB, 5,5′-dithiobis(2-nitrobenzoate)

0005-2744/81/0000–0000/$02 50

polypeptide chain of the dystrophic brain-type creatine kinase contained small but significant differences in its primary structure as compared to that of the normal brain-type isoenzyme, this was in contrast to the normal and dystrophic muscle types which appeared to be very similar physically, chemically and kinetically [3]

The suggestion was made [1] that X-linked progressive muscular dystrophy (Duchenne) might be an inborn error in the final development of the muscle tissue in the human male, such that it lacks the ability to switch off the synthesis of fetal-like proteins (e g , the brain-type creatine kinase) This, in turn, might lead to a fetal-like complement of proteins in the skeletal muscle, in the membrane of the muscle, or even within the membrane of, for example, the red cell

Our preliminary studies, coupled with those reported (e g , Refs 4–10), had led us to the hypothesis [11] that, at least in mammals, there may be two major forms of isoenzymes of adenylate kinase the cytoplasmic type, present largely in the skeletal muscle, and the mitochondrial type as represented by the liver This idea had prompted us to isolate in crystalline form the muscle type and the liver type from the same organism, viz , the calf [11] These two isoenzymes differed, for example, in their molecular weights, in their isoelectric points, in their inhibition by Ap_5A (with their K_i values almost 2 orders of magnitude larger for the liver type), and most significantly, in their immunological behavior towards a rabbit anti-muscle-type globulin [11] Thus, the anti-enzyme is unreactive as an inhibitor towards the calf liver-type enzyme, but is a powerful inhibitor of the calf muscle type (or the human muscle type) In addition, the liver type showed a unique inhibition by phospho*enol*pyruvate and several other distinguishing steady-state kinetic features from the calf muscle isoenzyme [12]

Recently, the muscle-type adenylate kinase and liver-type adenylate kinase have also been purified from the rat [13] and a study made of the ontogenesis of these two rat adenylate kinase isoenzymes [14] Finally, mention should be made of the genetically determined polymorphism described in human erythrocytes [15–24], one species of which, designated adenylate kinase$^1_1$, was sequenced [25] Schirmer and Thuma [26] postulated, on the basis of the SH-group reactivity and inhibition of the muscle-type adenylate kinase by DTNB, and the poor reactivity with the liver type by DTNB (cf Ref 8), that a liver-type isoenzyme of adenylate kinase was also present in dystrophic muscle in contrast to the normal human muscle which contained only muscle type

Within this report, we have confirmed the observations of Schirmer and Thuma [26], but are led to the conclusion that in the serum of patients with Duchenne dystrophy there is a muscle-type adenylate kinase, the DTNB-reactive groups of which have been altered to resemble those of the liver type, or in effect that an aberrant adenylate kinase has resulted, possibly due to a mutational event or events

Materials and Methods

Preparation of dystrophic sera and hemolysates and sources of various normal human tissues

For the anti-enzyme assays (see below) of the dystrophic serum and erythrocytes, samples of blood from patients with Duchenne dystrophy and Becker's dystrophy were obtained through the University of Utah Muscle Clinic The blood samples were collected in chilled silicone-treated tubes without anticoagulant and rapidly centrifuged at 3°C After washing the blood cells three times with 0 2 (Γ/2), Tris-NaCl buffer (50 mM Tris, 37 mM HCl, 163 mM NaCl, pH 7 4) containing 1 mM dithioerythritol and 1 mM EDTA, they were hemolyzed in 3–5 vol of 1 mM EDTA, pH 7 4, at 0°C The plasma was allowed to stand in an ice bath for several hours until coagulated and was then recentrifuged at 3°C to remove the fibrin clot

Aliquots of either serum or hemolysates were adjusted to 2 mM dithioerythritol by the addition of 0 1 vol of freshly prepared 22 mM dithioerythritol in 22 mM EDTA (pH 7 4) and allowed to stand in an ice bath for at least 3 h for adenylate kinase activity assays and 12 h for creatine kinase assays Total creatine kinase and adenylate kinase assays in the serum, and total adenylate kinase assays in the hemolysates, as well as anti-enzyme assays for the human muscle-type creatine kinase (M-M type) and for the muscle-type adenylate kinase in the serum, and muscle-type adenylate kinase activity in the hemolysates were conducted as described below Protein of

the sera was determined by the biuret procedure [27] and hemoglobin by the cyanomethemoglobin procedure as described in Ref 28

For the agarose gel electrophoresis studies and the inhibition tests (see below), heparinized blood was obtained from those patients with muscular dystrophy under clinical investigation at the Ehime University Muscle Clinic and Prefecture Serum adenylate kinase activitity was measured after reduction for at least 60 min at 0°C with 4 mM dithioerythritol (0 1 mM EDTA), pH 7 4 Normal human blood was obtained from the blood bank (outdated samples) and normal skeletal muscle and liver tissues were obtained through Ehime University Departments of Pathology at autopsy within a few hours after death

Other materials

Tris, dithioerythritol, NADP, NADH, the sodium salts of 5'-AMP, ADP and ATP, the lithium salt of Ap_5A, hexokinase, lactate dehydrogenase, pyruvate kinase and glucose-6-phosphate dehydrogenase were obtained from Sigma and crystalline bovine serum albumin from Armour (Reheis Chemical Co) All other reagents were the best available or analytical grade Twice-distilled deionized water, degassed (by boiling), was used for the preparation of all reagents

Methods

Measurements of enzymatic activities Spectrophotometric (coupled-enzyme assays [29]) procedures were employed for the assays of either total creatine kinase or total adenylate kinase activity For adenylate kinase, the initial velocity of the reverse direction [12] was measured with the following final concentrations in a 3 0 ml reaction mixture [11] 2 1 mM ADP, 2 1 mM $Mg(OAc)_2$, 6 7 mM glucose, 0 67 mM NADP, 4 U of hexokinase, 2 U of glucose-6-phosphate dehydrogenase, 50 mM Tris (OAc^-), pH 8 1, 30°C Dilutions of the enzyme, where necessary, were made in ice-cold 1 mM dithioerythritol, 1 mM EDTA, pH 7 4, with 1 mg/ml albumin In some cases, the forward direction [12] initial velocity was measured with the pyruvate kinase and lactate dehydrogenase coupled-enzyme assay for ADP, employing the reaction mixture as given in Ref 12

For creatine kinase, the coupled-enzyme assay for the reverse direction was followed as described by Okabe et al [30], with the 3 0 ml reaction mixture described by Kuby et al [1] 2 1 mM ADP, 4 3 mM $Mg(OAc)_2$, 3 3 mM creatine phosphate, 6 7 mM glucose, 0 67 mM NADP, 2 U of glucose-6-phosphate dehydrogenase, 4 U of hexokinase, 50 mM Tris (OAc^-), pH 7 4, 30°C After a preliminary temperature equilibration of the reaction mixture (minus creatine phosphate), during which time the trace ATP contamination (found in commercial samples of ADP) was converted to ADP, the enzyme-containing sample (e g , from serum) was added and any adenylate kinase activity in the sample measured, this was followed by the addition of creatine phosphate (usually in a 100 μl aliquot) to determine the apparent creatine kinase activity After subtraction of the initial adenylate kinase activity, the true creatine kinase activity was obtained For dystrophic serum samples, this technique was found to be superior to the conventional method of including approx 1 mM AMP to inhibit adenylate kinase activity, since invariably the addition of the AMP resulted in inhibition of both the serum creatine kinase activity and the serum adenylate kinase activity

Anti-enzyme assays

Erythrocyte adenylate kinase Aliquots (5–15 μl) of the hemolysate (reduced with 2 0 mM dithioerythritol, see above) containing 0 1–0 2 U of activity were added to two 10 × 75 mm tubes containing 1 100 μl (total volume) of 0 20 (Γ/2) Tris-NaCl, buffer, pH 7 4 (see above), 1 mg/ml bovine serum albumin, 2 mM dithioerythritol, 2 mM EDTA, and in one tube, 2 5 anti-enzyme U of purified rabbit anti-calf muscle adenylate kinase were present (see Ref 11), the second tube (the control) was without anti-enzyme activity After a minimum of 3 h at 25°C, an aliquot (100–200 μl) from each tube was added to each of two adenylate kinase assay cuvettes, and the percent inhibition of the erythrocyte adenylate kinase by the anti-muscle type was calculated from the relative activities to yield the percent muscle-type adenylate kinase

Serum adenylate kinase Since the rate of reaction of the anti-muscle-type adenylate kinase with the muscle-type adenylate kinase was exceedingly rapid (see Ref 11), the inhibition of the muscle type in the serum could be determined directly, without a prein-

cubation period, in the assay cuvette After an initial velocity was established with approx 50 mU of activity at 30°C, 2 5 anti-enzyme (in approx 15 μl) were added rapidly, and the decrease in velocity measured after several minutes of reaction to establish a new steady state The percent inhibition could then readily be calculated from the two slopes and was related to the percentage of the muscle type in the serum

Serum creatine kinase The procedure was identical to that employed for the erythrocyte adenylate kinase in two 10×75 mm tubes, an aliquot of reduced serum (reduced with 2 mM dithioerythritol, 2 mM EDTA, pH 7 4, for 12–24 h), containing approx 0 1 U, was added to 0 2 (Γ/2) Tris (OAc^-)-NaCl buffer, pH 7 4, with 1 mg/ml albumin and 2 mM dithioerythritol, 2 mM EDTA To one tube, purified rabbit anti-human muscle-type (M-M type) creatine kinase (2 4 anti-enzyme U) was added, with the second tube acting as a control After 18 h at 25°C, aliquots were removed from both tubes for determination of creatine kinase activity (see above) and the percent inhibition calculated, which in turn was related to the presence of M-M (plus a fraction of M-B) type present in the serum, but not to B-B type

Agarose gel electrophoresis Electrophoresis of the adenylate kinase isoenzymes was conducted on 1% (w/v) agarose gel film (Corning, containing 5% (w/v) sucrose, 0 035% (w/v) EDTA and 65 mM barbital buffer, pH 8 6) in a Corning cassette electrophoresis cell at 90 V for 45 min, at room temperature The electrophoresis buffer employed was Corning's 50 mM barbital, pH 8 6, containing 0 035% EDTA, and the coupled-enzyme dye stain for ATP, as described by Shaw and Koen [31], was used to visualize the bands of adenylate kinase activity

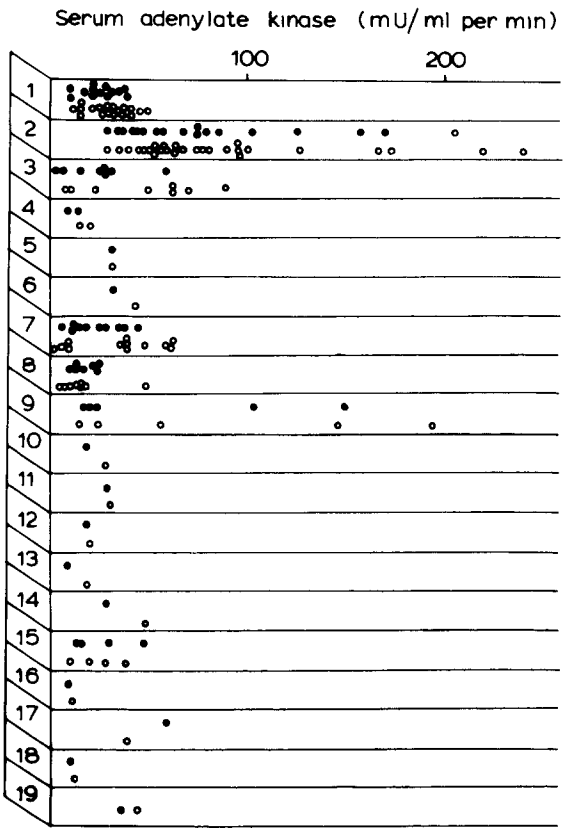

Fig 1 The distribution of adenylate kinase activity in individual human sera Open circles indicate the activity (mU/ml) of the forward direction (ν_0^f), solid circles show the activity of the reverse direction (ν_0^r) (Two high-activity cases in the Kugelberg-Welander disease were assayed in hemolyzed serum) In Table I are listed the mean values, standard deviations, and the number of cases measured for each disease (and normal) given here Rows 1, normal, 2, Duchenne type, 3, L-G type, 4, FSH type, 5, congenital type, 6, myotonic type, 7, spinal muscle type, 8, Charcot-Marie tooth disease, 9, Kugelberg-Welander disease 10, amyotrophic lateral sclerosis, 11, cervical spondylosis, 12, rheumatoid arthritis, 13, dermatomyositis, 14, cerebral palsy, 15, quadriceps contracture, 16, Perthe's disease, 17, Rousey-Levy disease, 18, generalized hypotony, 14, liver cirrhosis

Results and Discussion

Human serum adenylate kinase activity in normal and diseased states

In Fig 1 and Table I, the results obtained on the measured total adenylate kinase activity in the sera of normal human individuals are compared with those obtained on the sera from a variety of patients with several types of dystrophy, including the Duchenne type of muscular dystrophy, the facio-scapulohumeral (FSH) type and spinal muscular dystrophy In the case of the Duchenne muscular dystrophic patients (2–27-year-old males), the control blood measured was age matched to those of the patients, and the total serum adenylate kinase activity in both directions was measured at least 60 min after reduction at 0°C with 4 mM dithioerythritol (0 1 mM

TABLE I

A COMPARISON OF THE SERUM ADENYLATE KINASE ACTIVITY IN NORMAL AND DISEASED STATES

v_0^r, reverse reaction, v_0^f, forward reaction Activities are expressed in mU/ml per min, and are given as the mean ± S D

	Number of cases	v_0^r	v_0^f
Normal	18	27 54 ± 9 20	28 47 ± 13 95
Type of muscular dystrophy			
Duchenne	24	73 10 ± 37 14	91 77 ± 59 40 [a]
Limb-girdle	8	45 65 ± 22 52	42 28 ± 23 68
Fasciocapulohumeral	2	33 44 ± 3 64	44 16 ± 4 25
Congenital	1	35 37	27 65
Myotonic	1	36 01	47 59
Spinal	12	42 57 ± 15 45	42 23 ± 15 60
Charcot-Marie tooth disease	6	18 78 ± 5 38	15 83 ± 6 22
Kugelberg-Welander disease	3	22 15 ± 18 59	27 97 ± 25 16
Amyotrophic lateral sclerosis	1	25 69	26 94
Cervical spondylosis	1	14 79	11 58
Rheumatoid arthritis	1	12 22	10 29
Dermatomyositis	1	13 51	23 66
Cerebral palsy	1	29 74	21 95
Quadriceps contracture	2	33 92 ± 15 23	13 86 ± 4 30
Perthe's disease	1	25 69	21 17
Rousey-Levy disease	2	40 62 ± 7 43	34 73 ± 10 01
Generalized hypotony	1	14 79	16 40
Liver cirrhosis	1	51 41	73 95

[a] The activities in the Duchenne type were confirmed to be significantly increased by Student's t-test ($P < 0\ 005$)

EDTA), pH 7 5, to achieve full activity The total activity was very significantly enhanced in the Duchenne muscular dystrophic sera, whereas, in the neuronal type of dystrophy such an increase was not observed (see Fig 1 and Table I) Furthermore, in the FSH type, limb-girdle (L-G) type and spinal muscular dystrophy, this enhancement was not observed Thus, an enhanced total activity of adenylate kinase appears to be specific to Duchenne muscular dystrophic patients (compare also patients with a variety of other diseases, including rheumatoid arthritis, liver cirrhosis, cerebral palsy), and moreover, this increase in the serum adenylate kinase activity of Duchenne muscular dystrophics correlated very well with their dramatic increase in total creatine kinase activity (over the controls)

Electrophoresis on thin films of agarose gel of extracts from human tissues

As shown in Fig 2A and B, in the case of the normal human tissue extracts, electrophoretically distinguishable patterns of active enzyme species of adenylate kinase seem to be characteristic of the serum, muscle, liver and the erythrocyte, and this observation of tissue-specific patterns is in agreement with those previously reported (e g , Refs 9 and 10)

* The mean values of v_0^r and v_0^f listed in Table I in the case of the Duchenne dystrophic group of data included the entire set of 24 values measured These values ranged over the entire age span and course of the disease of these dystrophics At a relatively early age (e g , approx 10 years old) the highest activities of adenylate kinase were observed and these values then decreased with age (until approx 20 years old) The minimum and maximum values observed for 24 Duchenne dystrophics were v_0^r= 28 80 – 193 10, v_0^f= 29 58 – 249 40, and those for the set of 16 normal males were v_0^r= 14 79 – 45 66, v_0^f= 8 040 – 46 62 Such a range of values was not observed in other cases, except in the hemolyzed serum from the Kugelberg-Welander disease The mean values for the dystrophics is therefore a calculated value which tends to underestimate the relatively large differences observed between the Duchenne dystrophics and age-matched normal males Nevertheless, application of the Student's t-test (with $P < 0\ 005$) shows these mean values of v_0^r or v_0^f for Duchenne dystrophics to be very significantly higher than those for the normals

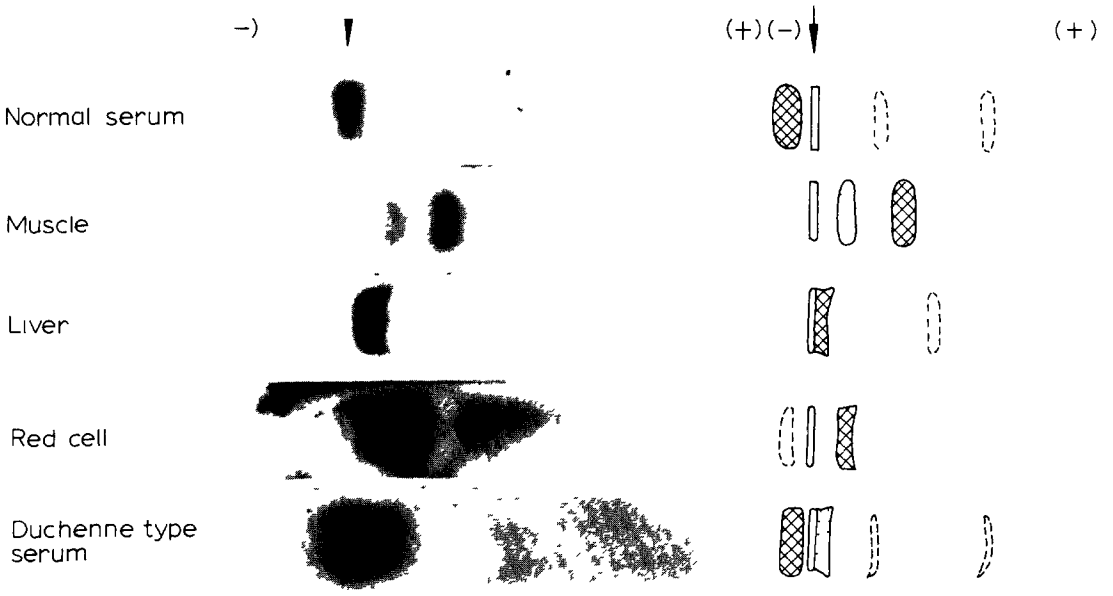

Fig 2 Thin-film agarose (1%) gel electrophoretic patterns of adenylate kinase activities from human tissues The gel buffer was 65 mM barbiturate-NaOH, pH 8 6, containing 0 35% Na_2 EDTA, 5% sucrose (Corning), and the bridge buffer was 50 mM barbiturate, pH 8 6 Electrophoresis was carried out at 90 V, 5 mA and 25°C for 45 min, and gels were stained for adenylate kinase activity [31] (A) Original photographs of the gels stained for activity (B) A composite drawing of a number of gels stained for activity

Curiously, the sera from the Duchenne muscular dystrophic patients contain an additional variant electrophoretic band compared to the normal serum pattern which appears to correspond to the liver type The normal adult muscle extract appears to contain two electrophoretic bands, a major and a minor component, which might correspond to the so-called adenylate $kinase_1$ and adenylate $kinase_{12}$ species, respectively, of Fildes and Harris [15] The electrophoretic position of the normal erythrocyte enzyme, of the individual shown in Fig 2, appears to be located between that of the liver type and the minor electrophoretic component of the muscle type

Interestingly, the additional serum electrophoretic component found in the case of the X-linked recessive Duchenne muscular dystrophic patients was not observed in the sera of the female carriers, nor in autosomal dominant dystrophics, such as FSH dystrophy and myotonic dystrophy Finally, normal human infants, of the same age as the Duchenne dystrophics, show only one electrophoretic band in their sera

Studies on the effect of phosphoenolpyruvate, DTNB and Ap_5A on the initial velocity of adenylate kinase from extracts of normal human and Duchenne dystrophic tissues

Effect of phosphoenolpyruvate As described by Kuby et al [11], in contrast to the crystalline calf muscle adenylate kinase, the crystalline liver adenylate kinase was sensitive to the presence of phospho*enol*pyruvate in the assay mixture for the forward direction and this led to a study of the inhibition by phospho*enol*pyruvate of the liver type ($K_i \simeq$ 1 2–5 6 mM)

In Fig 3, the normal human adenylate kinase activities from extracts of the muscle, hemolysates of the erythrocyte, and the serum appear to be unaffected by the presence of phospho*enol*pyruvate, whereas the normal human liver enzyme and the Duchenne dystrophic serum enzyme appear to be inhibited in a similar manner over the same range of phospho*enol*pyruvate concentrations

Effect of DTNB Schirmer and Thuma [26] reported that the adenylate kinase activity of extracts

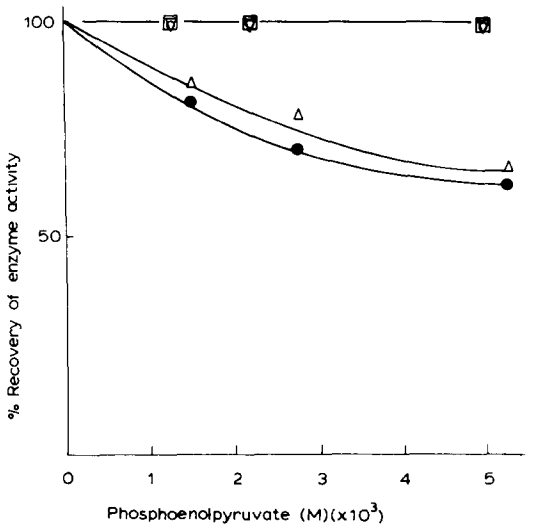

Fig 3 Inhibition by phospho*enol*pyruvate of the adenylate kinase activity (forward direction measured) in extracts of normal human liver, muscle and Duchenne type dystrophic serum The tissue extracts (200 mU of adenylate kinase/ml of 10 mM Tris-OAc, pH 8 1) were preincubated for 20 min at 30°C with various concentrations of phospho*enol*pyruvate For each sample, a control without phospho*enol*pyruvate was prepared and the samples were tested for adenylate kinase by the coupled-enzyme assay It should be noted that 100% enzyme activity is different for each tissue, the absolute values (expressed as mU/mg of protein) were 0 59 (normal serum), 83 6 (normal erythrocyte) 2710 (normal muscle), 340 (normal liver) and 0 97 (dystrophic serum) □———□, normal erythrocyte, ○———○, normal muscle, △———△, normal serum, △———△, normal liver, ●———●, Duchenne type serum

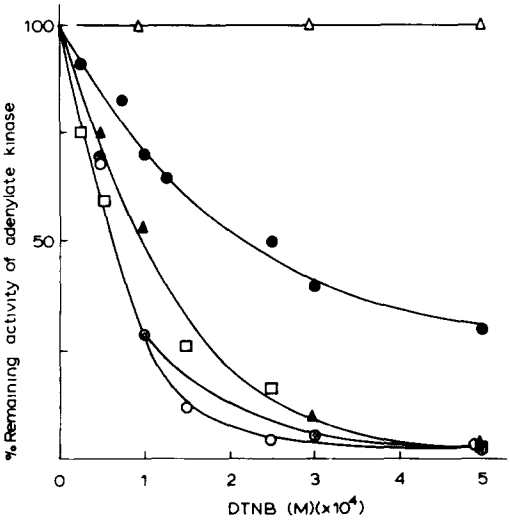

Fig 4 Inhibition by DTNB (in the absence of dithioerythritol) of adenylate kinase activities (reverse direction measured) in extracts of human muscle and liver, hemolyzed erythrocytes and sera The tissue extracts (approx 200 mU of adenylate kinase/ml of 10 mM Tris-OAc, pH 8 1) were preincubated for 20 min at 0°C with various concentrations of DTNB ○———○, normal muscle, ●———●, normal serum, □———□, normal erythrocyte, △———△, normal liver, ▲———▲, Duchenne type erythrocyte, ●———●, Duchenne type serum

of Duchenne dystrophic muscle was inhibited only 40–60% after treatment with 0 5 mM DTNB, and that in contrast to the muscle enzyme, the liver enzyme was insensitive to the reaction of DTNB (cf Ref 8) As shown in Fig 4, the adenylate kinase activity of extracts from normal human muscle, in contrast to that from normal human liver, was essentially inactivated after 20 min at 0°C with 0 5 mM DTNB Similarly, the enzyme activity from normal human serum and the normal erythrocyte, as well as that from the Duchenne type erythrocyte was inactivated at approx 0 5 mM DTNB, whereas the Duchenne type serum was 50% inhibited with 0 25 mM DTNB, and approx $\frac{1}{3}$ of the activity still remained after treatment with 0 5 mM DTNB

Effect of Ap_5A Kuby et al [11] had found Ap_5A to be a potent inhibitor of the crystalline rabbit and calf muscle adenylate kinases ($K_i \simeq 10^{-8}$ M) (cf Refs 33 and 34), whereas it proved to be only $\frac{1}{10}$ to $\frac{1}{100}$ as effective an inhibitor for the crystalline calf liver-type

In Table II, the inhibition of Ap_5A of the adenylate kinase enzyme activity from extracts of several human tissues is compared At 5 10^{-8} M Ap_5A, the normal human muscle enzyme was inhibited by 73%, compared to only 25% inhibition of the normal liver enzyme by Ap_5A at 5 10^{-6} M Both the normal serum and erythrocyte enzymes were inhibited to the extent of 60 and 47%, respectively, by 5 10^{-8} M Ap_5A

In comparison, the dystrophic serum enzyme was inhibited only 41%, whereas the corresponding red cell enzyme was 65% inhibited by 5 10^{-8} M Ap_5A

Curiously, the female carrier serum adenylate activity was intermediate in its behavior to the Ap_5A (cf 49% inhibition vs 60% for the normal), but this

TABLE II

EFFECT OF Ap_5A ON ADENYLATE KINASE ACTIVITY

Activity expressed as mμmol/min per mg (μmol/min per mg for normal muscle and liver)

Ap_5A	Normal		Dystrophic		Carrier		Normal	
	Serum	Erythrocyte	Serum	Erythrocyte	Serum	Erythrocyte	Muscle	Liver
–	0 59	83 63	0 97	452 8	0 75	83 56	2 71	0 34
5 44 10^{-8} M	0 25	43 91	0 57	159 2	0 38	36 80	0 56	0 26 [a]
% inhibition	60 30	47 49	41 11	64 83	49 23	55 96	72 96	24 56

[a] Ap_5A present at 5 44 10^{-6} M

should be repeated on a larger number of cases to evaluate its statistical significance

Heat stability In Table III, the heat stabilities of partially purified enzymes from several human tissues are compared Samples of the various enzymes, which were treated at 30, 55 or 60°C for 10 min, were immediately chilled and assayed

It is evident that the normal human liver type is thermally more stable than the normal muscle type The normal serum enzyme resembles the muscle type in that it is quite heat labile But the dystrophic serum enzyme appears to be more thermally stable than its normal human counterpart, and at 55°C, the female carrier serum enzyme lies intermediate in heat lability between the normal and the dystrophic serum Curiously, the female carrier erythrocyte enzyme proved to be more stable than the normal erythrocyte enzyme, which was extraordinarily heat labile, with the heat lability of the dystrophic erythrocyte enzyme lying in an intermediate position

TABLE III

HEAT STABILITY OF ADENYLATE KINASE

All data are expressed as relative percent recovery of activity, with the 30° C data set at 100%

Tissue	Recovery of activity	
	55° C	60° C
Normal		
Serum	19 5	16 0
Erythrocyte	5 2	1 4
Dystrophic		
Serum	28 6	24 0
Erythrocyte	8 8	5 6
Carrier		
Serum	24 2	11 6
Erythrocyte	19 2	15 8
Normal		
Muscle	20 1	17 0
Liver	52 3	22 3

Anti-enzyme assays In Table IV, data are presented on five Duchenne dystrophics and two Becker's dystrophics The five Duchenne dystrophics show the typically huge values of creatine kinase in the serum, huge compared to normal males of the same age group (approx 0 05–0 3 U/ml with our assays), and except for patient R N , the Duchenne dystrophics also show the typical inverse age-related value of creatine kinase in their serum In addition, their percent inhibition by anti-human muscle-type creatine kinase averages 89% (86–93% range), indicative of at least 11% of the other active isoenzymes (M-B (hybrid type) + B-B (brain type)) in their serum These data are in agreement with the findings of Kuby et al [1], who had found, by direct isolation, all three isoenzymes, M-M, M-B and B-B types in the autopsy samples of the atrophying muscle of terminal Duchenne dystrophics The two patients with Becker's dystrophy (brothers) also show 8–9 U/ml of creatine kinase, which also is inhibited only by approx 90% (89–90% range) by anti-M-M type human creatine kinase

However, as can be seen, erythrocyte adenylate kinase of these dystrophics (Table IV) is inhibited to the extent of 94–99% by anti-muscle-type adenylate kinase and their serum adenylate kinase 95–98% by anti-muscle adenylate kinase Therefore, by this assay, there is less than 1–6% liver-type adenylate kinase in their red cells and less than 3–5% liver-type

TABLE IV

ANTI-ENZYME ASSAYS OF DYSTROPHIC SERUM CREATINE KINASE AND OF DYSTROPHIC ERYTHROCYTE AND SERUM ADENYLATE KINASE

n d , not determined

Patient (male)	Age (years)	Diagnosis	Creatine kinase in serum	
			Creatine kinase (U/ml)	% inhibition of creatine kinase by ant-M-M type creatine kinase
E S	7½	Duchenne dystrophy	3 2	90 7
R N	7	Duchenne dystrophy	8 7	86 9
W S	5	Duchenne dystrophy	4 8	92 6
E C	2	Duchenne dystrophy	7 0	86 1
G R	4	Duchenne dystrophy	7 8	92 0
B C	3	Becker's dystrophy	8 7	89 4
C C	5	Becker's dystrophy	8 0	90 0
Controls	0 104 U of human M-M-type creatine kinase in test			100 0
	0 096 U of human B-B-type creatine kinase in test			0 0
	0 031 U of M-M-type + 0 052 U of B-B-type in test			39 4

Patient	Adenylate kinase in red cell		Adenylate kinase in serum		
	Adenylate kinase (U/mg Hb)	% inhibition by anti-muscle-type adenylate kinase	Adenylate kinase		% inhibition by anti-muscle-type adenylate kinase
			U/ml serum	U/mg protein	
E S	0 171	98 5	0 062	1 1 10^{-3}	95 1
R N	0 193	96 0	0 169	2 2 10^{-3}	96 9
W S	0 133	98 5	0 078	1 1 10^{-3}	96 1
E C	n d	n d	0 115	2 3 10^{-3}	97 7
G R	0 170	94 0	0 122	1 8 10^{-3}	96 7
B C	n d	n d	0 051	7 9 10^{-4}	95 0
C C	0 190	98 4	n d	n d	n d
Controls	0 090 U of muscle type in test	100 0			
	0 23 U of liver type in test	0 0			

adenylate kinase in the serum of these dystrophics

In summary, therefore, the inhibition patterns observed for the dystrophic serum adenylate kinase by DTNB, phospho*enol*pyruvate and Ap_5A, as well as the relative heat stability of both the dystrophic serum and erythrocyte adenylate kinase, all point to be presence of a liver type in the dystrophic serum (and possibly in the red cell), and the agarose gel electrophoresis patterns stained for adenylate kinase activity show the presence of an apparently normal serum adenylate kinase active enzyme, but also of a species which appears to correspond to the normal liver-type active enzyme

However, their kinetics (Hamada, M , unpublished observations) reveal the presence of an apparently abnormal adenylate kinase enzyme in the dystrophic erythrocyte, and possibly in the serum, with a relatively high value of $\bar{K}_{ADP^3-}$ and an unusually high V^f/Hb (but with a $V^r/V^f \simeq 1\ 1$), certainly more like the normal muscle type than the liver type [12]

Finally, by immunological anti-adenylate kinase inhibition titrations, there is no more than 1–6% liver-type adenylate kinase in the dystrophic (Duchenne or Becker's) red cells, and less than 3–5% liver-type adenylate kinase in their serum.

A logical conclusion which may be drawn from

these seemingly contradictory sets of data is that there is a variant or mutant adenylate kinase in the serum (and possibly in the erythocyte) of these dystrophics (Duchenne and Becker's) which resembles the liver type in its inhibition patterns, and possibly electrophoretically, but which immunologically reacts as if it were the muscle type

It is also likely that the Duchenne dystrophic serum adenylate kinase has leaked out of the atrophying muscle, either as a result of muscle fiber damage or as a consequence of a relatively leaky muscle membrane, a characterisitic of these Duchenne dystrophics Moreover, the red cell membrane of these Duchenne dystrophics appears to be more permeable to the diffusion of its adenylate kinase compared to normal males when placed in a hypotonic environment (Nesset, C , Manship, M and Kuby, S A , unpublished observations) Whether this is the result of an aberrant adenylate kinase with an abnormal net charge or an abnormal red cell membrane, or both, is the subject of future investigations Only by direct isolation and determination of the covalent structures of the adenylate kinases from the tissues of the Duchenne dystrophic will the final answer be provided to the postulate made here viz , that the Duchenne dystrophic serum contains an aberrant adenylate kinase enzyme, which might reflect a mutational event (or events) linked to human Duchenne progressive muscular dystrophy Whether or not this aberrant adenylate kinase enzyme is a fetal type will have to be answered by ontogenetic studies

Finally, mention should be made of the interesting work of Thomson and Smith [32], who have drawn attention to the severe deficiency of total muscle adenine nucleotides (ATP, ADP and AMP) in Duchenne muscular dystrophics, and the possibility that the underlying cause of muscular dystrophy may lie in this deficiency Aberrant adenylate kinases of unusual kinetic properties would, of course, contribute to this unusual deficiency Work is currently in progress to isolate the several types of adenylate kinases in normal and dystrophic man to establish the effects which atypical adenylate kinases might have when placed in the muscle milieu of the dystrophic individual

Acknowledgments

The authors wish to thank Drs Y Hatanaka, Y Kakimoto, H Mori, R Fukunishi and R Tabei of the Ehime University Departments of Pathology, and Dr W J Cathey of the University of Utah Department of Pathology for their assistance in supplying autopsy materials M H , H O , K O , T W , K U and M N were supported by the Scientific Research Fund of the Ministry of Welfare of Japan and by a grant from the Ehime Prefectural Medical Fund Organization S K , M M , E T and F Z were supported, in part, by grants from the National Institutes of Health, U S Public Health Service

References

1 Kuby, S A , Keutel, H J , Okabe, K , Jacobs, H K , Ziter, F , Gerber, D and Tyler, F H (1977) J Biol Chem 252, 8382–8390
2 Kuby, S A , Palmieri, R H , Okabe, K , Frischat, A and Cress, M C (1979) Arch Biochem Biophys 194, 336–353
3 Jacobs, H K and Kuby, S A (1980) J Biol Chem 255, 8477–8482
4 Markland, F S and Wadkins, C L (1966) J Biol Chem 241, 4124–4135
5 Markland, F S and Wadkins, C L (1960) J Biol Chem 241, 4136–4145
6 Criss, W E , Sapico, V and Litwak, G (1970) J Biol Chem 245, 6346–6351
7 Sapico, V , Litwak, G and Criss, W E (1972) Biochim Biophys Acta 258, 436–445
8 Chiga, M and Plaut, G W E (1960) J Biol Chem 235, 3260–3265
9 Klethi, J and Mandel, P (1968) Nature 218, 467–468
10 Russell, P J , Horenstein, J M , Goins, L , Jones, D and Laver, M (1974) J Biol Chem 249, 1874–1879
11 Kuby, S A , Hamada, M , Gerber, D , Tsai, W -C , Jacobs, H K , Cress, M C , Chua, G K , Fleming, G , Wu, L H , Fisher, A , Frischat, A , and Maland, L (1978) Arch Biochem Biophys 187, 34–52
12 Hamada, M and Kuby, S A (1978) Arch Biochem Biophys 190, 772–792
13 Tamura, T , Shiraki, H and Nakagawa, H (1980) Biochim Biophys Acta 612, 56–66
14 Carleer, J and Ansay, M (1980) Arch Biochem Biophys 199, 76–83
15 Fildes, R A and Harris, H (1966) Nature 209, 261–263
16 Rapley, S , Robson, E and Harris, H (1967) Ann Hum Genet 31, 237–242
17 Skude, G and Jakobsson, A (1970) Hum Hered 20, 319–324

18 Szeinberg, A , Kahana, D , Slava, G , Zaidman, J and Benezzer, J (1969) Acta Haematol 42, 111–126
19 Bowman, J , Frischer, H , Ajmar, F , Carson, P and Gower, M K (1967) Nature 214, 1156–1158
20 Rapley, S and Harris, H (1970) Ann Hum Genet 33, 361–364
21 Giblett, E R (1969) Genetic Markers in Human Blood, pp 512–519, F A Davis, Philadelphia
22 Brownson, C and Spencer, N (1972) Biochem J 130, 797–804
23 Brownson, C and Spencer, N (1972) Biochem J 130, 805–811
24 Tsuboi, K K and Chervenka, C H (1975) J Biol Chem 250, 132–140
25 Von Zabern, I , Wittmann-Liebold, B , Untucht-Grau, R , Schirmer, R H and Pai, E F (1976) Eur J Biochem. 68, 281–290
26 Schirmer, R H and Thuma, E (1972) Biochim Biophys Acta 268, 92–97
27 Gornall, A G , Bardawill, C J and David, M M (1949) J Biol Chem 177, 751
28 Manual of Clinical Laboratory Procedures (1970) (Faulkner, W R and King, J W , eds), pp 154–156, The Chemical Rubber Co , Cleveland, OH
29 Oliver, I T (1955) Biochem J 61, 116
30 Okabe, K , Jacobs, H K and Kuby, S A (1970) J Biol Chem 245, 6498–6510
31 Shaw, C R and Koen, A L (1968) in Chromatography and Electrophoretic Techniques (Smith, I , ed), 2nd edn , vol 2, pp 355–356, Interscience Publishers, New York
32 Thomson, W H S and Smith, I (1978) Metabolism 27, 151–163
33 Lienhard, G E and Secemski, I I (1973) J Biol Chem 248, 1121–1123
34 Feldhaus, P , Frohlich, T , Goody, R S , Isakov, M and Schirmer, R H (1975) Eur J Biochem 57, 197–204