BIOCHEMICAL STUDIES OF MUSCLE IN HEALTH AND DISEASE

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THESIS

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CONTENTS

LIST of TABLES

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ABBREVIATIONS

The following abbreviations are used throughout this thesis:-

ADP	Adenosine-5'-diphosphate
AK(I)	Adenylate kinase (increment)
ALD	Fructose-1,6-diphosphate aldolase
Alk P	Alkaline phosphatase
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
BMD	Becker muscular dystrophy
CP	Creatine phosphate
CPK	Creatine phosphokinase
CPK-Bg	Creatine phosphokinase (Boehringer system)
CPK-Bk	Creatine phosphokinase (Baker system)
DHAP	Dihydroxyacetone phosphate
DMD	Duchenne muscular dystrophy
ECG	Electrocardiogram
EDTA	Diaminoethanetetra-acetic acid
EMG	Elecromyography
F-1,6-P	Fructose-1,6-diphosphate
GA-3-P	Glyceraldehyde-3-phosphate
GDH	Glycerophosphate dehydrogenase
GOT	Aspartate aminotransferase
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GPT	Alanine aminotransferase
γ-GT	γ-glutamyl transpeptidase
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HK	Hexokinase

IMP	Inosine monophosphate
IU	International Unit
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
NA3	Nicotinamide-3'-adenylate
NAD(H)	Nicotinamide-adenine dinucleotide (reduced)
NADP(H)	Nicotinamide-adenine dinucleotide phosphate (reduced)
NCN	Non-collagen nitrogen
PA3	Procaine adenosine-3'-monophosphate
PA5	Procaine adenosine-5'-triphosphate
PEP	Phosphoenol pyruvate
PGI	Phosphoglucose isomerase
Pi	Inorganic phosphate
PK	Pyruvate kinase
PK-Ala	Alanine inhibited pyruvate kinase
PK-LM	Liver and muscle pyruvate kinase
PK-M	Muscle pyruvate kinase
PRPP	Phosphoribosylpyrophosphate
6-PG	6-phosphogluconate
rbcs	Erythrocytes (red blood cells)
TIM	Triosephosphate isomerase
wbcs	Leucocytes and platelets (white blood cells)

PUBLICATIONS

The following publications report some of the work contained in this thesis :-

Smith, I. and Thomson, W.H.S. (1975) Serum enzyme assays with commercial kits. Lancet (i), 1038.

Smith, I. and Thomson, W.H.S. (1977) Carrier detection in X-linked recessive (Duchenne) muscular dystrophy : Pyruvate kinase isoenzymes and creatine phosphokinase in serum and blood cells. Clinica Chimica Acta 78, 439-451. This paper was presented at the Seventh Symposium on Current Research in Muscular Dystrophy, held at the University of Birmingham, January 1977.

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SUMMARY

Reconstituted commercial kits for serum enzyme assay were found to be contaminated, apparently by the very enzyme to be measured. With multiple-assay kits using kinetic methods saline instead of serum gave blank values that could be subtracted from the readings obtained to give dependable results. However, with boxes of single-assay vials the between-vial variation in contamination was substantial and, in diagnosis, could lead to unfortunate clinical consequences.

Recently serum pyruvate kinase (PK) has been reported superior to creatine phosphokinase (CPK) in diagnosing Duchenne muscular dystrophy (DMD) patients and carriers. The allosteric properties of the PK isoenzyme abundant in muscle allowed both forms to assayed and compared with CPK. Normal ranges were obtained for CPK using 2 different thiol activators and for 4 PK modalities; both sex and seasonal differences were noted. CPK in clotted blood and serum retained its activity, on storage at room temperature and lower, far better than did PK, which lost activity when kept as serum though whole blood chilling caused its gross efflux from blood cells. CPK elevations in known DMD carriers were much more decisive than those of PK, which were frequently equivocal and only barely above the normal upper limit. CPK is accordingly markedly superior to PK in sensitivity, stability and convenience.

Deducting the adenylate kinase increment (AKI) further refined the CPK assay, eliminating the effect of haemolysis in diagnosis and enabling studies of blood cell contents. Differential centrifugation separated rbcs from wbcs; and although chilling and

disruption showed both had a high PK content, only the former contained an appreciable amount of CPK. The erythrocyte content of CPK appeared to match that of serum, as if by rapid passage across the cell membrane.

Reasons are given to suggest that DMD may have some origin in a severe deficiency of total muscle adenine nucleotides, and thus perhaps in some defect of purine metabolism. Using double-blind techniques this possibility was tested in 16 DMD patients by giving oral allopurinol, a synthetic inhibitor of the purine catabolic enzyme xanthine oxidase, in an attempt to increase the adenine nucleotides of muscle by better salvage and recycling of available purines. Sublingual procaine adenylate was also tested. Instances of clinical improvement quickly occurred, statistically significant and accompanied by significant increases in physical strength and urinary creatinine output, together with the expected decrease in both serum and urinary urate values. At muscle biopsy ATP was found to be reduced to 40% of normal in untreated patients, but after a year on daily allopurinol this more than doubled to 81% of normal, accompanied by an even larger increase in creatine phosphate (CP). As initial clinical improvements have been maintained for over a year, the view that DMD may be the expression of some defect in purine synthesis is plainly upheld. Indeed, sustained clinical arrest in this otherwise relentlessly progressive disease may even appear possible.

CHAPTER I

INTRODUCTION

Most of the work reported here is directly related to the Duchenne type of muscular dystrophy (DMD). By far the most common and most severe of the various dystrophies, DMD is inherited as an X-linked recessive gene, and estimates of incidence range from 13 to 33 per 100,000 live male births. This means that around 1 in 5,000 males are afflicted and that in the United Kingdom there may be as many as 5,000 patients at different stages of the disease. Duchenne de Boulogne (1868), in a series of 6 reports on the cases of 11 boys and 2 girls, was the first to describe DMD adequately. He gave the principal characteristics of the disease as: 1) a weakness of movement progressively worsening to an abolition of mobility, 2) an increase in volume of some of the "paralysed" muscles, and 3) an abundant production of fibrous tissue or fatty vesicles in the more advanced stages. Duchenne called the disease "psuedohypertrophic muscular paralysis", though this last word has since been correctly modified to "dystrophy".

Clinical evidence of the disease becomes apparent usually about the third year of life, though weakness may occur as early as the first or as late as the seventh. In fact Toop and Emery (1974) and Vassilopoulos and Emery (1977) even suggest that the disorder is manifest *in utero* by the second trimester of pregnancy. The boy is late in walking, and soon becomes unsteady, with frequent falls and increasing difficulties ascending stairs. This growing disability is occasionally erroneously attributed to a trivial

complaint such as flat feet or general laziness. The weakness is thus first apparent in the muscles of the pelvic girdle and the thigh. In 80% of the patients the calves appear to be enlarged (Walton and Nattrass, 1954) and other muscles, such as the glutei and those of mastication, may also show this pseudohypertrophic phase. These regions initially feel firm but later become soft though remaining relatively enlarged when compared with other wasted regions. The pseudohypertrophy may precede noticable weakness by many months, although it usually disappears as the disease progresses, and may even be mistaken for good muscular development.

Before long the boys have increasing difficulty in running and climbing stairs. They rise characteristically from a supine position by rolling over face-down to the prone position, and with both hands on the floor throughout, attain a kneeling posture then stand erect by using both hands to "climb up" their legs and support the weak pelvic extensor muscles. This manoeuvre, called Gowers' sign (Gowers, 1879), may be the earliest evidence of definite pelvic weakness in a child. Weakness of the muscles of the trunk, the shoulder girdle and the upper extremities is often not reported until up to 5 years after the onset, yet it can usually be found at examination long before this. Once the shoulder girdle muscles are noticably affected the scalpulae "wing" from the thorax, and the patient is too weak to keep his arms down when being lifted under the axillae.

At the age of 4 or 5 years growth may outstrip progress of the disease giving a false impression of improvement, otherwise deterioration is continuous. Wasting in all muscles generally

parallels weakness, becoming more severe proximally though the calf muscle may remain remarkably strong for several years. Tendon reflexes are often depressed more than the weakness would suggest, commonly being absent in muscles that can still overcome gravity and moderate resistance. Neck muscles usually become weak only in the later stages but those of the face are often not seriously affected.

In most patients muscle is replaced by fat and fibrous tissue, which later shrinks to give contractures of the large limb joints, with early progressive plantar-flexion and inversion of the feet and finally hip contractures. Contractures also develop eventually in the knee, elbow, shoulder and wrist joints. The early inversion of the feet prevents the patient bringing his heels to the floor, so that he walks on his toes, feet wide apart, with a pronounced waddle and lumbar lordosis due to weakness of the hip extensors.

Life in a wheelchair becomes necessary, usually between 7 and 11 years, when the patient can no longer walk at all. Confinement to bed leads to a rapid increase in weakness and a speedy decline to the wheelchair, and must be avoided, so that surgical procedures designed to lengthen the Achilles tendon are often disastrous because of this. Once chairbound the contractures develop more rapidly and weakness of the spinal muscles invariably leads to increasing scoliosis, with displacement of vital organs. Progressive deterioration of respiratory function occurs and may very occasionally lead to severe terminal carbon dioxide retention. Some boys become generally wasted as the disease progresses but others become very obese, presumably due to a

combination of excessive feeding and immobility, however studies of endocrine function have failed to reveal the origin of this difference (Walton and Gardner-Medwin, 1974). Sexual development is usually normal, though puberty may be delayed.

Eventually pronounced deformities by fixed contractures may make even a chairbound existence impossible and herald the final stages of DMD where the patient is confined to bed, just able to speak, swallow, breath and feebly grimace, but is otherwise all but helpless. Survival beyond the age of 20 years is unusual and beyond 25 years rare. Death usually results from inanition, chest infection or cardiac failure.

Intellectual impairment is common in DMD and was recognised by Duchenne in 5 of his 13 cases, leading him to believe that the disorder was cerebral in origin. About 10% to 25% of the patients are retarded with verbal ability the most severely affected; but progressive deterioration does not occur and there is no correlation between muscular and intellectual involvement. Skeletal deformity includes a narrowing of the shafts and rarefaction of the ends of the long bones giving coxa valga. At later stages there is severe scoliosis, widespread decalcification and disorganisation of the skeletal system. These changes render the affected bones liable to fracture on minimal trauma, when even a fall from the wheelchair may fracture a femur. Walton and Warrick (1954) concluded that these bone changes were the result of disuse and the absence of normal muscular stresses, compounded by the distorted posture due to the disease, and were not caused by a dystrophy of the bone itself.

Myocardial involvement is probably invariable in DMD although it may not be detectable in the early stages. Persistant tachycardia, arrhythmias and non-specific murmurs are common and sudden death from myocardial failure may occur (Berenbaum and Horowitz, 1956) though congestive cardiac failure is rare. The electrocardiogram (ECG) in DMD, which is characteristic, shows tall R waves in the right precordial leads with deep Q waves in the limb and left precordial leads. Emery (1972) has shown that this ECG pattern may be of diagnostic value in distinguishing DMD from other forms of muscular dystrophy.

DMD is also characterised by the diagnostically important elevations in the activity of some serum enzymes. Sibley and Lehninger (1949) first discovered elevated serum ALD activities in 2 patients with muscular dystrophy and confirmatory reports followed (Evans and Baker, 1957). Ebashi et al. (1959) were the first to report extremely high values of CPK activity in serum of DMD patients, now generally recognised as the most sensitive index of muscle disease, since CPK occurs almost exclusively in muscle (Colombo et al., 1962; Schmidt, 1964; Dawson and Fine, 1967; Smith, 1972) with small amounts in the brain and myocardium and only traces elsewhere. Gross elevations of the activity of CPK and other serum enzymes may be evident long before any clinical symptoms of DMD appear. These elevations have been monitored from birth in the brother of a DMD patient (Heyck et al., 1966). At birth the infant had values which were raised in comparison to those found in normal neonates. The elevations reached a peak between 14 and 22 months with a slow decline in the years thereafter and although the disease had already been confirmed at biopsy there was still no clinical indications of DMD at $2\frac{1}{2}$ years old.

Early in the course of DMD activities of many thousand units, several hundred times the normal upper limit, are commonly found. Thomson et al. (1974) found a significant steady decline of serum CPK as the age of the patients increased and muscle mass diminished. The rate of decline was the same for both ambulant and wheelchair boys, though the values were almost halved after confinement of the individual to the chair. Thus CPK in DMD specifically measures active muscle mass and may assess prospective therapy if activity and mass remain the same over a short period. There can be little doubt that the increased amounts of these serum enzymes originate mainly from muscle, though it has been suggested (Kleine, 1970) that part of the elevation is derived from other tissues. Nevertheless, the quantity of enzyme in the serum only represents a minute fraction of that found in muscle as 1g of normal human muscle has 10^4 times the activity of 1ml of serum (Dreyfus et al., 1956).

The fact that DMD is inherited as an X-linked recessive gene is confirmed by its appearance in 2 sons by different fathers of the same carrier mother (Thomson, 1971) and by its equally rare occurrence in 2 carrier daughters in the same way (Thomson,1975b). These carriers have an XX genotype, with one X-chromosome carrying the DMD gene abnormality which is masked by normal factors on the other X-chromosome. Moreover, the random fusion during early growth of mononucleate myoblasts from clones with one or other X-chromosome already randomly inactivated (Lyon, 1962) gives a dual population of nuclei regulated by either a normal or a dystrophic X-chromosome in the mature multinucleated cell, with different proportions of each in different individuals giving a uniform X-chromosomal mosaic (Emery, 1965). Since DMD seems

myogenic (Thomson et al., 1974) this implies a range of carrier manifestation from the undetectable, with few X-chromosomes carrying the DMD gene and no clinical or biochemical evidence of the carrier state, to the florid DMD itself with the gene present in most X-chromosomes. Undetectable obligate carriers do occur (Thomson, 1969a), as well as cases of marked disability (Moser and Emery, 1974) and even rapid crippling and early death (Frazer, 1963) of occasional females in typical pedigrees of male DMD.

Females carrying the gene would only manifest the condition fully if they were of XO genotype (Turner's syndrome) or homozygous. Both Walton (1956) and Ferrier et al. (1965) found DMD in girls with Turner's syndrome in accord with gene location on the X-chromosome. Girls homozygous for the gene are of extreme rarity as DMD patients are not likely to reproduce and there is little likelihood of a female carrier marrying a male in whom a gonadal mutation of the DMD gene has occurred. There are, nevertheless a number of instances of females described as cases of DMD where the pattern of inheritance has suggested autosomal recessive inheritance, Ionasescu and Zellweger (1974) extensively examined 4 such females who were clinically, histologically and enzymatically very similar to male DMD. However, no absolute determination of the mode of inheritance could be made in these cases, which may in fact be simply very severely manifesting carriers, as suggested by Thomson et al. (1975b).

DMD is sometimes referred to as the "malignant" or "fast" variant of pseudohypertrophic muscular dystrophy, whereas Becker (Becker, 1955; Becker and Kiener, 1955; Becker, 1957; Becker, 1962) later described a "benign" or "slow" X-linked recessive form,

Becker muscular dystrophy (BMD). Emery et al. (1968-1969) and Skinner et al. (1974) have reported evidence suggesting that the genes for BMD and DMD are at 2 different loci and that the 2 forms are quite distinct, with BMD not merely part of the spectrum of severity of DMD. BMD is clinically similar to DMD but it has a much slower course, with very gradual progression of weakness and wasting, apparent first in the pelvic and then in the pectoral muscles as in DMD. The age of onset is usually between 5 and 25 years and ability to walk is not lost until middle or even late adult life, some 25 years or more later. In most cases there is no cardiac involvement or contractures and deformities do not usually occur. The life span may even be normal, with patients fathering offspring and transmitting the disorder through obligate carrier daughters to grandsons, but never to sons. The biochemical identification of BMD carriers is uncertain and though some can be identified it seems probable that the detection rates are lower than for DMD carriers.

The question whether the primary disorder in DMD is located in voluntary muscle or elsewhere has not so far been resolved. Some 30 years ago a deficiency of vitamin E was thought responsible when it was noted that several animal species, when deprived of dietary vitamin E, developed an experimental muscular dystrophy closely resembling the human form. Hopes of a cure for DMD were raised when α-tocopherol produced a prompt and dramatic recovery in rabbits with this disease. However in DMD patients neither α-tocopherol nor its esters, administered orally or intramuscularly, had the least effect (Milhorat et al., 1945). It was then thought that vitamin E was either not absorbed or poorly utalized, and various derivatives were then tested. Though some results seemed

encouraging, no real success was achieved. Many other potential therapies were tried but in 1954 a comprehensive list of 82 treatments (Milhorat, 1954) reported that not one had shown any significant, lasting effect on the course of the disease. The fact that the pathogenesis of muscular dystrophy still remains unknown explains the great diversity of the medications tried. These ranged from vitamins, sugars and amino acids to enzymes, hormones and products of the gastrointestinal tract.

In 1955 a mutant strain of mouse, the Bar Harbor 129, afflicted with a hereditary disorder which resembled human muscular dystrophy, was bred (Michelson et al., 1955). The availability of this animal stimulated a great deal of work in which it was used as an experimental model for the human disease. However, Baker et al. (1958) found several differences between the human and murine disease with tremors, unusual reflexes of the head and hind leg and periocular inflamation frequently occurring in the latter. The serology is different (Schapira and Dreyfus, 1963) and the mode of inheritance is autosomal recessive (Stevens et al., 1957), not X-linked recessive as in DMD. Askanas and Hee (1974) suggest that the dystrophy in mice and chickens may be of neurogenic origin, but stress that analogies between animal and human muscular dystrophies can be very misleading and findings should only be related to the human disease with great caution. Hironaka and Miyata (1973) also found evidence that murine dystrophy may be neurogenic but work suggesting that DMD arises in the same way (McComas et al., 1970) is open to criticism (Scarpalezos and Panayitopoulos, 1973; Desmedt and Borenstein, 1973; Rosselle and Stevens, 1973) with Harriman (1976) finding no morphological evidence to support the concept of DMD as a neurogenic disease. Moreover Thomson et al.

(1974) made observations that cannot be explained by a neurogenic but only by a myogenic origin. Work with animal models must therefore be of limited use as there is no known hereditary or experimentally induced animal dystrophy which exactly resembles DMD or any other human dystrophy.

Therapies tested in more recent years include procaine-3'-adenylate as sub-lingual tablets (Bourne and Golarz, 1962) which appeared to have beneficial effects after a year, with patients showing no muscular deterioration for up to 4 years (Golarz and Bourne, 1962). There was, however, no follow-up to this preliminary investigation nor any precise diagnosis of the muscle disorder. Intravenous infusion of Laevadosin, a mixture of nucleotides and nucleosides did seem to help (Thomson and Guest, 1963), but Pearce et al. (1964) and Walton et al. (1965) found it to have no effect, though their methods were criticised (Thomson, 1964; Thomson, 1965). Anabolic steriods gave apparently encouraging results (Dowben, 1963) but Gamstorp (1964) repeated this work using oral methandrosenolone and found that although 75% of DMD patients showed moderate improvement this was followed by rapid deterioration, often with unpleasant side effects. After a year on androstenolone marked decreases in strength occurred in most DMD patients tested by Fowler et al. (1965) who believed the treatment to be positively harmful. More recent work has concentrated on treating only the consequences of the disease. Chou et al. (1975) reported the beneficial effect of penicillamine in chickens, which appears to inhibit collagen formation in inherited avian muscular dystrophy. A double blind controlled trial of penicillamine in DMD is presently in progress but after 6 months no definite improvement had been seen (Enomoto and Bradley, 1977),

though little could be expected so soon. Diethylstilbestrol lowers serum enzyme activities in DMD (Cohen and Morgan, 1976) but this is not associated with changes in weakness or muscle mass. Munsat and Bradley (1977) state that a reduction in CPK activity does not necessarily imply theraputic benefit. Prednisone, another steriod has been offered as a palliative treatment (Drachman et al., 1974; 1975) but this has been severely criticised (Munsat and Walton, 1975; Rowland, 1975).

Demos (1961a and b) advanced the vascular hypothesis suggesting that muscle degeneration in DMD was due to an abnormality of muscle blood flow. The use of a vasodilating agent, to test his supposition met with moderate success (Demos, 1963). Bradely et al. (1975) and Boyson and Engel (1975) however, both reported evidence contradicting the Demos hypothesis. A disorder of connective tissue has been proposed as another explanation for DMD. Bourne and Golarz (1959) and Golarz et al. (1961) found evidence of excess dephosphorylating activity in connective tissue; but their findings could not be substantiated by Engel (1962). More recently Ionasescu et al. (1971a) demonstrated a 4-5 fold increase in collagen synthesis by the heavy polyribosomes of dystrophic muscle compared with those of normal controls. A similar increased rate of collagen synthesis has also been found in DMD carriers (Ionasescu et al., 1973) and recently it has been suggested that the basic defect in DMD is misdirected regeneration with a tendency to synthesise more collagen than contractile proteins (Ionasescu, 1975). However the overproduction of connective tissue may not be the primary fault but rather a non-specific response to injury.

A cell membrane defect has also been invoked and many abnorm-

alities have indeed been found in the membranes of DMD muscle (Takagi et al., 1973; Mawatari et al., 1974) and erythrocytes, where; 1) the phospholipid content differs (Kunze et al., 1973; Kalofoutis et al., 1977), 2) potassium (Howland, 1974) and sodium contents (Sha'afi et al., 1975) are abnormal and 3) the membrane protein kinase also is abnormal (Roses et al., 1975). These studies suggest that the fault is expressed biochemically in membranes, but again this may be secondary rather than primary and it is still a matter for conjecture which defect in DMD so far observed is the cause and which is the result.

In the most widely accepted classification, carriers of the DMD gene are divided into 3 categories (Walton and Gardner-Medwin, 1974). Definite carriers are those mothers who have an afflicted son and another male relative with the disease in the female line of inheritance; for example a brother, maternal uncle or sister's son. Probable carriers are the mothers of 2 or more affected sons but no other affected relatives, and possible carriers are the mothers of isolated cases and the female relatives of affected males. Thomson (1969a) however, defined a female carrier by exclusively female transmission to affected male relatives in a generation (A) directly succeeding (a son or a daughter's son) or (B) indirectly succeeding or preceding (a brother or a sister's son or a mother's brother). An obligate carrier has both A and B often with further biochemical evidence, and a presumed carrier has A or B with biochemical evidence of the carrier state.

In some carriers a slight degree of myopathy may often be detected by investigation. Occasionally this appears as actual

weakness (Chung et al., 1960) with apparent hypertrophy of the calves (Emery, 1963) though the latter was shown by systematic measurement to be mere supposition (Thomson et al., 1975b). In the majority of cases carrier manifestition is subclinical.

Carriers are best detected, therefore by a manifestation of DMD which survives attenuation in the X-chromosomal carrier mosaic. This is found in the serum CPK activity originating from muscle, which in health shows very low values, in DMD gross elevations, and in carriers values ranging from low but undoubted elevations to those resembling actual DMD. Serum CPK can thus detect 50-88% of definite and probable carriers (Walton and Gardner-Medwin, 1974) though around 70% seems to be the best that can usually be expected. Thomson (1969a) in precisely defined circumstances identified 86% of inevitable carriers using one of the most sensitive of the many methods available to measure CPK activity. Several CPK determinations should be carried out before a female at risk of being a carrier is pronounced normal since Perry and Frazer (1973) found that in serial CPK estimations all 10 carriers tested had at least 1 normal value , with 3 giving several normal results. They recommended that 4 CPK determinations be made for each female at risk in order to maximise the chances of detection.

There have been numerous attempts to improve the CPK detection rate by assaying the enzyme after physical exertion. Emery (1967) found standardised exercise had little effect in normal women but may produce a significant increase in enzyme values in some carriers and be useful in the genetic counselling of suspected carriers with equivocal serum CPK values. Hughes et al. (1971)

however, could not improve the rate of carrier detection using CPK with controlled ischaemic exercise. Recently Danieli and Angelini (1976) measured haemopexin (a β-globulin) and increased the level of detection of definite and probable carriers by CPK determination from 70 to 80%.

Spurious elevation of serum CPK activity may occur after exertion (Griffiths, 1966a) and in small children after a brief struggle at venepuncture (Thomson, 1971). Pregnancy in normal women was reported by Emery and Pascasio (1965) as having little effect on CPK activity but King et al. (1972) found there were significantly lower values at 8-20 weeks gestation. 2 more investigations (Emery and King, 1971; Blyth and Hughes, 1971) found carriers with elevated values that had even been brought within the normal range by pregnancy.

Other methods of carrier detection are often more complex and usually less sensitive except for the measurement of ribosomal protein synthesis (Ionasescu et al., 1971b) by estimating amino acid incorporation by polysomes in vitro from biopsied muscle, when it was found to be significantly raised in 8 out of 10 suspected carriers, 5 of whom had elevated serum CPK values. Further work (Ionasescu et al., 1973; Ionasescu et al., 1976) confirmed the abnormality in a total of 57 out of 58 known carriers (98%), with CPK estimation detecting 36 (62%). Because of the complexity of this method its use is only justified after normal serum CPK values have been obtained on 3 separate occasions (Ionasescu et al., 1973). Again pregnancy is a complicating factor as normal pregnant women may also show elevated ribosomal protein synthesis (Ionasescu et al., 1974).

Gardner-Medwin (1968) thought electromyography (EMG) useful in identifying some carriers with normal CPK values and Moosa et al. (1972) refined the technique somewhat. A report by Valli et al. (1976) claims that EMG, with 3 muscles per patient tested, gave a detection rate of 82% though previously only 50-70% of definite carriers could be identified. Walton and Gardner-Medwin (1974) however, warn that a most careful comparison with normal controls is essential if errors are to be avoided.

The histology of muscle biopsy material has been examined, using the light microscope, by Emery (1965) and Pearce et al. (1966) and although obvious and diagnostic changes were seen in some carrier specimens most were either normal or showed minimal and non-specific changes. Electron microscopy is perhaps more successful. Fisher et al. (1972) reported abnormalities in 19 carriers, but without mentioning CPK activity, and Afifi et al. (1973) in a study of 5 carriers found that 2 were abnormal under the light microscope, 4 had elevated CPK activities, but all 5 were abnormal using the electron microscope.

Nagai et al. (1969) described how the biological half-life of Rb^{86} was diminished in all carriers tested, though Bradley et al. (1971) and Thomson et al. (1975b) could not substantiate this, perhaps because the original comparison had been with normal males and not females as in the 2 other investigations. Using the same technique, whole body counting, Blahd et al. (1967) found significant reductions in total body potassium and although Thomson et al. (1975b) also found similar reductions the complexity of the method and the expensive instrumentation required severely limits its usefulness. Another extremely complex method which is not a

practical proposition in most laboratories is the measurement of limb blood flow which Demos et al. (1962) found to be significantly altered in a proportion of carriers. Askanas (1967), using immunoelectrophoresis, found abnormalities in the β-globulin fraction of serum proteins in some carriers, but this work could not be confirmed by Emery (1971). Emery (1969) also studied the ECG of carriers, and although certain irregularities were found its use is likely to be limited to differentiating the severely manifesting DMD carrier from the limb-girdle type of muscular dystrophy where the ECG is normal.

Using a scanning electron microscope Matheson and Howland (1974) found that carriers had an increased proportion of deformed erythrocytes (echinocytes); further work by Lumb and Emery (1975) failed to obtain a clear cut normal range, however, and although Howells (1976) reported automated methods for discriminating echinocytes from erythrocytes Matheson et al. (1976) could not reproduce the original work, as they found the erythrocytes very sensitive to different handling procedures. Manual muscle testing by Roses et al. (1977) demonstrated a pattern of proximal muscle weakness in carriers which they found a valuable adjunct to other methods of carrier detection. Very good results have been obtained by the use of a combination of several methods for each potential carrier. Radu et al. (1968), using serum enzymes before and after exercise, with EMG and muscle biopsy, detected 53 out of the 56 carriers tested (95%); and Gardner-Medwin et al. (1971), even although they found CPK to be by far the most superior single method, recommend that a battery of tests be used to obtain the best results.

Abnormal results in any of these investigations indicates the carrier state. However, when the result is normal difficulties arise since no result is invariably positive for all carriers. It has been suggested that, of these phenotypically normal heterozygous carriers, the percentage undetectable by laboratory tests may be 6-20% (Graham et al., 1975), so that complete detection is approached but never attained. When a suspected carrier does not give any positive result advice may still be given in terms of probability which can be estimated using the precise value of CPK in serum, within the normal range, together with the application of Bayes' theorem to her pedigree (Emery and Morton, 1968). Studies on the lethal X-linked recessive gene indicate that the proportion of new female mutants may be as high as one half (Franke et al., 1976). Roses (1976) and Roses et al. (1976) presented evidence suggesting that new mutations producing DMD males, are rare and, though Zatz et al. (1977) could not verify these results in a study of Brazilian pedigrees, differences in reproductive performance may explain the contradictions. These findings, whereby many DMD boys are born to apparently sporadic carriers, indicate a pressing need by the genetic advisory service for even more accurate routine methods of carrier detection.

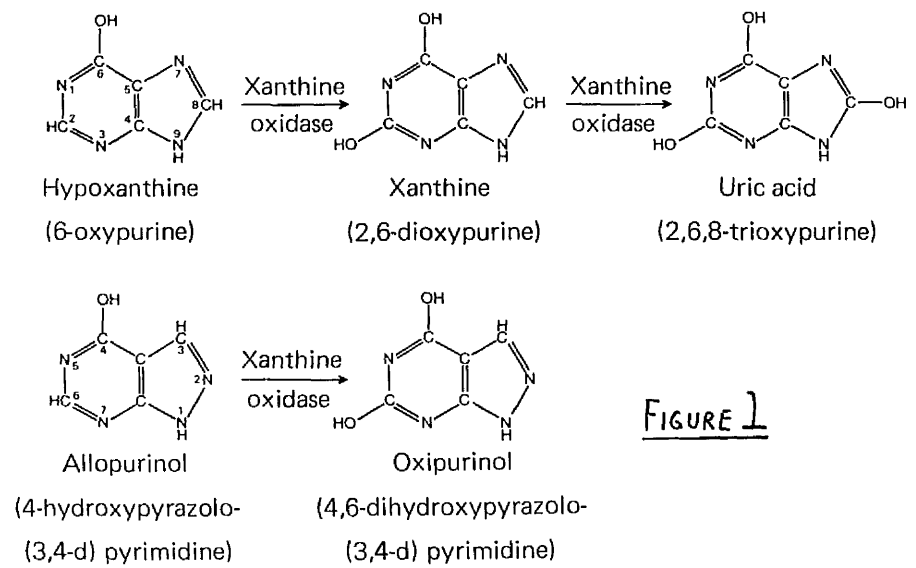
Serum PK, however, has recently been compared with CPK, so far the simplest and most sensitive single test available, and reported to be markedly superior, even without recourse to its specific isoenzymes (Harano et al., 1973; Alberts and Samaha, 1974). These reports merit careful examination with complete analysis throughout for tissue specificity, and extensive comparative studies have accordingly been undertaken.

There is evidence that ATP mediates the retention of enzyme molecules in the intact cell, thereby influencing mean serum values. Enzyme efflux *in vitro* is increased by metabolic inhibitors, lack of glucose, anoxia and stimulation (Zieler, 1958). Similarly *in vivo* excessive amounts of skeletal muscle CPK and other enzymes appear in the serum after hypoxia at rest (Griffiths, 1966b; Highman and Altman, 1960) and after prolonged exertion in health (Griffiths, 1966a; Sanders and Bloor, 1975) with rapid restoration afterwards and neither evidence nor likelihood of muscle cell damage. These different causes of enzyme efflux share a common tendency to diminish intracellular ATP. Sweetin and Thomson (1973b) showed that a decline in plasma glucose caused failure of the erythrocyte membrane (NaK)-ATPase, which has an absolute requirement for ATP (Glynn, 1968), allowing sudden effux of intracellular K^+ closely followed by that of ALD and LDH, suggesting that their retention also required ATP. This work received direct support from Wilkinson and Robinson (1974). Evidence of muscle enzyme efflux into the circulation after stimulation was described in the intact cat by Thomson *et al*. (1975a). Efflux occurred only after indications of exhaustion of ATP.

In DMD there is a 30-50% reduction in muscle ATP, part of a marked depletion of total adenine nucleotides (Vignos and Warner, 1963; Stengel-Rutkowski and Barthelmai, 1973), essential to every aspect of cell metabolism. This may cause chronic dilution of the cell contents in body fluids beyond prolonged survival, with the typical gross serum enzyme elevations proportional to the decreasing muscle mass. If the basic defect in DMD is a chronic lack of muscle ATP an explanation is found, not only for the dilution of cell content, but for many other apparently unrelated disorders

of DMD muscle. The delayed motor development in male DMD infants could be due to weakness, caused by a lack of ATP, before real muscle loss begins; likewise the rapid failure on standard exercise of symptomatic DMD carriers with apparently adequate contractile equipment is explained (Thomson et al., 1975b). Brief bed rest, which spares muscle ATP, greatly reduces efflux and serum enzyme values both in DMD patients and carriers (Thomson and Guest, 1963; Thomson, 1962; Stephens and Lewin, 1965); and likewise wheelchair confinement immediately halves CPK efflux in DMD patients (Thomson et al., 1974).

Vignos and Warner (1963) found a reduction of 30% in ATP, unchanged ADP values and no AMP at all in the muscle of DMD patients; whereas Stengel-Rutkowski and Barthelmai (1973), using more precise methods, found reductions of 50, 70 and 80% in ATP, ADP and AMP respectively in DMD muscle compared with normal. Barak et al. (1974) reported decreased ATP levels in dystrophic mice. Diminished muscle ATP is also a principal feature in the reversible myopathy of vitamin E deficiency in rats, with a 22% reduction accompanied by a 118% rise in the less abundant ADP, though the total muscle adenine nucleotides alter only slightly (Dhalla et al., 1971). Thus the formation of ATP from ADP seems defective in this myopathy. This does not appear to be the case in DMD, since the normal performance of DMD mitochondria in a suitable medium (Ionasescu et al., 1967; Peter, 1968; Peter et al., 1970) suggests that the cause of the ATP deficiency lies elsewhere, perhaps in disordered muscle purine metabolism by either excessive breakdown or insufficient synthesis. Elevated serum and urinary uric acid values, the end product of purine catabolism in man, were reported by Láhoda (1972) but this present work refutes



Hypoxanthine (6-oxypurine)

Xanthine (2,6-dioxypurine)

Uric acid (2,6,8-trioxypurine)

Allopurinol (4-hydroxypyrazolo-(3,4-d) pyrimidine)

Oxipurinol (4,6-dihydroxypyrazolo-(3,4-d) pyrimidine)

FIGURE 1

these findings and indicates that the opposite is the case (see later), so that a defect in purine synthesis seems the more likely possibility. In fact, the serum uric acid values reported by Láhoda (1972) were above the level at which tophi formation could be expected (Seegmiller, 1965), a symptom never found in DMD. The DMD gene in the dystrophic nucleus may thus exert its whole effect by insufficient provision of essential muscle purines.

Smellie et al. (1956) suggest there might be 2 types of tissue; those that, like liver, can make sufficient purine nucleotide for their requirements, and those, like bone marrow, that depend on a supply of purines formed in other tissues. Much of the de novo purine synthesis seems to occur in the liver with some peripheral tissues dependant on the salvage of erythrocyte-borne hypoxanthine (Murray et al., 1970). This purine salvage pathway does in fact require only 1 molecule of ATP to produce inosinic acid, whereas de novo synthesis, using more basic substrates, requires no less than 5 (Fox, 1975). Purine metabolism in muscle itself does not appear to have been investigated.

Allopurinol, a synthetic isomer of hypoxanthine, and its metabolite oxipurinol (Figure 1) inhibits xanthine oxidase (xanthine : oxygen oxidoreductase, E.C. 1.2.3.2.) (Chalmers et al., 1968), thus reducing irreversible purine loss as uric acid. In a detailed study of xanthine oxidase inhibition Elion et al. (1966) found allopurinol to be a more potent competitive inhibitor than oxipurinol (structurally similar to xanthine). Synthesis of the enzyme is neither induced nor depressed by the subnormal activity caused by the presence of an inhibitor. Also, allopurinol is cleared from man at a rate similar to that of hypoxanthine and

xanthine but oxipurinol is cleared much more slowly with some accumulation, and probably plays a significant rôle in the theraputic effect of allopurinol. Recent work indicates there is a slow reaction whereby oxipurinol complexes with molybdenum at the active site of xanthine oxidase (Spector, 1977), indicating a further reason for the efficacy of allopurinol.

Allopurinol markedly enhances incorporation of hypoxanthine into nucleotides and nucleic acids in mice (Pomales et al., 1963), as well as increasing the exogenous hypoxanthine metabolised in man by 40% (Rundles et al., 1966), and its administration to healthy human subjects raises in vivo erythrocyte ATP levels by 30% within 3 days (Manzke and Dörner, 1975). Experimentally induced haemorrhagic shock in animals causes a reduction in tissue adenine nucleotides, particularly ATP (Chaundry et al., 1974), but pretreatment with allopurinol greatly increases the survival rate (Baker, 1972) probably by preventing the purine loss which made the shock irreversible (Crowell et al., 1969). The actual values of liver adenine nucleotides return to normal far more quickly in dogs treated with allopurinol than in untreated controls (Hopkins et al., 1975). Similar observations were made in other viscera (Keaveny et al., 1975), as in the ischaemic kidney of rats (Cunningham et al., 1974); and pretreatment with allopurinol for 3 days induced marked increases of ATP in the canine myocardium after operative arrest (Lindsay et al., 1975). These findings encourage expectations of similar effects in DMD muscle after allopurinol, with accompanying clinical improvement dependent on the mass of muscle remaining. Allopurinol has the added benefit of being an exceptionally well tolerated drug (Rundles et al., 1963)

since toxic reactions , usually mild cutaneous appearances which quickly subside when treatment is discontinued, are very rare, occurring only in patients with severe gout or impaired renal function. Sublingual PA3, NA3 and placebo tablets were administered to imperfectly diagnosed DMD patients (Golarz and Bourne, 1962; Bourne and Golarz, 1962) but slight beneficial effects were observed only after 1 year and only with PA3. The ineffectiveness of NA3 may be explained by Gershon and Fox (1974) who found reduced phosphoribosylpyrophosphate (PRPP) values after oral nicotinic acid. The rate of de novo purine synthesis is, in part, determined by the PRPP concentration (Fox and Kelley, 1971). Bourne and Golarz (1962) found it necessary to use PA3 since the proliferating endomysium dephosphorylated the 5'- but not the 3'-adenylate, the latter then being converted on absorption to the active 5'-adenylate. Various nucleotides and nucleosides given by intravenous infusion likewise had a slight beneficial effect (Thomson and Guest, 1963).

This study tests the hypothesis of a purine metabolic defect in DMD by a double-blind trial of allopurinol, used to promote salvage and re-cycling of what purines are available, and of PA3 and PA5 superimposed on allopurinol. A separate study of the adenine nucleotide content of DMD muscle, before and after treatment with allopurinol, compared with that of healthy controls, was also undertaken.

CHAPTER II

METHODS AND MATERIALS

Pyrex borosilicate glassware and Grade A soda glass pipettes were used throughout; these were cleaned by steeping for at least a week in 30% AR nitric acid, repeated rinsing in glass-distilled water then drying in covered beakers at 125°C or for pipettes, by AR acetone and suction. The silica photometric cells, of 1 cm light path, were cleaned by repeated rinsing in distilled water when re-used on the same day, with immersion in nitric acid overnight. Detergents or chromate, giving ions liable to firm adsorption on glass surfaces, were avoided.

Serum and plasma assays

Serum and plasma enzyme activities were continuously measured as linear absorbance changes against time, using a Unicam SP 800 flat-bed recording double-beam spectrophotometer with fixed wavelength attachment (SP 820), automatic re-cycling cell changer (SP 830) and programme controller (SP 825). 4 reactions could be followed simultaneously against 4 blanks in the constant temperature water-jacketed cell housing (SP 874), thermostatically controlled by circulated water from a constant-temperature bath regulated by a Circotherm turbine-heater working against a cooling coil bearing a slow current from the cold-water rising main.

Whole blood was taken from an antecubital vein using a 1½ inch 21-gauge needle and sterile disposable syringe, isopropanol skin toilet, minimal venous stasis and gentle handling to avoid

haemolysis. All venepunctures were performed by Dr. W.H.S. Thomson. The blood was left to clot at room temperature for up to an hour in a clean dry glass container. Serum was used throughout except later in a separation of erythrocytes (rbcs) from leucocytes and platelets (wbcs). Serum or plasma was separated by centrifugation at 2,000g, and again at 3,200g to remove the last blood cells. These procedures were always carried out in subdued light as direct sunlight, even for short periods, can cause a loss of up to 25% of the CPK activity (Thomson, 1969b). Where necessary distribution before clotting, into stoppered graduated Pyrex tubes, was direct from the syringe or by a marked wide-bore Pasteur pipette.

Treatment of blood and serum specimens included incubation for 24 hours at 25±0.1°C in a water bath and at 1.5°C±0.1 in a 25% ethylene glycol bath, using a Grant CCK cooling coil and 2.5 cm expanded polyurethane insulation, or successively for 1 or 6 hours at 1.5°C then at 25°C to complete the 24 hour period. Certain clotted or heparinised specimens were vortex mixed (Whirlimixer) for 60 seconds for mechanical haemolysis after 24 hours at 1.5°C, to increase rbc fragility. Serum was also stored in a cold room (24 hour range -3 to +8°C, mean 4°C) or deep-frozen at -21.5°C.

Subjects were normal healthy adult males and females with no family history of DMD, DMD patients, female DMD carriers and other females from DMD pedigrees, as well as patients with the similar but far milder X-linked BMD and female BMD carriers. Classically affected male relatives of carriers of DMD and BMD are denoned by; s, son; b, brother; mb, mother's brother; ss, sister's son; mss, mother's sister's son; and in BMD where affected males may reproduce unlike DMD, additionally by; f, father; and mf, mother's father.

Cases of myocardial infarction and viral hepatitis A were also included.

The normal ranges of some serum enzymes were ascertained in July and again in November of 1975, using subjects in good health and active daily employment, and with DMD free pedigrees. On each occasion 24 males (aged 18.14 to 61.92 years) and 24 females (aged 17.41 to 65.56 years) were bled, many individual being common to both series. In July, blood specimens were taken at intervals over 2 successive days from subjects randomly divided in 2 equal groups. In November all subjects were bled at intervals on the same day.

Rbcs and wbcs were separated in blood from 3 healthy subjects, 40-50 ml blood was taken and divided between 4 glass vials coated inside with ion-free heparin (Evans "Pullarin") and promptly mixed by gentle rotation before combining into 2 equal volumes of heparinised blood. These were centrifuged for 15 minutes at 400g and the resulting cloudy wbc-rich supernatants were pipetted into 2 other tubes and centrifuged at 3,200g for 5 minutes to give a pellet of wbcs in clear plasma. This plasma was then carefully flushed through the respective packed rbcs and the whole process repeated 5 or 6 times. After the last spin 1 of the supernatants was restored to its packed rbcs (the other rbcs were discarded) and this rbc-rich suspension was well mixed and divided into 3 portions - for plasma assay forthwith, after chilling for 24 hours at 1.5°C and after cell disruption (Whirlimixer) for 60 seconds following 24 hours at 1.5°C. A portion of the other supernatant was retained for assay forthwith, and the remainder was flushed through all the wbcs. As most of the wbcs had adhered to each

other, making uniform dispersion impossible without causing cell damage, the whole was chilled at 1.5°C for 24 hours, then centrifuged at 3,200g to allow removal of an aliquot of cell free plasma before cell disruption (as before). The necessary corrections of the results were made in respect of the same number of wbcs in different known volumes of plasma.

The effect of exercise was studied in a healthy male. He was bled at 10am and the 3x2 ml portions of clotted blood taken had serum enzyme assays performed forthwith, after 24 hours at 1.5°C and also on cell disruption after this chilling. That same evening the subject undertook 2 hours of continuous severe physical exertion. Venepunctures were performed at the same time on the following 5 consecutive mornings and the specimens obtained were likewise divided and treated before serum assay.

Creatine phosphokinase (ATP : creatine phosphotransferase, E.C. 2.7.3.2.; CPK) was measured by the method of Oliver (1955), modified by Rosalki (1967):-

$$\text{ADP} + \text{CP} \xrightarrow{\text{CPK}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH}$$

CPK acts in its optimal direction at near-physiological pH, and, with HK, G-6-PDH, ADP and NADP in excess, is rate limiting for the production of indicator NADPH. CPK activity was measured by rate of increase in absorbance at 340 nm, linear during the 15 minute period of observation. 2 reconstituted commercial kits were used; that of Boehringer Mannheim GmbH (The Boehringer Corporation (London) Ltd.) (CPK-Bg), with glutathione as CPK activator and 10 mM AMP to inhibit adenylate kinase (ATP : AMP phosphotransferase,

E.C. 2.7.4.3.; AK) and that of J.T. Baker Cemicals B.V. Deventer, Netherlands (Diamed Diagnostics, Liverpool) (CPK-Bk), with dithiothreitol and 11.5 mM AMP. AK is not completely inhibited by the addition of AMP to the CPK assay mixture and so may falsely elevate CPK activity by supplying ATP in the presence of excess ADP:-

$$2\ ADP \xrightarrow{AK} AMP + ATP$$

This increment (AKI) was measured using the Baker kit which has the CP separate, allowing its substitution with saline so that the actual CPK activity (nett CPK-Bk) could be obtained by subtracting AKI from each CPK-Bk assay. Activity, expressed as International Units per litre at 25°C, was measured against air using 0.1 ml serum to give a final volume of 2.72 ml (CPK-Bg) or 2.3 ml (CPK-Bk and AKI). CPK was also measured using the Boehringer and Baker single-assay vials. Test concentrations were the same as the multiple-assay kits of both systems. The Baker procedure was unchanged but with the Boehringer kit 0.1 ml serum was added to a vial whose contents had previously been dissolved in 2.5 ml of prepared buffer, the whole was then transferred to a cuvette for assay.

Aldolase (fructose-1,6-diphosphate : D-glyceraldehyde-3-phosphate-lyase, E.C. 4.1.2.7.; ALD) was measured by the slightly modified method of Ludvigsen (1963):-

$$F\text{-}1,6\text{-}P \xrightarrow{ALD} DHAP + GA\text{-}3\text{-}P$$

$$GA\text{-}3\text{-}P \xrightarrow{TIM} DHAP$$

$$2\ DHAP + 2\ NADH \xrightarrow{GDH} 2\ \alpha\text{-glycerophosphate} + 2\ NAD$$

With ALD rate limiting for the disappearance of indicator NADH, since TIM, GDH, F-1,6-P and NADH are in excess, ALD activity was

measured by a linear decrease in absorbance against time at 340 nm for 20 minutes. In the Boehringer kit 0.2 ml serum gave a final volume of 2.76 ml and activity, expressed as IU/l at 25°C, was measured at 37°C against a blank of 0.2 ml serum in saline.

Lactate dehydrogenase (L-lactate : NAD oxidoreductase, E.C. 1.1.1.27.; LDH) was measured by the method of Wróblewski and LaDue (1955):-

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}$$

LDH acts in its optimal direction and activity was measured by the disappearance of indicator NADH, using excess pyruvate and NADH, as a linear decrease in absorbance against time at 340 nm for 10 minutes. The Boehringer "optimized" kit was used, with 0.1 ml serum added to give a final volume of 3.15 ml with activity, expressed as IU/l at 25°C, measured against air. LDH was also measured using the Boehringer single-assay vial. The test concentration remained the same, and 0.1 ml serum was added to the vial with its contents already dissolved in 2.5 ml of buffer. The whole was then transferred at once to a cuvette for assay.

Aspartate aminotransferase, formerly glutamic-oxaloacetic transaminase, (L-aspartate : 2-oxoglutarate aminotransferase, E.C. 2.6.1.1.; GOT) measurement was based on the method of Karmen (1955):-

$$\text{aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{GOT}} \text{glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}$$

GOT activity was measured by the disappearance of indicator NADH, in the presence of excess aspartate, 2-oxoglutarate, NADH and MDH, as a linear decrease in absorbance against time at 340 nm for 10 minutes. The Boehringer "optimized" kit was used, with 0.5 ml serum giving a final volume of 3.7 ml with GOT activity, measured

against air, expressed as IU/L at 25°C.

Alanine aminotransferase, formerly glutamic-pyruvic transaminase (L-alanine : 2-oxoglutarate aminotransferase, E.C. 2.6.1.2.; GPT) was measured by the method of Wróblewski and LaDue (1956):-

alanine + 2-oxoglutarate $\xrightarrow{GPT}$ glutamate + pyruvate

pyruvate + NADH $\xrightarrow{LDH}$ lactate + NAD

GPT activity was measured by the disappearance of indicator NADH, with alanine, 2-oxoglutarate, NADH and LDH in excess, as a linear decrease in absorbance against time at 340 nm for 10 minutes. The single-assay vial Boehringer "optimized" kit was used, with 0.5 ml serum added to the vial, the contents of which had already been dissolved in 3.0 ml buffer, and the whole was transferred immediately to a cuvette for measurement of activity against air expressed as IU/l at 25°C. GOT activity was also measured using single-assay vials, by the same procedure.

γ-glutamyl transferase (amino acid : γ-L-glutamyl transferase, E.C. 2.3.2.2.; γ-GT) measurement was based on the method of Szasz (1969):-

glycylglycine + L-γ-glutamyl-p-nitroaniline

$\xrightarrow{\gamma\text{-GT}}$ glutamylglycylglycine + p-nitroaniline

γ-GT activity was measured by the increasing appearance of coloured p-nitroaniline, with glycylglycine and γ-L-glutamyl-p-nitroaniline in excess, as a linear increase in absorbance against time at 405 nm for 10 minutes. The Boehringer single-assay vial was used, with 0.2 ml serum added to the vial, the contents of which had already been dissolved in 3.0 ml buffer, and the whole was transferred at once to a cuvette for measurement of activity, against air expressed as IU/l at 25°C.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1.; Alk P) was measured by the method of Bessey et al. (1946):-

p-nitrophenylphosphate $\xrightarrow{\text{Alk P}}$ p-nitrophenol + Pi

Alk P activity was measured by the increasing appearance of coloured p-nitrophenol, with p-nitrophenolphosphate in excess, as a linear increase in absorbance against time at 405 nm for 10 minutes. The Boehringer kit was used with 0.05 ml serum in 3.0 ml of buffered substrate; activity, measured against air, was expressed as IU/l at 25°C.

Pyruvate kinase (ATP : puruvate phosphotransferase, E.C. 2.7.1.40.; PK) measurement was based on the method of Beisenherz et al. (1953):-

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}$$

With PK rate limiting for the disappearance of indicator NADH, since PEP, ADP, NADH and LDH are in excess, activity was measured by a linear decrease in absorbance against time at 340 nm for 10 minutes, self-corrected by reading for 10 minutes before adding the ADP to start the PK reaction. The Boehringer kit was used, with a test concentration of 1.0 mM PEP and 0.5 ml serum added to give a final volume of 3.25 ml. PK activity was expressed as IU/l at 25°C, and measured against air. To estimate the contribution made by each isoenzyme (i) liver and erythrocyte PK was inhibited (Seubert et al., 1967; Llorente et al., 1970; Harano et al., 1973) by adding 2.0 mM alanine to the reaction mix to give PK-Ala, (ii) the muscle form, being isosteric, had adequate activity at 0.2 mM PEP giving PK-M, unlike the liver form where allosterism and sigmoid kinetics gave very little activity below 0.3 mM PEP

(Tanaka et al., 1967a; Llorente et al., 1970; Harano et al., 1973), and (iii) both forms of PK were measured at 2.0 mM PEP to give PK-LM. From PK and PK-Ala the percentage inhibition by alanine was calculated; likewise the ratio of PK-M to PK-LM. The alanine was added to the standard kit in 0.01 ml and different PEP solutions (monosodium salt, Boehringer) were added to otherwise unchanged assay conditions. The allosteric properties of liver and erythrocyte PK are lost on chilling but recover rapidly at ambient temperatures (Llorente et al., 1970; Harano et al., 1973), therefore all frozen or chilled sera were left at room temperature for at least 2 hours before assay.

Glucose was measured by the method of Schmidt (1961):-

$$\text{glucose} \xrightarrow{\text{HK}} \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH}$$

Glucose concentration, expressed as mg/100 ml, was measured by the increase in absorption at 340 nm (Unicam SP 500 Series 2 spectrophotometer) by NADPH, formed in the presence of excess HK, G-6-PDH and NADP. The Boehringer kit was used with 0.2 ml of a 1:10 dilution of serum in saline giving a final volume of 2.90 ml The reaction was started by adding 0.02 ml of mixed enzymes and followed to the end point, usually within 10 minutes.

Sodium (Na^+) and potassium (K^+) were measured on an Instrumentation Laboratory 143 flame photometer, with inbuilt lithium standard, digital read-out and requiring only 0.1 ml serum. Values are expressed as mequiv/l and the analyses were carried out by the University Department of Chemical Pathology, Royal Hospital for Sick Children, Yorkhill, Glasgow.

Uric acid was measured by the method of Kageyama (1971):-

$$\text{uric acid} \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{methanol} \xrightarrow{\text{catalase}} 2H_2O + \text{formaldehyde}$$

$$\text{formaldehyde} + \text{acetylacetone} + NH_3 \longrightarrow 3,5\text{-diacetyl-1,4-dihydroxylutidine}$$

Uric acid concentration is directly measured as mg/100 ml by increased absorbance of indicator diacetyl-dihydroxylutidine with uricase, catalase, methanol, acetylacetone and ammonia present in excess. The Boehringer Urica-quant kit was used with 0.5 ml serum added to a 5.0 ml reagent volume and incubated at 37°C for 60 minutes before measuring the increase in absorbance at 410 nm (Unicam SP 500 Series 2 spectrophotometer). This method was chosen as others, which do not use the very specific uricase, are not so precise (Müller et al., 1974) and are prone to serious interference with occasional false elevations (Lum and Gambino, 1973). Uricase was first used by Kalchar (1947) and later by Praetorius and Poulsen (1953) whereby uric acid was determined, directly at 290 nm, by the decrease in absorbance after uricase action. The Kageyama (1971) method, with uric acid breakdown coupled to the formation of a coloured product, excludes deproteinisation and is therefore much simpler; it is also free from turbidity errors that may occur when a wavelength in the ultra-violet range is used.

24 hour urinary outputs

24 hour urine collections were arranged at home between 8 am Sunday and 8 am Monday using a polythene funnel and a 2.5 litre (Winchester) amber bottle containing 4 ml toluene (AR) as a presrvative and 10 ml saturated aqueous lithium carbonate (AR)

to prevent urate precipitation, so enabling the urine to be stored at room temperature for up to 3 days before analysis (Chalmers and Watts, 1968; Liddle et al., 1959).

After 10 fold dilution with distilled water, urinary uric acid was measured as serum, and output expressed in mg/24 hours. Urinary creatinine was measured by the method of Folin and Wu (1919) based on the Jaffé reaction (creatinine/alkaline picrate) and adapted to Auto Analyser; output was expressed in mg/24 hours. The determinations were routinely made by the Biochemistry Department, Glasgow Royal Infirmary.

Clinical design of double-blind trial

The effects of allopurinol and later, sublingual procaine adenylates, were tested by double-blind techniques. 16 boys (aged 3.39 to 14.29 years) took part in the trial; 9 were ambulant and 7 confined to the wheelchair. All had clinically classical DMD confirmed by grossly elevated serum enzyme values, notably of CPK, and additionally in all but 3 by characteristic histology at muscle biopsy and/or genetically, with brothers, maternal uncles or mothers carrying the gene.

In each patient the maximum height in cm was found to which he could, using one and/or both hands, in a single continued exhaustive attempt, raise the mercury in a laboratory manometer by rapidly pumping air from a small rubber hand-bulb into a 500 ml resevoir connected to the manometer. In addition, each ambulant patient was timed (in seconds) ascending the same flight of 10 stairs, as fast as possible.

Clinical status was measured by Table XIX. Repeated examination of the patients gave this 16 point scale of observed disabilities, beginning with the worst and ending with the least. Each patient was placed on the scale at the point of his maximum capability, confirming always that he could perform every action up to and including this point, but none beyond it. For each patient a change in position on the scale merely indicated an improvement (+) or no improvement (0) for statistical purposes only and could not be used to quantify the degree of physical improvement.

ECG leads I, II, III, V4R, VI and V6 were recorded in all patients and also aVR, aVL and aVF in the older children (Mrs. McLean, Knightswood Hospital, Glasgow).

Allopurinol tablets (100 mg), commercial but unmarked, were allocated at random to 8 of 16 numbered bottles, and inert matching placebo tablets to the rest. Allocations were carried out by the Wellcome Foundation Ltd., who provided the tablets and kept the key to the numbering. Procaine adenylate tablets were prepared using the modified procedure of Ruskin (1963). To 46.6 g of powdered procaine base (Sigma (London) Chemical Co. Ltd.) suspended in 900 ml distilled water at room temperature was added, with stirring, an equimolar amount (100 g) of ATP (Cambrian Chemicals Ltd.) in successive portions. The pale pink opalescent solution quickly obtained was filtered and lyophilised in an oil pump vacuum against EtOH/solid CO_2 to a pale pink microcrystalline solid, m.pt. 130-140°C (decomp.). This was ground and dried <u>in vacuo</u> to give procaine adenosine-5'-triphosphate (PA5) as an almost colourless powder. In the same way procaine adenosine-3'-monophosphate (PA3) was obtained as an exceedingly hygroscopic powder, m.pt. 88-93°C

from equimolar amounts of procaine base and adenosine-3'-monophosphoric acid (BDH Chemicals Ltd.). These procedures were carried out by Dr. W.H.S. Thomson.

Atomic absorption spectrophotometry (Mr. A.G. Hill, BDH) showed less than 1, 1, 5 and 0.5 parts per million of barium, cadmium, lead and arsenic respectively in both preparations, and semi-quantitative arc emission spectrography likewise confirmed the absence of toxic elements.

Sub-lingual tablets, peppermint flavoured and with a dissolution time of 20-30 minutes, each contained 150 mg PA3 or PA5 (about 100 mg adenosine phosphate) and were prepared by Arthur Cox and Co., Brighton. In matching placebo tablets lactose replaced PA3 and PA5, with a small amount of anhydrous citric acid and "Bitrex" solution added to give a sourness and bitterness matching that of the active tablets. This nasty taste could be somewhat masked by peppermint sweets, though most patients soon became used to it.

PA3 tablets were allocated at random to 4 of 8 numbered bottles, and placebo tablets to the rest; this was repeated in 8 more bottles for PA5. Allocations were made by the pharmacist at Knightswood Hospital, Glasgow, who kept the key to the numbering.

Patients were divided into 4 groups and examined before noon on the same day each week for 18 consecutive weeks. The first 6 weeks (period A), in which nothing was given, was used to assess clinical status of the patients and to determine the mean of enzyme activities and metabolite values. For the next 6 weeks (period B) each patient, irrespective of age or weight, took one

tablet daily from the allopurinol/placebo bottle allocated to him. The same dose was given to every patient as the preparation of a multiplicity of tablets precisely correct for each individual would have made the conduct of a double-blind procedure impossible. Since clinical change was not quantified, but measured as improvement (+) or no improvement (0), there was no threshold below which allopurinol would not have an effect. Variations in dose with respect to age or weight randomly affected both groups, and were without statistical consequences. The contents of the bottles were revealed at the end of period B and for the last 6 weeks period C all patients took one unmarked allopurinol tablet daily; in addition, those who already had allopurinol in period B received one of the PA5/placebo bottles and those who were on the placebo received one of the PA3/placebo bottles. Each patient took 4 sub-lingual tablets daily during period C after which the contents of the bottles were again revealed.

At weekly examination, venepuncture for serum enzymes (CPK-Bk, AKI, ALD, GOT and δ-GT) and urate was followed by testing performance using manometry and stairs. The 24 hour urine collection for urate and creatinine output was also received. After each 6 week period clinical status was assessed in terms of the scale in Table XIX. The activity of serum CPK-Bk in DMD patients is so high that dilution of the serum with saline is necessary, and since activity may vary considerably with the degree of dilution (Thomson, 1969b; Snehalatha et al., 1973) repeated estimations in each individual were always carried out at the same dilution. The ECG was taken at the end of each period.

Assay of metabolites in muscle

The amounts of ATP, ADP, AMP, CP and G-6-P were determined in muscle of a) 5 DMD patients (aged 3.67 to 10.14 years) immediately before and, in 4 of these 5, 6 months after taking allopurinol daily, b) 5 DMD patients (aged 4.57 to 9.46 years) after daily allopurinol for approximately a year, and c) 5 healthy male controls (aged 12.51 to 56.32 years) undergoing surgical repair.

DMD patients (all ambulant) were admitted to hospital at 9 am, no premedication was given and the subjects were anaesthetised with N_2O/O_2/halothane. At open biopsy 1-2 g of muscle was quickly dissected from the vastus lateralis (Dr. Alex McQueen, Department of Dermatology, Glasgow University). Within 15-30 seconds the tissue was freeze-clamped and immersed in liquid N_2 in a Dewar flask. Less than 30 minutes later in the laboratory the specimen was rapidly weighed and powdered under liquid N_2 in a pre-cooled mortar and pestle. An aliquot (0.3 to 0.9 g) of powdered muscle was taken, weighed and kept at -21.5°C until the non-collagen nitrogen (NCN) estimation could be carried out.

5.0 ml 6% perchloric acid was added in 1 ml lots to the remaining muscle powder under liquid N_2 in the mortar and the whole ground up together. The N_2 was then allowed to evaporate, and the powdered perchloric acid/tissue immediately transferred to a large tube and left to thaw on ice; protein precipitation would occur simultaneously with tissue warming as perchloric acid thaws first. The resulting preparation was finely homogenised (Ultra-Turrax) on ice for 30 seconds, centrifuged at 3,200 g for 10 minutes. After pipetting off the supernatant the pellet was resuspended in 5.0 ml

3% perchloric acid and recentrifuged. The 2 supernatants were combined, filtered, and two 2 ml portions neutralised for duplicate determination (pH 6.95-7.05 by 10 and 0.1 M KOH using a Pye Dynacap pH meter and glass electrode). The exact volume of KOH added was noted. This neutralised extract was left on ice for 10 minutes, decanted from the sediment of $KClO_4$ and analysed forthwith.

The following assay conditions were based on those used by Stengel-Rutkowski and Barthelmai (1973) and Bergmeyer (1963); and G-6-P, ATP and CP were measured sequentially by addition of the appropriate substrates and enzymes to the test mixture in a cuvette.

Test concentrations:-

Triethanolamine hydrochloride buffer, 90 mM, pH 7.6

Diaminoethanetetra-acetic acid, disodium salt (EDTA), 9 mM

$MgSO_4$, 12.5 mM

Nicotinamide adenine dinucleotide phosphate, oxidised form, disodium salt (NADP), 0.4 mM

D-glucose, 1.3 mM

Adenosine diphosphate, disodium salt (ADP), 0.4 mM (ATP contamination<1%)

Glucose-6-phosphate dehydrogenase, grade I from yeast (G-6-PDH), 0.14 IU

Hexokinase, from yeast (HK), 5.6 IU

Creatine phosphokinase, from rabbit muscle (CPK), 1.8 IU

All biochemicals were supplied by Boehringer Mannheim GmbH.

Principle of the assays:-

$$CP + ADP \xrightarrow{CPK} \text{creatine} + ATP$$

$$ATP + \text{glucose} \xrightarrow{HK} ADP + \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH}$$

In the presence of sufficient NADP, G-6-PDH reacts quantitatively with G-6-P to form indicator NADPH. The addition of HK ensures phosphorylation of added glucose, using the ATP of the sample, to G-6-P which is then oxidised, producing indicator NADPH. If ADP and CPK are then added all 3 coupled reactions proceed quantitatively allowing the assay of CP.

The following were therefore pipetted into a cuvette:-

Solution	Conc. of stock soln.	Volume (ml)	
Buffer	0.12 M	2.5	
EDTA	0.15 M	0.2	
$MgSO_4$	0.21 M	0.2	
NADP	12.5 mM	0.1	
Sample		0.1	
			Absorbance$_1$: A_1
G-6-PDH	7 IU/ml	0.02	
			Test Volume 3.12 ml : A_2
			$A_2-A_1 = \Delta A_{G-6-P}$
Glucose	22.0 mM	0.2	
			A_3
HK	280 IU/ml	0.02	
			Test Volume 3.34 ml : A_4
			$A_4-A_3 = \Delta A_{ATP}$
ADP	27.6 mM	0.05	
			A_5
CPK	90 IU/ml	0.02	
			Test Volume 3.41 ml : A_6
			$A_6-A_5 = \Delta A_{CP}$

The initial absorbance was read, before any adition of enzymes, for a period of 3-5 minutes (A_1). With G-6-PDH added the change in absorbance was followed until the reaction stopped (A_2). The difference between A_2 and A_1 (ΔA_{G-6-P}) was due to the G-6-P content of the sample. If the G-6-PDH preparation had been contaminated with PGI the result would be an estimation of the total hexose monophosphate present and not just G-6-P. This was not the case, however, as a reaction did occur when, after reading A_2, PGI was added. This indicated that no PGI was originally present and that A_2-A_1 was in fact a measure of G-6-P only.

A_3 was obtained within 30 seconds of the addition of glucose, and HK, to start the ATP reaction, was then quickly added. These 2 precations prevent any significant reaction of ATP with the HK contaminating the G-6-PDH preparation; for the same reason glucose was not added until after the initial reaction was complete. The absorbance was followed until there was no further reaction of ATP (A_4), and the difference between A_4 and A_3 (ΔA_{ATP}) gave a measure of the ATP present. ADP was added to the test mix and, after the contaminating ATP had reacted, the absorbance (A_5) was noted before the final addition, that of CPK, was made. Again the reaction was followed to completion (A_6) and the difference between A_6 and A_5 (ΔA_{CP}) measured the CP content of the sample.

ADP and AMP were also measured sequentially.

Test concentrations:-

Triethanolamine hydrochloride buffer, 90 mM, pH 7.6

EDTA, 9 mM

$MgSO_4$, 12.5 mM

Nicotinamide adenine dinucleotide, reduced form, disodium salt (NADH), 0.4 mM

Phosphoenol pyruvate, monosodium salt (PEP), 12.5 mM

Lactate dehydrogenase, from rabbit muscle (LDH), 3.6 IU

Pyruvate kinase, from rabbit muscle (PK), 1.2 IU

Adenylate kinase, from rabbit muscle (AK), 1.4 IU

All biochemicals were supplied by Boehringer Mannheim GmbH.

Principle of the assays:-

$$AMP + ATP \xrightarrow{AK} 2\ ADP$$

$$ADP + PEP \xrightarrow{PK} ATP + pyruvate$$

$$pyruvate + NADH \xrightarrow{LDH} lactate + NAD$$

In the presence of sufficient PEP, PK reacts with ADP to form pyruvate which, with excess NADH and LDH, quantitatively diminishes the amount of indicator NADH. If ATP and AK are then added in excess the 3 coupled reactions will proceed sequentially allowing the assay of AMP.

The following were therefore pipetted into a cuvette:-

Solution	Conc. of stock soln.	Volume (ml)
Buffer	0.12 M	2.5
EDTA	0.15 M	0.2
$MgSO_4$	0.21 M	0.2
PEP	0.21 M	0.2
NADH	13.5 mM	0.1
LDH	180 IU/ml	0.02
Sample		0.1
		A_1
PK	60 IU/ml	0.02
		Test Volume 3.34 : A_2
		$A_2-A_1 = \Delta A_{ADP}$

AK 70 IU/ml 0.02

Test Volume 3.36 ml : A_3

$A_3 - A_2 = \Delta A_{AMP}$

The indicator reaction with LDH was allowed to settle for 3-5 minutes and, after the absorbance was read (A_1), PK was added to start the ADP reaction which was then followed to completion (A_2). The difference between A_2 and A_1 (ΔA_{ADP}) was due to the ADP content of the sample. AK was added and the change in absorbance was followed until the AMP reaction stopped, when the difference between A_3 and A_2 (ΔA_{AMP}) measured the AMP present in the sample.

All these estimations of muscle metabolite contents were made, in duplicate, on a Unicam SP 500 Series 2 spectrophotometer at 340 nm against the same reaction mix with saline replacing the sample. The results are expressed in moles x 10^{-6}/g of muscle (wet weight) and moles x 10^{-5}/g of non-collagen nitrogen (NCN).

In the calculation of these results the following formula was used:-

$$\frac{\Delta A \times V_K \times V_2 \times V_4}{E \times d \times V_1 \times V_3 \times V_5} = \text{moles} \times 10^{-6}/\text{g of muscle (wet weight)}$$

Where:-

V_K = volume in cuvette after the addition of enzyme to start the reaction (test volume, ml)

V_1 = weight of the muscle sample (g)

V_2 = V_1 + total weight of perchloric acid used for the deproteinisation (g)

V_3 = volume of deproteinised extract before neutralisation (ml)

V_4 = volume of deproteinised extract after neutralisation (ml) (i.e. V_3 + volume of KOH required for neutralisation)

V_5 = volume of extract added to the cuvette (ml)

E = extinction coefficient of NADH and NADPH; E = 6.22 cm^2/mole x 10^{-6}

d = light path (1 cm)

The AMP calculation was altered to take into account the oxidation of 2 moles of indicator NADH by 1 mole of AMP.

Though the absorbance usually remained constant after completion of a reaction, there was occasionally a small progressive change. This drift, which appeared always to be linear with time, was assumed to be superimposed throughout the whole reaction, and was corrected by extrapolating to the start of the reaction. To ensure the greatest accuracy the plot was estimated from the regression line, calculated by the method of least squares. The drift was found only in the assay of ATP and CP, and may be explained by contamination of both HK and CPK by AK. A small amount of AK could cause the ADP present to dismute to AMP and ATP, the latter then being introduced to the coupled reactions to give a small but steady formation of NADPH, linear with time and actually measuring the slight activity of AK. No other reasons are apparent.

To determine the NCN content the powdered frozen muscle set aside was removed from the deep-freeze and allowed to thaw. The sample was homogenised (Ultra-Turrax) in a known volume of distilled water (about 10% w/v). 1 volume of this homogenate was added to 10 volumes of 0.05 M NaOH, stoppered, mixed and allowed to stand for 18 hours at room temperature before filtration (Whatman No. 1 paper) to give the alkali digest, according to the method of Lilienthal *et al*. (1950). The nitrogen content of this digest was estimated, (Miss Moira Young, Department of Animal Husbandry,

Glasgow University) by micro-Kjeldahl digest and titration, since colourimetry (Nessler) proved unsatisfactory. It is expressed as mg/g of muscle (wet weight).

Statistics

For brevity, statistical significance may be denoted by *($P<0.05$), **($P<0.01$) and ***($P<0.001$), NS denoting no significance. Unless otherwise stated significant differences between means were estimated using Student's "t" test.

CHAPTER III

SERUM ENZYMES AND DMD CARRIER DETECTION : RESULTS AND DISCUSSION

Serum enzyme assays with commercial kits

Owing to increasing diversity of serum enzyme assays in diagnosis, many laboratories now depend on reconstituted commercial kits. Those measuring reaction rates spectrophotometrically at 340 nm may give erroneous results. Occasional omissions, such as cofactors, are immediately evident; but unsuspected contamination, apparently by a small quantity of the very enzyme to be measured, is increasingly common, though far less obvious, and may be due to its presence in accessory indicator enzymes prepared from the same parent tissue.

Contamination blank values (Table I) were obtained, as perfectly linear readings over 20 minutes, when normal saline was used instead of serum in freshly prepared multiple-assay kits from a well-known manufacturer (Boehringer Mannheim GmbH). In the case of CPK, however, the blank value for a particular kit, read in quadruplicate weekly for 4 weeks storage at $4^{o}C$, did remain constant at 8.16, so that subtraction of this blank from values using serum gave very close correlation (CPK-Bg, Table VII) with those obtained from the same serum using the J.T. Baker multiple-assay vial (CPK-Bk), which had no blank value at all (though that of their single-assay vial ranged from nil to 2.04). Thus where specimens go to the same laboratory performing frequent CPK assays, a simple correction gives dependable results. This correction was made throughout the work reported here.

Table I opposite

Contamination blank values, expressed as i.u./l at 25°C, obtained when saline was substituted for serum in commercial multiple-assay kits used for the determination of serum enzyme activities.

Table II opposite

Contamination blank values, expressed as i.u./l at 25°C, obtained when saline was substituted for serum in commercial single-assay vials used for the determination of serum enzyme activities.

Table III opposite

Normal ranges in serum of enzymes (in i.u./l at 25°C) and of electrolytes (in mequiv/l) determined previously by other workers.

TABLE I

CONTAMINATION BLANK VALUES (Multiple-assay kits)

Assay	Catalogue No.	Contamination
CPK-Bg	15926	11.66, 9.33, 5.25 (3 kits, same batch no.) 8.46, 6.71 (2 kits, same batch no.)
ALD	15974	0.43, 0.39 (2 kits, same batch no.)
GOT	15923	2.14
GPT	15925	1.01
LDH	15977	Nil
PK	15985	Nil

TABLE II

CONTAMINATION BLANK VALUES (Single-assay vials, boxes of 20)

Assay	Catalogue No.	Mean	Recorded range	Mean ± 2 S.D.
CPK-Bg	15970	2.13	0.42 - 4.18	0 - 4.30 } different
		3.08	1.25 - 6.06	0.96 - 5.21 } batch nos.
GOT	15564	1.45	0.51 - 2.25	0.71 - 2.16
GPT	15565	1.71	1.18 - 2.70	0.94 - 2.48
LDH	15561	7.55	2.99 - 23.43	0 - 17.36

TABLE III

NORMAL RANGES

Assay	Normal males	Normal females	
Na^+	135.10 - 143.33	135.20 - 144.44	(Sweetin and Thomson, 1973a)
K^+	3.55 - 4.61	3.71 - 4.72	
ALD	1.07 - 2.34	0.81 - 2.27	
LDH	95.17 - 166.13	80.16 - 178.26	
GOT	6.90 - 15.47	6.78 - 12.61	
GPT	5.12 - 23.56	4.40 - 15.95	
γ-GT	4.20 - 31.19	2.75 - 16.97	
Alk P	63 - 160	41 - 154	(Hausamen et al., 1967)

In small laboratories where CPK is rarely requested, however, boxes of 20 lyophilised single-assay vials (Boehringer) are used instead of multiple-assay kits. Since a saline blank in such a vial distorts conditions for subsequent serum assay, it might be assumed no blank is necessary, or that the blank value is the same in all vials from the same box and with the same batch number. Individual blank values, which varied widely, were nevertheless obtained (as above) from each single-assay vial in complete boxes of 20 (Table II), and these varying values are greatly multiplied if diluted serum is used. In GOT and GPT the contamination is small, with little variation between vials, and presents no problem; but blank values from the inexplicable LDH vials, which contain only pyruvate, NADH and phosphate buffer, vary by as many as 20 units and might easily influence diagnosis in the anginal infarct seen too late for other enzymes. In CPK, however, even a few units above or below the female upper limit (Table IV) may confirm or deny a high risk of the carrier state for females in pedigrees of DMD; so that an unluckly CPK vial that adds or subtracts a random gratuitous 6 units or so might decide a needless abstinence from reproduction, or worse, the appearance of dystrophic offspring in families falsely reassured. In such circumstances the single-assay vials should be avoided and, since CPK survives overnight posting in a filled tube of clotted blood (Sweetin and Thomson, 1973a), these specimens should be sent to a suitable laboratory where the workload justifies use of multiple-assay kits in which a constant blank value is readily determined.

Comparative studies of pyruvate kinase and creatine phosphokinase

Table III quotes normal ranges used in this work. Normal values

in healthy adults (Table IV) show that in both sexes PK and PK-LM, each measuring the combined liver and muscle isoenzymes, are very similar since a PEP concentration greater than 1 mM affects each form only slightly (Tanaka et al., 1967a). Similarly PK-Ala (liver form inhibited) closely corresponds with PK-M (liver form not active). CPK-Bk appears much more sensitive than CPK-Bg, probably due to dithiothreitol in the Baker kit being a better CPK activator than the glutathione of Boehringer (Warren, 1972; Miyada et al., 1975). Further, the female normal range for CPK-Bk was estimated in July and again in November; 7 subjects were common to both series and 17 were not. For those 17, although the means were not significantly different between series the variances were**, and in the paired observations there was a significant correlation* between the mean value of, and the signed difference between, July and November as tested by non-parametric rank correlation. The interpretation of this data is that females with CPK above the mean do tend to have higher values in summer than in winter, whereas those below the mean tend to have lower values then. A similar but opposite finding in males was found not significant. Such markedly seasonal sex differences are difficult to explain and provide yet another complication in the detection of DMD carriers. Normal AKI is small in both sexes.

Significant sex differences (Table V) occur only in values of serum enzymes originating in muscle (PK-Ala, PK-M, % alanine inhibition), with very large increases in male CPK, particularly in November. In females arranged in 3 groups according to the stage of their menstrual cycle (Table VI) the means for post-menopausal women were higher than the others, though not significantly so, perhaps due to the known decline of ovarian oestrogens.

Table IV opposite

Levels of serum enzymes (in i.u. /l at 25°C) determined in 24 normal males (aged 18 to 62 years) and 24 normal females (aged 17 to 66 years).

TABLE IV

NORMAL VALUES

Sex	Assay	Mean	Standard deviation	Recorded range	Mean ± 2 S.D.
Male	PK	17.55	4.09	9.37 - 25.19	9.36 - 25.73
	PK-LM	18.62	4.48	10.24 - 27.80	9.66 - 27.58
	PK-Ala	11.69	2.81	6.38 - 18.29	6.06 - 17.31
	PK-M	10.43	2.76	4.60 - 15.68	4.91 - 15.95
	% alanine inhibn.	33.19	5.68	22.83 - 43.49	21.83 - 44.55
	Ratio PK-M/PK-LM	0.56	0.07	0.44 - 0.69	0.43 - 0.69
	CPK-Bg (July)	35.97	14.01	17.78 - 69.68	7.95 - 63.99
	CPK-Bk (July)	44.54	16.31	21.46 - 81.40	11.92 - 77.16
	CPK-Bk (Nov.)	47.46	22.02	20.23 - 96.45	3.42 - 91.51
	AKI (Nov.)	0.44	0.46	0 - 1.73	0 - 1.36
	Nett CPK-Bk (Nov.)	47.02	22.00	18.50 - 96.20	3.01 - 91.04
Female	PK	15.53	3.19	9.41 - 19.44	9.16 - 21.90
	PK-LM	16.42	3.42	9.72 - 24.14	9.58 - 23.26
	PK-Ala	9.45	1.99	5.43 - 13.48	5.48 - 13.43
	PK-M	8.64	1.85	4.81 - 13.59	4.94 - 12.34
	% alanine inhibn.	38.72	8.03	25.00 - 54.27	22.65 - 54.79
	Ratio PK-M/PK-LM	0.53	0.08	0.34 - 0.66	0.36 - 0.69
	CPK-Bg (July)	21.09	10.72	1.17 - 45.48	0 - 42.52
	CPK-Bk (July)	27.37	11.90	3.70 - 53.28	3.57 - 51.17
	CPK-Bk (Nov.)	24.06	6.27	14.55 - 34.29	11.52 - 36.60
	AKI (Nov.)	0.53	0.56	0 - 1.48	0 - 1.66
	Nett CPK-Bk (Nov.)	23.53	6.38	14.31 - 34.04	10.77 - 36.29

Table V opposite

Percentage differences in mean serum enzyme activities between the normal males and normal females shown in Table IV. In each case the difference is shown as a percentage of the level in females.

Table VI opposite

Comparisons of the mean serum enzyme activities (in i.u./l at 25°C) of the normal females shown in Table IV grouped according to the stage of their menstrual cycles.

TABLE V

% DIFFERENCES OF MALE FROM FEMALE MEANS

PK	+12.40
PK-LM	+13.39
PK-Ala	+23.63 **
PK-M	+20.65 *
% alanine inhibn.	-14.28 **
Ratio PK-M/PK-LM	+5.66
CPK-Bg (July)	+70.56 ***
CPK-Bk (July)	+62.75 ***
CPK-Bk (Nov.)	+97.33 ***
AKI (Nov.)	-22.22
Nett CPK-Bk (Nov.)	+99.79 ***

TABLE VI

MENSTRUAL CYCLE AND MEANS IN NORMAL FEMALES

Stage of cycle	Number	PK	PK-LM	PK-Ala	PK-M
1- 14 days	14	15.18	15.71	9.50	8.73
15- 28 days	4	14.94	16.38	8.42	8.02
Post-menopausal	6	15.74	18.10	10.05	8.85

		% alanine inhibn.	Ratio PK-M/PK-LM	CPK-Bg (July)	CPK-Bk (July)
1- 14 days	14	36.88	0.55	19.39	25.53
15- 28 days	4	42.74	0.50	18.59	24.67
Post menopausal	6	40.33	0.49	26.72	33.46

		CPK-Bk (Nov.)	AKI (Nov.)	Nett CPK-Bk (Nov.)
1- 14 days	9	24.06	0.52	23.54
15- 28 days	7	23.65	0.56	23.08
Post-menopausal	8	24.42	0.52	23.90

Sweetin and Thomson (1973a) did, in fact, find this difference significant for certain enzymes, and dearth of these hormones in males, together with the larger muscle bulk, may explain the sex differences noted. All modalities of PK correlate very highly in males (Table VII) but slightly less well in females. The modest correlations in males between CPK and PK, better with PK-Ala and PK-M than PK and PK-LM, are absent in females except of low significance only with the 2 muscle forms; and the more sensitive CPK-Bk usually gave a higher correlation. No correlation between CPK and AKI could be found in normal subjects.

No relation between age and any serum enzyme value was found in either sex (Table VIII) but a small decline of AKI with time of day (Table IX) did occur in males. A similar decline of AKI in females conflicts with the findings of Juchems et al. (1968) who reported that AKI (not inhibited by AMP, however) tended to increase at night. CPK in females showed a highly significant increase with time of day on one occasion in July, and, though the changes mentioned may have masked a similar relationship in November, these observations tend to confirm late afternoon as the most informative time for carrier detection (Thomson, 1968). Further, the decline with time of day of female % alanine inhibition indicates an increasing proportion of muscle PK.

In normal serum stored for 24 hours the activity of all PK modalities declined very rapidly at 25°C (Table X) and only a little less rapidly at 4°C. After only 24 hours at these temperatures PK and PK-LM declined to only a fraction of their original value. Since PK-Ala and PK-M did not decrease so far, the liver isoenzyme may be the more labile; and this difference in stability

Table VII opposite

Correlation matrices between activities of different serum enzymes in the groups of normal males and females shown in Table IV. This Table is in two halves, an upper and a lower, as indicated. The groups in the two halves were <u>not</u> identical though some males were common to both male groups and some females were common to both female groups.

TABLE VII

CORRELATION MATRICES

	PK	PK-LM	PK-Ala	PK-M	CPK-Bg
Males (22 df)					
PK-LM	0.9699***				
PK-Ala	0.9263***	0.9004***			
PK-M	0.9006***	0.8935***	0.9186***		
CPK-Bg (July)	0.5197**	0.5038**	0.5711**	0.6273***	
CPK-Bk (July)	0.5825**	0.5825**	0.6115***	0.6436***	0.9720***
Females (22 df)					
PK-LM	0.9450***				
PK-Ala	0.7993***	0.7386***			
PK-M	0.8203***	0.7886***	0.7991***		
CPK-Bg (July)	0.2131	0.2136	0.3758*	0.3768*	
CPK-Bk (July)	0.2239	0.2185	0.3515*	0.3919*	0.9860***

	CPK-Bk (Nov.)	AKI (Nov.)
Males (22 df)		
AKI (Nov.)	0.0442	
Nett CPK-Bk (Nov.)	0.9998***	-0.0233
Females (22 df)		
AKI (Nov.)	-0.1520	
Nett CPK-Bk (Nov.)	0.9962***	-0.2371

Table VIII opposite

Regressions of serum enzyme activities (E) in i.u./l at 25°C, on age (A) in years, in the 24 normal males and 24 normal females shown in Table IV.

TABLE VIII

REGRESSIONS OF SERUM ENZYME VALUES (E) ON AGE (A) (n=24; 22 df)

Males		
PK	$E = 0.1865 - 5.1296 \times 10^{-4} A$	(t=0.9365)
PK-LM	$E = 0.1977 - 5.3907 \times 10^{-4} A$	(t=0.8974)
PK-Ala	$E = 0.1304 - 5.1410 \times 10^{-4} A$	(t=1.3969)
PK-M	$E = 0.1211 - 5.9063 \times 10^{-4} A$	(t=1.6630)
% alanine inhibn.	$E = 29.9791 + 8.8760 \times 10^{-2} A$	(t=1.1257)
Ratio PK-M/PK-LM	$E = 0.6217 - 1.7176 \times 10^{-3} A$	(t=1.9571)
CPK-Bg (July)	$E = 0.1101 + 3.6636 \times 10^{-4} A$	(t=0.5379)
CPK-Bk (July)	$E = 0.1532 + 7.5729 \times 10^{-4} A$	(t=0.8148)
CPK-Bk (Nov.)	$E = 0.1832 + 2.2954 \times 10^{-4} A$	(t=0.1883)
AKI (Nov.)	$E = 0.0022 - 1.0290 \times 10^{-5} A$	(t=0.4063)
Nett CPK-Bk (Nov.)	$E = 0.1810 + 2.3983 \times 10^{-4} A$	(t=0.1969)
Females		
PK	$E = 0.1502 - 4.0260 \times 10^{-5} A$	(t=0.0765)
PK-LM	$E = 0.1500 + 1.7666 \times 10^{-4} A$	(t=0.3130)
PK-Ala	$E = 0.0943 - 9.4320 \times 10^{-5} A$	(t=0.2878)
PK-M	$E = 0.0862 - 8.7190 \times 10^{-5} A$	(t=0.2856)
% alanine inhibn.	$E = 36.0262 + 6.6984 \times 10^{-2} A$	(t=0.4852)
Ratio PK-M/PK-LM	$E = 0.5894 - 1.5900 \times 10^{-3} A$	(t=1.1651)
CPK-Bg (July)	$E = 0.0278 + 1.1070 \times 10^{-3} A$	(t=1.8782)
CPK-Bk (July)	$E = 0.0512 + 1.4864 \times 10^{-3} A$	(t=1.9284)
CPK-Bk (Nov.)	$E = 0.1002 - 6.3990 \times 10^{-5} A$	(t=0.1335)
AKI (Nov.)	$E = 0.0015 + 1.5910 \times 10^{-5} A$	(t=0.3724)
Nett CPK-Bk (Nov.)	$E = 0.0988 - 7.9910 \times 10^{-5} A$	(t=0.1639)

Table IX opposite

Regressions of serum enzyme activities (E) in i.u./l at 25°C on time of day (T) expressed as minutes after 9.00 a.m. in groups of 12 and 24 normal males. For all enzymes except CPK-Bk (Nov.), AKI (Nov.) and Nett CPK-Bk (Nov.) regressions are shown for two experiments carried out on different groups and different days.

TABLE IX

REGRESSIONS OF SERUM ENZYME VALUES (E)
ON TIME OF DAY (T MINS AFTER 9AM)

Males (n=12; 10 df)		
PK	$E = 0.1813 + 0.0851 \times 10^{-4} T$	(t=0.0991)
	$E = 0.1446 + 0.3284 \times 10^{-4} T$	(t=0.4518)
PK-LM	$E = 0.1938 + 0.0710 \times 10^{-4} T$	(t=0.0708)
	$E = 0.1455 + 0.5670 \times 10^{-4} T$	(t=0.7874)
PK-Ala	$E = 0.1207 + 0.0422 \times 10^{-4} T$	(t=0.0636)
	$E = 0.0821 + 0.4296 \times 10^{-4} T$	(t=0.7627)
PK-M	$E = 0.1062 + 0.1290 \times 10^{-4} T$	(t=0.2075)
	$E = 0.0891 + 0.0346 \times 10^{-4} T$	(t=0.0750)
% alanine inhibn.	$E = 34.1783 - 1.5044 \times 10^{-3} T$	(t=0.0041)
	$E = 27.9594 + 1.9892 \times 10^{-2} T$	(t=0.0499)
Ratio PK-M/PK-LM	$E = 0.5509 + 0.4084 \times 10^{-4} T$	(t=0.0080)
	$E = 0.6025 - 1.9310 \times 10^{-4} T$	(t=0.0514)
CPK-Bg (July)	$E = 0.1314 + 0.2109 \times 10^{-4} T$	(t=0.1870)
	$E = 0.0881 + 0.9319 \times 10^{-4} T$	(t=1.0614)
CPK-Bk (July)	$E = 0.1832 + 0.7257 \times 10^{-4} T$	(t=0.4875)
	$E = 0.1264 + 1.3946 \times 10^{-4} T$	(t=1.1669)
Males (n=24; 22 df)		
CPK-Bk (Nov.)	$E = 0.1800 + 0.4627 \times 10^{-4} T$	(t=0.3348)
AKI (Nov.)	$E = 0.0034 - 0.0607 \times 10^{-4} T$	(t=2.3514 *)
Nett CPK-Bk (Nov.)	$E = 0.1766 + 0.5235 \times 10^{-4} T$	(t=0.3801)

Continued...

Table IX (Continued) opposite

Regressions of serum enzyme activities (E) in i.u./l at $25^{o}C$ on time of day (T) expressed as minutes after 9.00 a.m. in groups of 12 and 24 normal females. For all enzymes except CPK-Bk (Nov.), AKI (Nov.) and Nett CPK-Bk (Nov.) regressions are shown for two experiments carried out on different groups and different days.

TABLE IX (Continued)

Females (n=12; 10 df)		
PK	$E = 0.1347 + 0.4118 \times 10^{-4} T$	(t=0.5572)
	$E = 0.1501 + 0.0907 \times 10^{-4} T$	(t=0.1629)
PK-LM	$E = 0.1335 + 0.7079 \times 10^{-4} T$	(t=0.9492)
	$E = 0.1586 + 0.1838 \times 10^{-4} T$	(t=0.2923)
PK-Ala	$E = 0.0882 + 0.0624 \times 10^{-4} T$	(t=0.1467)
	$E = 0.0746 + 0.6750 \times 10^{-4} T$	(t=1.7977)
PK-M	$E = 0.0745 + 0.1632 \times 10^{-4} T$	(t=0.3930)
	$E = 0.0816 + 0.2126 \times 10^{-4} T$	(t=0.6638)
% alanine inhibn.	$E = 34.3924 + 1.1261 \times 10^{-2} T$	(t=0.8410)
	$E = 49.5600 - 3.8809 \times 10^{-2} T$	(t=2.9519 *)
Ratio PK-M/PK-LM	$E = 0.5847 - 2.8099 \times 10^{-4} T$	(t=1.5648)
	$E = 0.5181 + 0.7217 \times 10^{-4} T$	(t=0.4894)
CPK-Bg (July)	$E = 0.0760 - 0.2506 \times 10^{-4} T$	(t=0.2594)
	$E = 0.0042 + 2.8729 \times 10^{-4} T$	(t=4.9637 ***)
CPK-Bk (July)	$E = 0.1159 - 0.2942 \times 10^{-4} T$	(t=0.3673)
	$E = 0.0268 + 3.5146 \times 10^{-4} T$	(t=4.3056 **)
Females (n=24; 22 df)		
CPK-Bk (Nov.)	$E = 0.1004 - 0.1100 \times 10^{-4} T$	(t=0.3015)
AKI (Nov.)	$E = 0.0041 - 0.0739 \times 10^{-4} T$	(t=2.5722 *)
Nett CPK-Bk (Nov.)	$E = 0.0963 - 0.0361 \times 10^{-4} T$	(t=0.0971)

also explains the decrease in % alanine inhibition and the corresponding increase in ratio PK-M/PK-LM. Even at -21.5°C much PK activity may be lost after 4 weeks, whereas CPK was comparatively stable in the same circumstances (Table XI) and only after 13 weeks at -21.5°C did its activity decline appreciably in both CPK-Bg and CPK-Bk. Storage for 24 hours at 25°C gave a small reduction in CPK activity, allowing overnight mailing of serum before assay, though prompt analysis is always preferable. PK, however, is not at all suitable for overnight posting, since a large loss of activity could bring an otherwise abnormal carrier within the normal range, or, as with 3 and 4 in Table X the % alanine inhibition could become spuriously abnormal.

In whole blood stored at 25°C for 24 hours CPK survives well (allowing overnight mailing) and may even increase slightly (Table XII), especially after chilling for an hour, as if there was slight efflux from blood cells containing more CPK than serum. The frank efflux of LDH after 24 hours at 25°C usually increased very markedly with chilling, though in DMD patient 11 a decrease in serum activity after 25°C for 24 hours (with or without a preliminary hour at 1.5°C), suggested movement into blood cells from an elevated serum value; efflux occurred only after prolonged chilling. In other subjects efflux was slight (3 and 9), moderate, or gross (1, 6 and 10) and apparently quite individual and independent of sex or health. PK efflux was mainly type L, as indicated by the changes in % alanine inhibition and ratio M/LM, and was of similar degree to that of LDH.

In contrast to LDH (Table XII) PK generally decreased after 24 hours at 25°C, the loss of activity due to its instability

Table X opposite

Activity of pyruvate kinase (in i.u./l at 25°C) in serum separated from clotted blood of 2 normal males and 2 normal females. In each case the blood was centrifuged one hour after being withdrawn. The enzyme activity in the serum was then assayed

(a) immediately

(b) after storage at 25°C for 24 hours

(c) after storage at 4°C for 24 hours

(d) after storage at 4°C for 1 week

(e) after storage at -21.5°C for 24 hours

(f) after storage at -21.5°C for 1 week

(g) after storage at -21.5°C for 4 weeks.

TABLE X

ENZYME STABILITIES IN STORED NORMAL SERA - PYRUVATE KINASE

Procedures: (a) Forthwith (b) 24 hours at 25°C
(c) 24 hours at 4°C (d) 1 week at 4°C
(e) 24 hours at -21.5°C (f) 1 week at -21.5°C
(g) 4 weeks at -21.5°C

% inhn - % alanine inhibition; Ratio - Ratio PK-M/PK-LM.

Number	Sex	Age (yrs)	Assay	(a)	(b)	(c)	(d)
1	M	20.06	PK	17.03	13.17	13.06	7.32
			PK-LM	20.76	13.27	14.21	7.73
			PK-Ala	12.33	10.45	10.24	7.00
			PK-M	10.66	8.15	9.20	5.23
			% inhn	27.6	20.6	21.6	4.3
			Ratio	0.51	0.61	0.65	0.68
				(e)	(f)	(g)	
			PK	16.51	14.52	13.79	
			PK-LM	17.45	16.41	15.47	
			PK-Ala	11.50	10.35	9.51	
			PK-M	10.14	8.15	9.72	
			% inhn	30.1	28.8	31.1	
			Ratio	0.58	0.50	0.63	
				(a)	(b)	(c)	(d)
2	M	22.63	PK	15.47	12.02	12.22	7.52
			PK-LM	17.97	12.57	13.06	8.47
			PK-Ala	11.70	9.41	8.88	6.48
			PK-M	9.82	8.57	8.47	5.96
			% inhn	24.3	21.7	26.7	13.9
			Ratio	0.55	0.67	0.65	0.70
				(e)	(f)	(g)	
			PK	16.20	14.53	11.70	
			PK-LM	16.09	16.09	12.65	
			PK-Ala	10.35	8.78	8.99	
			PK-M	8.99	7.94	8.67	
			% inhn	36.1	39.6	23.2	
			Ratio	0.56	0.49	0.69	

Continued ...

TABLE X (Continued)

Number	Sex	Age (yrs)	Assay	(a)	(b)	(c)	(d)
3	F	27.04	PK	14.11	10.35	11.39	4.91
			PK-LM	16.83	10.97	13.79	4.39
			PK-Ala	10.47	8.15	7.32	3.45
			PK-M	8.78	6.90	6.06	3.03
			% inhn	25.6	21.2	35.8	29.8
			Ratio	0.52	0.62	0.44	0.69
				(e)	(f)	(g)	
			PK	13.38	12.33	10.97	
			PK-LM	15.88	13.69	12.85	
			PK-Ala	9.61	9.30	6.69	
			PK-M	9.09	7.73	6.58	
			% inhn	28.1	24.6	39.1	
			Ratio	0.57	0.56	0.51	
				(a)	(b)	(c)	(d)
4	F	39.98	PK	15.47	10.87	11.60	6.58
			PK-LM	17.03	11.70	13.48	5.85
			PK-Ala	11.60	9.09	8.99	5.43
			PK-M	9.93	7.73	7.63	3.87
			% inhn	25.0	16.3	22.5	17.5
			Ratio	0.58	0.66	0.57	0.66
				(e)	(f)	(g)	
			PK	13.79	13.69	11.91	
			PK-LM	16.62	14.63	12.33	
			PK-Ala	10.03	10.55	8.47	
			PK-M	9.30	8.05	5.02	
			% inhn	27.3	22.9	28.9	
			Ratio	0.56	0.55	0.41	

Table XI opposite

Activity of creatine phosphokinase (in i.u./l at 25°C) in serum separated from the clotted blood of 3 normal males and 3 normal females. In each case the blood was centrifuged one hour after being withdrawn. The enzyme activity in the serum was then assayed

(a) immediately

(b) after storage at 25°C for 24 hours

(c) after storage at 1.5°C for 24 hours

(d) after storage at -21.5°C for 24 hours

(e) after storage at -21.5°C for 1 week

(f) after storage at -21.5°C for 4 weeks

(g) after storage at -21.5°C for 13 weeks.

TABLE XI

ENZYME STABILITIES IN STORED NORMAL SERA - CREATINE PHOSPHOKINASE

Procedures: (a) Forthwith (b) 24 hours at 25°C
(c) 24 hours at 1.5°C (d) 24 hours at -21.5°C
(e) 1 week at -21.5°C (f) 4 weeks at -21.5°C
(g) 13 weeks at -21.5°C

Each value given is the mean of two estimations.

Number	Sex	Age (yrs)	Assay	(a)	(b)	(c)	
1	M	20.41	CPK-Bg	44.61	43.00	40.82	
			CPK-Bk	56.24	54.51	54.27	
				(d)	(e)	(f)	(g)
			CPK-Bg	42.71	42.86	44.46	43.00
			CPK-Bk	55.13	54.76	52.42	49.46
				(a)	(b)	(c)	
2	M	21.54	CPK-Bg	27.84	25.66	26.53	
			CPK-Bk	34.16	32.19	34.16	
				(d)	(e)	(f)	(g)
			CPK-Bg	23.76	22.74	24.34	24.78
			CPK-Bk	32.68	32.92	33.42	30.09
				(a)	(b)	(c)	
3	M	23.15	CPK-Bg	23.61	22.16	21.72	
			CPK-Bk	30.34	28.24	27.75	
				(d)	(e)	(f)	(g)
			CPK-Bg	22.16	22.01	22.74	18.37
			CPK-Bk	29.97	29.48	29.48	25.28

Continued ...

TABLE XI (Continued)

Number	Sex	Age (yrs)	Assay	(a)	(b)	(c)	
4	F	21.85	CPK-Bg	26,24	23.32	22.89	
			CPK-Bk	30.71	29.11	29.11	
				(d)	(e)	(f)	(g)
			CPK-Bg	23.61	24.05	21.43	21.28
			CPK-Bk	31.94	28.00	26.89	27.26
				(a)	(b)	(c)	
5	F	27.33	CPK-Bg	13.99	12.24	12.10	
			CPK-Bk	15.05	14.80	15.42	
				(d)	(e)	(f)	(g)
			CPK-Bg	11.37	10.79	10.64	9.77
			CPK-Bk	14.68	14.80	14.92	13.69
				(a)	(b)	(c)	
6	F	40.28	CPK-Bg	28.13	27.84	28.57	
			CPK-Bk	36.75	33.30	33.92	
				(d)	(e)	(f)	(g)
			CPK-Bg	26.97	25.66	24.78	24.78
			CPK-Bk	33.55	34.29	32.68	32.56

Table XII opposite

Enzyme activities (in i.u./l at 25°C) in serum separated from the clotted blood of 6 healthy subjects (3 male and 3 female), 3 (female) DMD carriers and 2 (male) DMD patients. The blood was subjected to the following treatments

(a) allowed to clot at room temperature for one hour before centrifugation

(b) stored at 25°C for 24 hours before centrifugation

(c) stored at 1.5°C for 1 hour then 25°C for 23 hours before centrifugation

(d) stored at 1.5°C for 6 hours then 25°C for 18 hours before centrifugation

(e) stored at 1.5°C for 24 hours before centrifugation

In each case the enzyme activities of the serum were assayed immediately on separation.

TABLE XII

EFFECTS OF CHILLING CLOTTED BLOOD FROM HEALTHY SUBJECTS, DMD CARRIERS AND PATIENTS

Procedures: (a) Forthwith (b) 24 hours at 25°C
(c) 1 hour at 1.5°C, 23 hours at 25°C
(d) 6 hours at 1.5°C, 18 hours at 25°C
(e) 24 hours at 1.5°C

	Assay	(a)	(b)	(c)	(d)	(e)
No. 1 20.94 years Normal male	PK	40.65	38.35	65.00	106.6	183.4
	PK-LM	42.95	35.53	69.60	125.0	215.8
	PK-Ala	31.98	31.14	40.55	58.10	59.04
	PK-M	27.48	24.45	34.90	49.12	60.61
	% inhn	21.3	18.8	37.6	45.5	67.8
	Ratio	0.64	0.69	0.50	0.39	0.28
	CPK-Bg	221.1	191.5	205.5	202.0	204.4
	LDH	216.1	226.4	268.9	547.9	549.4
No. 1 20.96 years	PK	29.37	29.37	38.35	61.76	123.8
	PK-LM	30.20	29.37	40.96	69.60	152.7
	PK-Ala	24.24	25.08	27.48	34.49	48.28
	PK-M	20.80	19.44	22.36	31.66	42.01
	% inhn	17.4	14.6	28.3	44.2	61.0
	Ratio	0.69	0.66	0.55	0.46	0.28
	CPK-Bg	62.10	75.22	77.55	76.38	78.13
	LDH	140.3	171.2	226.9	379.8	315.0
No. 2 22.84 years Normal male	PK	16.51	12.54	15.99	31.04	58.73
	PK-LM	17.03	13.80	17.87	38.14	73.99
	PK-Ala	10.73	8.67	10.24	16.41	20.06
	PK-M	10.45	7.63	8.99	14.94	18.60
	% inhn	33.5	30.8	35.9	47.1	65.8
	Ratio	0.61	0.57	0.50	0.39	0.25
	CPK-Bg	23.32	23.61	22.74	22.74	22.74
	LDH	91.15	117.5	143.3	231.4	255.7
No. 3 23.72 years Normal male	PK	18.81	17.66	19.44	19.44	21.01
	PK-LM	19.33	18.18	18.50	20.17	22.89
	PK-Ala	15.36	13.17	14.21	14.74	14.00
	PK-M	13.06	11.27	12.12	12.74	10.76
	% inhn	18.3	25.4	26.9	24.2	33.3
	Ratio	0.68	0.62	0.66	0.63	0.47
	CPK-Bg	58.60	66.18	72.01	66.18	71.13
	LDH	155.0	173.7	179.2	172.7	172.7

Continued ...

TABLE XII (Continued)

	Assay	(a)	(b)	(c)	(d)	(e)
No. 4 27.26 years	PK	13.17	10.87	13.79	16.83	32.92
Normal female	PK-LM	13.06	10.14	14.00	21.21	42.95
	PK-Ala	8.36	7.84	8.88	10.24	15.05
	PK-M	5.33	6.27	7.52	8.78	12.23
	% inhn	36.5	27.9	35.6	39.1	54.3
	Ratio	0.41	0.62	0.54	0.41	0.28
	CPK-Bg	12.54	15.45	16.91	15.45	15.45
	LDH	83.05	106.9	127.1	165.1	202.1
No. 5 40.14 years	PK	15.36	13.79	15.05	14.84	19.44
Normal female	PK-LM	16.09	15.99	14.42	16.72	22.15
	PK-Ala	11.39	10.76	10.03	8.57	10.87
	PK-M	8.99	7.73	8.47	8.57	10.66
	% inhn	25.8	22.0	33.3	42.3	44.1
	Ratio	0.56	0.48	0.59	0.51	0.48
	CPK-Bg	20.12	21.87	23.03	23.03	22.74
	LDH	110.9	133.7	199.0	152.4	148.9
No. 6 59.09 years	PK	14.32	10.66	25.08	50.94	131.0
Normal female	PK-LM	15.02	11.50	29.68	66.88	156.8
	PK-Ala	8.15	6.27	10.87	21.95	28.84
	PK-M	6.27	5.85	11.50	20.90	36.05
	% inhn	43.1	41.2	56.7	56.9	78.0
	Ratio	0.42	0.51	0.39	0.31	0.23
	CPK-Bg	11.95	11.37	12.24	10.93	13.12
	LDH	197.5	187.4	245.1	393.7	483.1
No. 6 59.15 years	PK	20.80	10.66	28.22	69.39	112.8
	PK-LM	22.99	12.65	35.43	86.53	142.3
	PK-Ala	10.03	6.90	13.59	26.75	31.04
	PK-M	8.67	5.85	10.56	23.20	29.16
	% inhn	51.8	35.3	51.2	61.5	72.5
	Ratio	0.38	0.46	0.30	0.27	0.20
	CPK-Bg	13.12	15.16	15.45	17.49	17.49
	LDH	230.9	209.1	300.8	457.3	440.6
No. 7 28.59 years	PK	67.72	73.67	82.45	81.20	85.57
DMD carrier 2s	PK-LM	68.34	70.85	84.65	86.53	98.75
	PK-Ala	62.07	59.25	63.95	60.82	58.63
	PK-M	52.04	52.67	56.43	53.30	61.76
	% inhn	8.3	19.6	22.4	25.1	31.5
	Ratio	0.76	0.74	0.67	0.62	0.63
	CPK-Bg	575.5	574.6	627.9	599.1	600.9
	LDH	258.3	279.5	305.4	352.5	347.9

Continued ...

TABLE XII (Continued)

	Assay	(a)	(b)	(c)	(d)	(e)
No. 8 36.98 years	PK	10.97	10.66	13.38	19.44	23.77
DMD carrier 1s	PK-LM	11.91	12.44	17.56	22.63	27.17
	PK-Ala	6.48	6.90	8.78	11.29	10.19
	PK-M	5.96	6.48	8.78	8.57	9.67
	% inhn	41.0	35.3	34.4	41.9	57.1
	Ratio	0.50	0.52	0.50	0.38	0.36
	CPK-Bg	11.66	11.37	13.12	11.66	9.91
	LDH	169.6	150.4	199.0	181.3	164.1
No. 9 42.54 years	PK	23.93	18.29	22.29	23.69	28.74
DMD carrier 2b; 3s	PK-LM	23.30	19.02	23.86	23.86	34.14
	PK-Ala	17.35	13.90	16.55	14.81	17.42
	PK-M	15.36	12.23	14.98	14.63	16.20
	% inhn	27.5	24.0	25.8	37.5	39.4
	Ratio	0.66	0.64	0.63	0.61	0.47
	CPK-Bg	141.1	142.6	151.9	146.2	145.2
	LDH	165.1	194.5	209.7	223.3	223.8
No. 10 6.88 years	PK	535.0	533.9	552.8	562.2	543.4
DMD patient (Ambulant)	PK-LM	530.9	531.9	543.4	555.9	549.7
	PK-Ala	491.2	481.7	492.2	476.5	428.5
	PK-M	426.4	401.3	428.5	420.1	392.9
	% inhn	8.2	9.8	11.0	15.2	21.2
	Ratio	0.80	0.75	0.79	0.76	0.71
	CPK-Bg	3358	3408	3528	3495	3723
	LDH	1291	1377	1493	1681	2350
No. 11 12.56 years	PK	270.6	256.5	262.8	281.6	298.9
DMD patient (Ambulant)	PK-LM	264.9	278.0	270.7	279.0	316.1
	PK-Ala	246.1	224.7	231.9	233.6	240.4
	PK-M	190.7	204.8	198.6	199.6	197.0
	% inhn	9.1	12.4	11.7	17.1	19.6
	Ratio	0.72	0.74	0.73	0.72	0.62
	CPK-Bg	1673	1759	1842	1768	1803
	LDH	703.9	650.7	660.9	729.2	830.7

apparently exceeding any prospective efflux. When chilled, PK and PK-LM showed larger increases than other PK modalities, since they both measure type M (muscle) and type L (liver and erythrocyte) PK. Although the liver and erythrocyte forms have identical electrophoretic properties (Etiemble and Biovin, 1976) they are kinetically different as liver isoenzyme is activated by fructose diphosphate but erythrocyte PK is not (Hess et al., 1966; Tanaka et al., 1967b). Both behave identically, however, under the conditions of this assay. As expected, PK-Ala and PK-M show only modest increases at 1.5°C, and occasionally none at all of PK-Ala (3, 5, 7, 9, 10 and 11) and of PK-M only after prolonged chilling (5, 9 and 10). These differences in behavior between PK-Ala and PK-M, with the higher value of the former, suggest that PK-M should give a more accurate estimation of muscle PK since it shows the smallest increment of type L, and since the inhibition of liver PK by alanine is far from complete. This individual variation of efflux renders delayed assay of any PK modality valueless in DMD carrier detection, as for example in normal females 4 and 6 who show definite elevations after chilling for 24 hours. Assay of PK in serum separated forthwith is therefore obligatory.

Analysis of serum from normal male 1 at his first venepuncture gave abnormally high values for all enzymes, due no doubt to a vigorous soccer game the previous evening, since severe exercise is known to cause enzyme efflux (Griffiths, 1966a). In serum obtained a week later all values had returned to normal. In the original specimen, the fall in CPK by some 30 units after 24 hours at 25°C (20 units after chilling), suggests some movement into blood cells from the serum elevation.

Of 11 known carriers examined (Table XIII, 1-11) 2 were obligate (5 and 11) but hitherto always undetectable, in accord with the natural distribution of carrier manifestation (Thomson et al., 1975b); and though 11 was doubtfully detected by a single, barely elevated finding (ratio 0.70 with upper limit 0.69) all other readings in both were normal. CPK decisively detected all 9 remaining DMD carriers (3 to 20 fold elevations), LDH only 3 clearly (less than 2 fold elevations) and 4 doubtfully, while PK detected 5 clearly (about 2 to 6 fold elevations) and 4 very doubtfully, with elevations of only a unit or so. PK-LM, PK-M and PK-Ala gave similar results in the same individuals, with % alanine inhibition and ratio even less effective, detecting 7 (only 2 decisively) and 5 (only 1 decisively) respectively. Of the 3 possible carriers, all values were normal except for the PK modalities in 12, each with elevations of only a few units.

Storage of clotted blood for 24 hours at 25°C (Table XIII) gave small changes in the already marked CPK elevations of known carriers but several marginal elevations of PK, PK-LM and ratio M/LM became normal, as did PK and PK-LM of possible carrier 12; while LDH behaved quite irregularly. Unlike normal females (Table VII), DMD carriers (Table XIII) showed a high correlation between serum PK and CPK, suggesting a higher content of muscle isoenzymes. LDH correlated only with CPK, though again not in normal females (Sweetin and Thomson, 1973a) and perhaps for the same reason.

In male DMD patients (Table XIII) all values were grossly elevated throughout, again with high correlation between CPK and PK but of LDH with neither. Further, the change in CPK after 24 hours at 25°C (17 and 18) suggests its passage through the blood

Table XIII opposite

Enzyme activities (in i.u./l at 25°C) in serum from 11 known (female) DMD carriers, 3 possible (female) DMD carriers, 4 (male) DMD patients, 2 (female) BMD carriers and 1 (male) BMD patient. Serum was separated from aliquots of clotted blood after 1 hour at room temperature and (activities given in brackets) from further aliquots kept at 25°C for 24 hours before separation.

TABLE XIII

CARRIERS AND PATIENTS: ENZYMES IN SERA FROM CLOTTED BLOOD SPUN FORTHWITH AND (IN BRACKETS) AFTER 24 HOURS AT 25°C

Assays; (a) PK (b) PK-LM (c) PK-Ala (d) PK-M
(e) % alanine inhibn (f) Ratio PK-M/PK-LM
(g) CPK-Bg (h) LDH

No.	Age (yrs)	Male relats.				
Known DMD carriers			(a)	(b)	(c)	(d)
1	16.58	1b	31.04	35.01	25.92	22.05
			(31.25)	(28.63)	(26.54)	(22.68)
			(e)	(f)	(g)	(h)
			16.5	0.63	127.4	132.7
			(15.1)	(0.79)	(132.7)	(144.8)
			(a)	(b)	(c)	(d)
2	16.90	2b	132.6	134.8	117.6	106.3
			(130.4)	(138.3)	(116.6)	(106.9)
			(e)	(f)	(g)	(h)
			11.3	0.79	473.2	186.4
			(10.6)	(0.77)	(472.3)	(190.9)
			(a)	(b)	(c)	(d)
3	21.42	1b	98.44	104.1	84.02	75.87
			(88.41)	(104.7)	(84.02)	(70.85)
			(e)	(f)	(g)	(h)
			14.6	0.73	851.9	350.9
			(5.0)	(0.68)	(745.2)	(284.1)
			(a)	(b)	(c)	(d)
4	28.59	2s	72.73	71.48	60.82	52.67
			(73.67)	(70.85)	(59.25)	(52.67)
			(e)	(f)	(g)	(h)
			16.4	0.74	575.5	258.3
			(19.6)	(0.74)	(574.6)	(279.5)
			(a)	(b)	(c)	(d)
5	36.98	2b; 1s	10.97	11.91	6.48	5.96
			(10.66)	(12.44)	(6.90)	(6.48)
			(e)	(f)	(g)	(h)
			41.0	0.50	11.66	169.6
			(35.3)	(0.52)	(11.37)	(150.4)
			(a)	(b)	(c)	(d)
6	39.77	2ss	27.69	27.38	21.63	12.54
			(27.07)	(26.44)	(22.15)	(18.92)
			(e)	(f)	(g)	(h)
			21.9	0.46	176.7	225.9
			(18.1)	(0.72)	(173.8)	(318.5)
			(a)	(b)	(c)	(d)
7	42.31	2b; 3s	22.15	24.04	18.50	16.93
			(21.53)	(24.24)	(17.35)	(15.26)
			(e)	(f)	(g)	(h)
			16.5	0.70	155.1	177.7
			(19.4)	(0.63)	(147.8)	(169.1)

Continued ...

TABLE XIII (Continued)

No.	Age (yrs)	Male relats.				
Known DMD carriers			(a)	(b)	(c)	(d)
8	46.24	1s	26.65	27.69	18.39	17.03
			(21.11)	(20.90)	(15.26)	(13.38)
			(e)	(f)	(g)	(h)
			31.0	0.62	100.3	192.4
			(27.7)	(0.64)	(94.17)	(158.5)
			(a)	(b)	(c)	(d)
9	52.44	2s	38.46	41.80	30.20	27.38
			(34.69)	(38.14)	(29.26)	(26.44)
			(e)	(f)	(g)	(h)
			21.5	0.66	280.5	233.5
			(15.7)	(0.69)	(277.8)	(237.0)
			(a)	(b)	(c)	(d)
10	53.87	1s	23.20	25.19	15.57	15.88
			(21.53)	(23.20)	(15.57)	(15.26)
			(e)	(f)	(g)	(h)
			32.9	0.62	204.7	269.4
			(27.7)	(0.66)	(201.5)	(269.9)
			(a)	(b)	(c)	(d)
11	55.13	1b; 2s	13.69	14.42	10.14	10.14
			(13.59)	(13.79)	(11.08)	(9.82)
			(e)	(f)	(g)	(h)
			26.0	0.70	26.52	168.1
			(18.5)	(0.71)	(29.45)	(169.6)

Correlations (9 df): PK/CPK=0.8312**; PK/LDH= 0.4123;
CPK/LDH= 0.7856**

Possible DMD carriers			(a)	(b)	(c)	(d)
12	11.10	1b	22.68	24.98	17.03	16.51
			(18.50)	(19.23)	(15.78)	(13.38)
			(e)	(f)	(g)	(h)
			24.9	0.66	32.94	185.8
			(14.8)	(0.70)	(33.82)	(211.7)
			(a)	(b)	(c)	(d)
13	36.05	1s	12.02	12.85	8.88	8.15
			(12.33)	(14.21)	(8.67)	(6.38)
			(e)	(f)	(g)	(h)
			26.1	0.63	25.07	104.3
			(29.7)	(0.45)	(30.03)	(142.8)
			(a)	(b)	(c)	(d)
14	47.48	1s	12.96	19.75	7.21	6.69
			(7.52)	(8.36)	(5.43)	(2.93)
			(e)	(f)	(g)	(h)
			44.4	0.34	10.50	132.2
			(27.8)	(0.35)	(10.50)	(136.7)

Continued ...

TABLE XIII(Continued)

No.	Age (yrs)	Male relats.		(A = ambulant; W = wheelchair)			
DMD patients				(a)	(b)	(c)	(d)
15	6.83	Nil	(A)	694.9	687.6	622.8	542.4
				(662.5)	(685.5)	(600.9)	(531.9)
				(e)	(f)	(g)	(h)
				10.4	0.79	3504	612.7
				(1.3)	(0.78)	(3528)	(1337)
				(a)	(b)	(c)	(d)
16	10.22	Nil	(W)	158.8	154.6	144.2	140.0
				(185.0)	(192.3)	(179.7)	(153.6)
				(e)	(f)	(g)	(h)
				9.2	0.91	1673	805.2
				(2.8)	(0.80)	(1668)	(1529)
				(a)	(b)	(c)	(d)
17	12.56	2mb	(A)	270.7	264.9	246.1	190.7
				(256.5)	(278.0)	(224.7)	(204.8
				(e)	(f)	(g)	(h)
				9.1	0.72	1673	703.9
				(12.4)	(0.74)	(1759)	(650.7)
				(a)	(b)	(c)	(d)
18	14.53	Nil	(W)	136.9	137.9	120.2	99.28
				(135.9)	(136.9)	(123.3)	(119.1)
				(e)	(f)	(g)	(h)
				12.2	0.72	1875	440.6
				(9.2)	(0.87)	(1694)	(379.8)

Correlations (2 df): PK/CPK= 0.9536*; PK/LDH= -0.0172
CPK/LDH= -0.2147

BMD carriers				(a)	(b)	(c)	(d)
19	18.23	2b		25.08	27.48	19.12	17.77
(Known carrier)				(21.11)	(23.51)	(16.93)	(15.15)
				(e)	(f)	(g)	(h)
				23.7	0.65	70.84	196.5
				(19.8)	(0.64)	(69.39)	(183.3)
				(a)	(b)	(c)	(d)
20	51.77	2s		11.81	14.74	9.30	7.73
(Possible carrier)				(10.87)	(13.75)	(7.11)	(6.58)
				(e)	(f)	(g)	(h)
				21.2	0.52	12.82	181.8
				(34.6)	(0.48)	(16.33)	(227.8)
BMD patient				(a)	(b)	(c)	(d)
21	10.05	1b	(A)	597.7	620.7	543.4	458.8
				(587.8)	(608.2)	(528.3)	(471.8)
				(e)	(f)	(g)	(h)
				9.1	0.74	3690	1003
				(10.2)	(0.78)	(3612)	(614.0)

cell membrane, but could equally be due to its adhesion to the cell wall. The slowly progressing BMD patient 21 had values similar to the youngest DMD patient 15 and, as before, CPK detected BMD carrier 19 more confidently than any other reading; on the other hand, possible BMD carrier 20 had normal CPK but slight abnormalities of % alanine inhibition and LDH.

A technical complexity arose in the assay of serum PK from whole blood kept at 25°C, with or without prechilling for 1 or 6 hours. A considerable reduction in the initial extinction of the test mix occurred, dependent on the length of incubation at 25°C, and presumably caused by a fall in NADH concentration. This seemed due to a rise in serum pyruvate, which, in the presence of LDH, is rapidly converted to lactate at the expense of added NADH. The test mix absorbance in these circumstances would have lost a good deal of NADH even before recording could be started.

Contrary to earlier reports (Harano et al.,1973; Alberts and Samaha, 1974) that PK is much more sensitive than CPK, this work has shown that CPK is superior. Not only are its elevations in DMD patients and carriers much greater than those of PK but CPK is far more stable, allowing overnight posting of sera for CPK assay, but not for that of the labile PK. Moreover, the assay of PK is more time consuming and complex, as well as being prone to complications.

The work of Alberts and Samaha (1974) must come under criticism since they reported a striking superiority of PK in carrier detection without stating that their detection rate was only 58%, whereas Walton and Gardner-Medwin (1974) found that CPK usually gave

around 70% detection and, in precisely defined circumstances, up to 86% (Thomson, 1969a) of carriers tested have been identified. This contrasts very markedly with their recording a 19% detection rate for CPK. It must be assumed, therefore, that their method of assay, though nowhere defined, was one of the less sensitive of the many available. Harano et al. (1973) also failed to state their method of CPK assay and the use of an insensitive procedure might explain the inability to find a correlation with PK in DMD patients (Table XIII). Their finding that DMD serum contained virtually no liver isoenzyme (ratio M/LM of 0.99 and 0% alanine inhibition) is also surprising.

Nevertheless, despite all these imperfections, PK modalities did show abnormalities in carriers when CPK did not and, if assayed forthwith, PK may yet prove to be a valuable adjunct to CPK in DMD carrier detection.

Adenylate kinase increment in creatine phosphokinase assay and studies of enzyme movements in blood cells

The changes in CPK-Bg noted previously were explained by a movement of the enzyme across blood cell membranes. It is, however, generally assumed that CPK is absent from rbcs (Pennington, 1974; Blum and Brauman, 1975), a belief stemming apparently from reports that CPK occurs in muscle and myocardium, with small amounts in brain and only traces elsewhere (Colombo et al., 1962; Schmidt, 1964; Dawson and Fine, 1967; Smith, 1972). Unfortunately, although the CPK content of blood cells was never actually investigated by any of these workers, Pennington (1974) and Blum and Brauman (1975) may have supposed that this had been done.

Mueller et al. (1975) reported that Alk P, if present in abnormally high quantities, could depress CPK values. This, in conjunction with the action of rbc glutathione reductase to remove the CPK-Bg activator glutathione (Weidman, 1973), may further explain the CPK-Bg variations found. To clarify the situation a study of the behaviour of clotted blood and serum in healthy subjects was carried out. CPK was assayed, using different activators, and also Alk P, and LDH to indicate the degree of mechanical haemolysis (Table XIV). Alk P was found normal in all subjects, but with little change after any procedure except a slight fall in activity on haemolysis, apparently indicating little or no blood cell Alk P. LDH as expected showed efflux into serum on chilling with additional large increases after haemolysis of the LDH-containing blood cells. CPK-Bg and CPK-Bk gave comparable results, except in 2 where CPK-Bk showed a much larger increase on haemolysis, perhaps due to the action of glutathione reductase masking a similar increase in CPK-Bg. Several subjects (1, 2, 3, 6, 7 and 8) showed a notable fall of CPK values in stored serum compared to the parent whole blood similarly treated. Storage as whole blood thus apparently preserves CPK-Bg and CPK-Bk, but storage as serum does not. That, blood cells are implicated is borne out by the increase in CPK activity after haemolysis. In unhaemolysed serum the type of activator had little effect on CPK values, nor were they influenced by those of Alk P, a finding confirmed by Wearne et al. (1975) and Tsung (1976) although Wearne et al. (1975) did find a "spurious CPK isoenzyme" on electrophoresis still active even when CP was omitted from the assay mix. This activity could only be due to AK converting excess ADP to AMP and ATP, of which the last then assayed as CPK to give an AK increment (AKI). Both CPK-Bg and CPK-Bk have added AMP to inhibit AK but apparently inhibition is

Table XIV opposite

Enzyme activities (in i.u./l at 25°C) in serum separated from the clotted blood of 9 healthy subjects (3 male and 6 female) after the following procedures

(a) separation after being allowed to clot at room temperature for 1 hour

(b) separation after storage at 25°C for 24 hours

(c) separation after storage at 1.5°C for 1 hour and 25°C for 23 hours

(d) separation after storage at 1.5°C for 6 hours and 25°C for 18 hours

(e) separation after storage at 1.5°C for 24 hours

(f) separation after storage at 1.5°C for 24 hours and subjection to 1 minute of vortex mixing to disrupt the blood cells

The additional activities given in brackets in columns (b), (c), (d) and (e) are those of an aliquot of serum separated after blood was allowed to clot at room temperature for 1 hour but before being subjected to the respective storage procedures. Each CPK activity given is the mean of two estimations.

TABLE XIV

BEHAVIOUR OF CLOTTED BLOOD AND (IN BRACKETS) SERUM FROM HEALTHY SUBJECTS

Procedures: (a) Forthwith (b) 24 hours at 25°C
(c) 1 hour at 1.5°C, 23 hours at 25°C
(d) 6 hours at 1.5°C, 18 hours at 25°C
(e) 24 hours at 1.5°C
(f) 24 hours at 1.5°C, 1 min. in vortex mixer to haemolyse

Each CPK value given is the mean of two estimations.

No.	Age (yrs)	Assay	(a)	(b)	(c)	(d)	(e)	(f)
Males								
1	19.55	CPK-Bg	27.84	28.86 (25.66)	28.28 (25.66)	27.70 (27.26)	29.45 (26.53)	31.78
		CPK-Bk	34.16	34.66 (32.19)	35.27 (28.98)	35.89 (32.56)	37.49 (34.16)	41.32
		Alk P	128.4	126.4 (120.8)	128.4 (123.8)	127.7 (121.4)	122.1 (125.1)	105.3
		LDH	106.3				159.5	224.8
2	20.42	CPK-Bg	44.61	46.79 (43.00)	46.79 (42.56)	44.46 (42.56)	46.21 (40.82)	47.67
		CPK-Bk	56.24	54.88 (54.51)	59.46 (51.68)	57.44 (54.27)	58.58 (54.27)	64.87
		Alk P	120.8	123.1 (121.4)	116.5 (119.1)	118.8 (122.4)	116.8 (116.2)	117.8
		LDH	130.7				156.5	322.1
3	23.15	CPK-Bg	23.61	25.07 (22.24)	27.40 (22.89)	25.07 (20.89)	24.18 (21.72)	33.38
		CPK-Bk	30.34	30.09 (28.24)	33.42 (28.61)	32.31 (28.48)	34.90 (27.75)	47.24
		Alk P	46.86	46.53 (46.53)	48.51 (45.21)	48.84 (47.19)	48.18 (44.55)	43.23
		LDH	129.1				236.0	415.8
Females								
4	27.33	CPK-Bg	13.99	11.57 (12.24)	12.97 (13.41)	13.99 (13.32)	12.39 (12.10)	20.99
		CPK-Bk	15.05	14.80 (14.80)	18.62 (13.69)	19.49 (14.92)	16.16 (15.42)	32.07
		Alk P	62.04	59.40 (59.73)	60.06 (57.42)	58.74 (60.72)	59.07 (59.73)	53.46
5	40.28	CPK-Bg	28.13	28.28 (27.84)	28.43 (27.55)	26.53 (26.97)	28.43 (28.57)	40.67
		CPK-Bk	36.75	35.52 (33.30)	35.89 (31.82)	35.77 (35.03)	33.67 (33.92)	
		Alk P	100.7	101.6 (96.69)	98.01 (94.38)	96.03 (96.03)	98.67 (94.05)	84.15

Continued ...

TABLE XIV (Continued)

No.	Age (yrs)	Assay	(a)	(b)	(c)	(d)	(e)	(f)
Females								
6	21.85	CPK-Bg	26.24	24.05 (23.32)	24.49 (22.59)	25.22 (22.74)	26.09 (22.89)	34.40
		CPK-Bk	30.71	32.44 (29.11)	29.35 (28.49)	27.63 (28.86)	33.67 (29.11)	
		Alk P	81.18	86.79 (83.82)	83.82 (82.50)	83.82 (82.50)	83.82 (81.19)	72.27
		LDH	103.8				176.7	694.3
7	37.84	CPK-Bg	20.85	19.53 (15.74)	19.10 (16.62)	19.82 (19.68)	18.95 (17.93)	33.06
		CPK-Bk	24.54	24.17 (20.72)	24.54 (22.57)	24.42 (22.08)	24.30 (22.32)	41.19
		Alk P	101.3	102.0 (103.0)	105.3 (103.6)	101.0 (104.6)	103.0 (99.66)	94.38
		LDH	108.8				131.2	353.9
8	41.83	CPK-Bg	20.12	22.30 (18.66)	22.74 (20.41)	23.32 (19.53)	20.55 (18.08)	27.99
		CPK-Bk	24.67	27.87 (24.30)	28.98 (24.05)	29.72 (24.67)	28.24 (25.65)	37.37
		Alk P	82.17	86.13 (89.43)	86.13 (85.47)	87.45 (87.12)	87.45 (84.81)	81.84
		LDH	62.29				86.09	218.7
9	54.65	CPK-Bg	56.41	49.27 (49.42)	48.69 (51.46)	51.75 (50.87)	55.54 (51.16)	60.35
		CPK-Bk	67.46	62.07 (65.38)	65.49 (65.00)	68.70 (63.76)	71.78 (67.97)	83.50
		Alk P	112.9	118.4 (116.5)	117.2 (118.5)	113.2 (117.2)	119.3 (113.5)	114.8
		LDH	187.9				271.4	488.7

incomplete; in fact, Szasz et al. (1976a) later showed that with 10 mM AMP there was still 8% of AK activity remaining.

True CPK (nett CPK-Bk) was therefore measured by deducting this AKI (Table XV), allowing some clarification of the distribution and behaviour of CPK in serum and whole blood, particularly after haemolysis. Dithiothreitol was used as the CPK activator instead of glutathione vulnerable to erythrocyte glutathione reductase. Any differences between CPK values in serum from treated whole blood and serum separated forthwith, must therefore arise from blood cells. Thus after 24 hours at 25°C the value of nett CPK-Bk in whole blood changed only slightly in most subjects; 3 and 4 showed definite decreases however, though there was no comparative treatment of serum, and others (2, 8 and 10) and especially DMD patient 11, actually showed a reduction when compared with similarly treated serum. Thus blood cells may even have allowed uptake of CPK by passage through the intact cell membrane. The same treatment also caused slight rises in LDH and AKI in most, with a fall in glucose (Danowski, 1941; Eckel et al., 1966) consumed to fuel the erythrocyte (NaK)-ATPase which retains K^+ and expells Na^+ (Glynn, 1968).

Marked increases in AKI and especially LDH occurred in whole blood after 24 hours at 1.5°C (Table XV), with AKI obviously a major component of the apparent CPK efflux seen previously. The sparing of glucose at 1.5°C was due to failure of the temperature dependent (NaK)-ATPase (Wood and Beutler, 1967), with K^+ rising as Na^+ fell; these values remained unchanged when no blood cells were present. Nett CPK-Bk changed little on chilling of whole blood except for a slight rise in 4 and a more pronounced increase

Table XV opposite

Enzyme activities (in i.u./l at 25°C), and concentrations of glucose (in mg/100ml) and electrolytes (in mequiv/l), in serum separated from the clotted blood of 6 healthy subjects (2 male and 4 female), 3 (female) DMD carriers, 3 (male) DMD patients, 1 (female) BMD carrier and 1 (male) BMD patient, after the following procedures

(a) separation after being allowed to clot at room temperature for 1 hour

(b) separation after storage at 25°C for 24 hours

(c) separation after storage at 1.5°C for 24 hours

(d) separation after storage at 1.5°C for 24 hours and subjection to 1 minute of vortex mixing to disrupt the blood cells

The additional activities given in brackets in columns (b) and (c) are those of an aliquot of serum separated after blood was allowed to clot at room temperature for 1 hour but before being subjected to the respective storage procedures. Each CPK or AKI activity given is the mean of two estimations.

TABLE XV

BEHAVIOUR OF CLOTTED BLOOD AND (IN BRACKETS) SERUM FROM HEALTHY SUBJECTS, CARRIERS AND PATIENTS

Procedures: (a) Forthwith (b) 24 hours at 25°C (c) 24 hours at 1.5°C (d) 24 hours at 1.5°C, 1 min. in vortex mixer to haemolyse

Each CPK and AKI value is the mean of two estimations.

No.	Sex	Age (yrs)	DMD	Assay	(a)	(b)	(c)	(d)
1	M	20.72	Nil	CPK-Bk	34.41	35.77 (33.42)	36.51 (34.53)	45.88
				AKI	0.49	2.96 (0.74)	3.95 (0.49)	10.98
				Nett CPK-Bk	33.92	32.81 (32.68)	32.56 (34.04)	34.90
				LDH	112.4	117.0 (110.4)	172.2 (115.5)	339.8
				Alk P	112.5	124.4 (115.2)	111.5 (116.8)	116.2
				Glucose	89.54	36.41 (84.73)	75.57 (86.79)	72.36
				Na^+	140	144 (141)	136 (141)	135
				K^+	4.3	4.4 (4.2)	9.5 (4.3)	10.5
2	M	24.14	Nil	CPK-Bk	89.17	85.10 (86.83)	87.44 (87.44)	97.43
				AKI	0.99	1.36 (1.60)	0.99 (0.74)	13.94
				Nett CPK-Bk	88.18	83.74 (85.22)	86.46 (86.70)	83.50
				LDH	159.5	174.7 (167.7)	179.3 (159.5)	421.8
				Alk P	70.62	71.94 (71.28)	70.95 (70.20)	66.00
				Glucose	84.04	18.32 (84.50)	75.57 (84.04)	75.80
				Na^+	143	144 (142)	137 (142)	135
				K^+	4.3	4.4 (4.3)	9.9 (4.3)	11.3

Continued ...

TABLE XV (Continued)

No.	Sex	Age (yrs)	DMD	Assay	(a)	(b)	(c)	(d)
3	F	27.68	Nil	CPK-Bk	24.17	18.75	21.71 (19.49)	28.86
				AKI	1.97	1.73	0.74 (0)	11.59
				Nett CPK-Bk	22.20	17.02	20.97 (19.49)	17.27
				LDH	107.9	113.4	182.8 (110.4)	545.9
				Alk P	66.66	67.32	66.00 (67.32)	59.07
				Glucose	106.9	53.36	97.55 (108.5)	89.54
				Na^+	143	144	134 (142)	132
				K^+	3.9	3.6	11.2 (3.8)	13.0
4	F	40.62	Nil	CPK-Bk	37.25	34.29	35.27 (31.57)	50.07
				AKI	1.73	2.22	1.73 (0)	15.79
				Nett CPK-Bk	35.52	32.07	33.55 (31.57)	34.29
				LDH	126.1	146.4	164.1 (129.1)	606.2
				Alk P	99.00	98.34	93.39 (96.69)	90.42
				Glucose	92.97	39.16	81.75 (90.68)	77.73
				Na^+	141	144	137 (142)	134
				K^+	4.1	4.1	8.6 (4.1)	10.5
5	F	42.14	Nil	CPK-Bk	28.61	28.98 (26.52)	28.86 (28.49)	33.42
				AKI	0.99	1.48 (0)	1.73 (0)	4.93
				Nett CPK-Bk	27.63	27.50 (26.52)	27.13 (28.49)	28.49
				LDH	66.85	84.06 (67.86)	90.65 (61.76)	188.9
				Alk P	81.84	87.45 (86.46)	85.80 (83.49)	86.46
				Glucose	76.72	28.85 (75.34)	71.22 (74.88)	65.04
				Na^+	142	144 (142)	136 (142)	133
				K^+	4.4	4.7 (4.4)	10.4 (4.3)	13.0

Continued ...

TABLE XV (Continued)

No.	Sex	Age (yrs)	DMD	Assay	(a)	(b)	(c)	(d)
6	F	49.43	Nil	CPK-Bk	15.42	14.68 (12.83)	21.83 (14.55)	28.24
				AKI	1.11	1.97 (0)	7.03 (0.37)	15.54
				Nett CPK-Bk	14.31	12.70 (12.83)	14.80 (14.18)	12.70
				LDH	119.0	152.9 (121.0)	311.9 (108.9)	584.4
				Alk P	96.03	104.9 (94.38)	98.34 (98.01)	99.99
				Glucose	94.58	46.94 (91.60)	84.04 (95.26)	75.11
				Na^+	139	140 (139)	132 (138)	128
				K^+	4.4	5.6 (4.3)	12.2 (4.4)	14.5
7	F	29.18	2s	CPK-Bk	413.2	403.3 (418.1)	439.1 (433.5)	444.0
				AKI	2.47	2.34 (2.96)	4.07 (1.48)	7.89
				Nett CPK-Bk	410.7	401.0 (415.1)	435.0 (432.0)	436.1
				LDH	192.4	250.7 (219.0)	339.3 (176.0)	481.1
				Alk P	83.16	84.48 (78.54)	79.53 (78.87)	77.88
				Glucose	101.7	31.83 (101.5)	90.68 (100.5)	86.10
				Na^+	140	141 (140)	134 (139)	132
				K^+	3.8	3.3 (3.8)	9.3 (3.8)	10.0
8	F	34.28	1s	CPK-Bk	263.8	251.7 (255.2)	272.2 (267.4)	280.7
				AKI	2.59	2.84 (0.99)	9.13 (3.21)	25.28
				Nett CPK-Bk	261.2	248.9 (254.2)	263.1 (264.2)	255.4
				LDH	150.4	217.8 (214.7)	392.5 (190.4)	696.8
				Alk P	112.5	107.9 (105.9)	107.3 (104.0)	97.68
				Na^+	142	146 (139)	134 (140)	129
				K^+	4.3	4.5 (4.2)	11.9 (4.3)	14.4
				Glucose	94.81	16.95 (89.77)	76.03 (81.98)	66.87

Continued ...

TABLE XV (Continued)

No.	Sex	Age (yrs)	DMD	Assay	(a)	(b)	(c)	(d)
9	F	47.01	ls	CPK-Bk	88.68		93.12	106.2
				AKI	5.43		7.40	17.27
				Nett CPK-Bk	83.25		85.72	88.92
				LDH	119.5		263.8	514.5
				Alk P	106.3		101.0	93.72
				Glucose	88.52		80.84	70.53
				Na^+	138		127	122
				K^+	3.9		14.4	18.2
10	M	11.06	DMD	CPK-Bk	2084	1962 (2039)	2237 (2054)	2193
				AKI	3.21	3.58 (2.59)	8.39 (2.96)	34.29
				Nett CPK-Bk	2081	1958 (2036)	2229 (2051)	2159
				LDH	850.8	850.8 (840.6)	1074 (845.7)	1550
				Alk P	257.4	264.0 (267.6)	280.5 (269.6)	260.7
				Glucose	84.96	25.41 (81.07)	65.04 (84.21)	57.94
				Na^+	140	141 (139)	135 (139)	132
				K^+	3.8	4.2 (3.8)	9.1 (3.8)	10.6
11	M	14.03	DMD	CPK-Bk	2163	1935 (2165)	2179 (2189)	2160
				AKI	1.48	4.81 (2.71)	11.22 (2.34)	35.64
				Nett CPK-Bk	2162	1930 (2162)	2168 (2187)	2124
				LDH	334.2	476.0 (481.1)	931.8 (471.0)	1539
				Alk P	230.0	238.9 (240.6)	241.2 (240.6)	211.2
				Glucose	95.95	31.83 (91.60)	79.69 (92.52)	76.72
				Na^+	139	142 (141)	134 (142)	130
				K^+	3.8	4.1 (3.9)	11.4 (3.8)	13.9

Continued ...

TABLE XV (Continued)

No.	Sex	Age (yrs)	DMD	Assay	(a)	(b)	(c)	(d)
12	M	15.30	DMD	CPK-Bk	521.7		738.8	753.6
				AKI	5.92		3.35	10.61
				Nett CPK-Bk	515.8		735.4	743.0
				LDH	180.7		288.1	523.6
				Alk P	187.1		199.7	188.4
				Glucose	104.0		95.26	87.48
				Na^+	139		130	128
				K^+	3.7		11.7	12.6
			BMD					
13	F	18.94	2b	CPK-Bk	67.59		69.31	85.72
				AKI	3.44		4.93	21.95
				Nett CPK-Bk	64.15		64.38	63.76
				LDH	172.2		245.6	515.5
				Alk P	76.56		87.78	72.27
				Glucose	80.61		80.61	73.28
				Na^+	139		139	132
				K^+	4.1		9.6	12.1
14	M	10.77	BMD	CPK-Bk	2851		2805	2807
				AKI	2.71		3.70	19.98
				Nett CPK-Bk	2849		2801	2787
				LDH	633.0		605.7	820.4
				Alk P	208.9		225.7	203.0
				Glucose	76.50		68.70	62.29
				Na^+	138		133	131
				K^+	4.2		7.9	9.5

in 10, even when compared to simultaneously treated serum, and again in 12, though without a serum blank. Mechanical haemolysis after chilling then added the intact blood cell contents to serum, giving the expected further increase of AKI, LDH and K^+, and with carriers and dystrophic patients usually showing greater increases in these enzymes than normal individuals, perhaps due to a higher blood cell content matching that of serum. In most subjects a fall in Na^+, glucose and a previously unchanged Alk P indicated their dilution on haemolysis, and although this sometimes occurred with true CPK, several subjects showed virtually no change, whereas others (1, 9 and 12 especially), showed a clear increase in nett CPK-Bk as if blood cell often contained as much or even more CPK than serum.

In a second series of 9 known DMD carriers (Table XVI) nett CPK-Bk detected 8 decisively (almost 2 to 50 fold elevations) and 1 barely, but ALD only 3 clearly (3 to 13 fold elevations) and 2 marginally. AKI was slightly elevated in only 4 instances and therefore of little use in carrier detection, to be expected due to AMP suppression and its irregular release from platelets on clotting (Todd *et al.*, 1964). Further, although CPK and ALD correlated very highly indeed, there was no correlation between either and AKI. A high AKI therefore, could occur with a marginal CPK elevation to give a false carrier diagnosis unless first subtracted. True CPK and ALD were grossly elevated in all DMD patients, but AKI rather less so; even in AK assay without AMP inhibition the elevations are much less than those of CPK (Kleine and Chlond, 1966). CPK and ALD correlated highly, contrary to the report of Harano *et al.* (1973), and AKI only with CPK. Values in the BMD patient 19 were similar, but even CPK failed to detect

Table XVI opposite

Enzyme activities (in i.u./l at 25°C) in serum from 9 (female) DMD carriers, 8 (male) DMD patients, 1 (female) BMD carrier, 1 (male) BMD patient, 7 patients with hepatitis-A and 2 patients after a myocardial infarction. In each case the blood was allowed to clot at room temperature for 1 hour. It was then centrifuged and the serum was assayed immediately on separation.

TABLE XVI

CPK, AKI & ALD IN DMD & BMD CARRIERS AND PATIENTS, HEPATITIS-A AND MYOCARDIAL INFARCTION

No.	Age (yrs)	Affected Relatives	CPK-Bk	AKI	Nett CPK-Bk	ALD		
DMD carrier females								
1	8.60	1b	2541	2.47	2538	28.87		
2	9.92	2b	1256	1.73	1254	14.44		
3	28.12	1b; 1s	118.4	0.74	117.7	1.48		
4	29.18	2s	413.2	2.47	410.7	4.04		
5	34.28	1s	263.8	2.59	261.2	2.45		
6	38.99	1s	53.77	1.23	52.54	1.04		
7	46.79	2b; 1s	88.06	1.48	86.58	1.86		
8	53.16	1b; 2s	109.0	0.25	108.8	1.75		
9	54.84	1mss; 2mb; 2s	197.3	0.74	196.6	6.61		
	Correlations (7 df):		CPK/AKI = 0.5122					
			CPK/ALD = 0.9875 ***					
			ALD/AKI = 0.4378					
DMD male patients (A = ambulant; W = wheelchair)								
10	3.12	1b (A)	1766	2.71	1764	32.37		
11	3.24	Nil (A)	6882	24.91	6857	58.41		
12	3.82	Nil (A)	4925	4.19	4921	66.74		
13	6.41	1mb (A)	4775	4.69	4771	68.92		
14	10.12	Nil (W)	2062	4.19	2058	18.46		
15	11.06	Nil (W)	2084	3.21	2081	14.71		
16	12.38	Nil (W)	965.0	3.95	961.0	7.95		
17	14.03	Nil (W)	2163	1.48	2162	14.98		
	Correlations (6 df):		CPK/AKI = 0.7573 *					
			CPK/ALD = 0.8733 **					
			ALD/AKI = 0.4197					
BMD carrier female								
18	33.23	1s; f	35.52	0.74	34.78	2.11		
BMD male patient								
19	10.65	mf	2131	2.22	2129	33.41		
Hepatitis-A patients							GOT	GPT
20	8.44		5.92	4.19	1.73	28.28	1141	1553
21	11.41		4.93	2.71	2.22	2.91	48.60	179.1
22	11.56		14.06	0.49	13.57	2.27	31.73	200.3
23	12.85		16.28	0.99	15.29	6.04	122.9	628.4
24	16.33		9.62	1.93	7.65	29.74	644.6	1754
25	18.01		10.61	3.21	7.40	19.23	787.7	1918
26	27.39		7.89	2.96	4.93	26.56	901.8	1373
Myocardial infarction								
27	46.24	5 hours after	77.70	0.99	76.71	1.61	8.44	6.08
		24 hours after	762.2	2.96	759.2	5.74	70.76	16.99
28	51.49	12 hours sfter	299.5	2.96	296.5	1.66	26.44	6.64

his carrier mother 18.

All hepatitis A patients (20-26, Table XVI) had elevated aminotransferases and ALD, but only slightly raised AKI. Nett CPK-Bk was extremely low, however, and, in all patients but one, even below the lowest limit of the normal range (Table IV). The presence of a dissociable inhibitor of CPK was therefore investigated. Mixing sera from hepatitis A patients and normal individuals gave only the expected CPK activity. Nor was any difference found between sera dialysed overnight and control sera, and the dialysate had the same effect as saline when used to dilute normal serum. Frozen hepatitis serum showed no increase in activity when stored for up to 4 weeks. Thus these very low CPK values, which seem typical of the condition (Thomson, 1971), and may indeed be associated with the unusually marked physical weakness of the early stages of the disease, may be due to a cold-stable, firmly bound inhibitor. In myocardial infarction patients 27 and 28 AKI had begun to show only slight elevations 12 hours after the event, and other enzyme values were as expected.

Differential centrifugation of heparinised blood from 3 subjects (Table XVII) enabled some formal assignation of enzyme changes to rbcs or wbcs. The relatively unchanged Na^+ and K^+ in the wbc suspension after chilling and vortex mixing indicates disruption of only a small volume of cells, but the large increases in PK (comparable to those arising from rbcs, and chiefly of type L (subject 2) raising % alanine inhibition and lowering M/LM ratio) suggest a very high PK content in the small number of wbcs present. In contrast LDH increases from wbcs were much more modest compared with those from rbcs. There was a slight fall in true CPK (subject 3)

Table XVII opposite

Enzyme activities (in i.u./l at 25°C), and concentrations of glucose (in mg/100ml) and electrolytes (in mequiv/l), in plasma separated from erythrocyte and leucocyte suspensions prepared from the blood of 3 healthy subjects (1 female and 2 male) as described on P. 25. The plasma was separated

(a) immediately after the preparation of the suspensions

(b) after storage of the suspensions at 1.5°C for 24 hours

(c) after storage of the suspensions at 1.5°C for 24 hours and subjection to 1 minute of vortex mixing to disrupt the blood cells.

TABLE XVII

BLOOD CELL CONTENTS OF HEALTHY SUBJECTS

Procedures: (a) Plasma separated forthwith
(b) 24 hours at 1.5°C, plasma separated
(c) 24 hours at 1.5°C, 1 min. in vortex mixer to disrupt cells, plasma separated

Assay	RBC suspension (a)	(b)	(c)	WBC suspension (a)	(b)	(c)
No. 1 Female	Aged 32.05 years					
PK	17.45	18.60	40.13	15.36	18.60	52.10
PK-LM	21.01	21.84	47.03	16.93		
PK-Ala	9.72	8.26	17.24	8.47		
PK-M	8.36	6.06	13.17	8.05		
% inhn	44.31	55.62	57.03	45.61		
Ratio	0.40	0.28	0.28	0.48		
CPK-Bg	19.24	19.82	32.94	17.49	17.93	19.14
CPK-Bk	24.67	24.67	44.15	24.42	24.67	25.41
LDH	91.15	139.6	347.4	85.08	104.6	177.9
Alk P	76.89	77.88	64.68	77.55		
Glucose	70.99	63.66	55.88	77.40		
Na^+	140	133	126	140	140	140
K^+	4.3	10.9	16.6	4.2	4.5	4.8
No. 2 Male	Aged 23.17 years					
PK	12.44	38.77	121.7	12.12		54.18
PK-LM	13.69	46.50	144.7	12.96		74.04
PK-Ala	8.57	12.96	50.68	8.15		23.25
PK-M	8.15	12.44	30.31	8.26		19.23
% inhn	31.09	66.58	58.37	32.75		57.09
Ratio	0.60	0.27	0.21	0.64		0.26
CPK-Bg	22.45	20.41	105.8	22.45		21.87
CPK-Bk	24.17	27.87	126.8	24.67		27.26
LDH	179.8	188.4	1117	148.4		274.7
Alk P	45.21	46.53	37.95	45.54		44.72
Glucose	63.20	55.42	46.26	65.95		62.06
Na^+	140	137	124	139		139
K^+	4.0	7.2	15.6	4.0		4.4

Continued ...

TABLE XVII (Continued)

Assay	RBC suspension (a)	(b)	(c)	WBC suspension (a)	(b)	(c)
No. 3 Male	Aged 23.27 years					
PK	14.42	25.81	93.53	12.75	12.12	69.70
PK-LM	13.79	33.02	122.8	14.74		
PK-Ala	9.93	8.88	18.29	9.09		
PK-M	8.15	10.03	24.04	8.88		
% inhn	31.16	65.59	80.45	28.69		
Ratio	0.59	0.30	0.20	0.60		
CPK-Bg	18.08	15.74	145.8	18.08	18.08	19.68
CPK-Bk	28.86	26.15	176.8	28.86	26.72	26.89
AKI	1.73	0.99	145.5	0.74	0.41	1.11
Nett CPK-Bk	27.13	25.16	30.83	28.12	26.31	25.78
LDH	107.4	252.2	1808	109.9	122.0	351.9
Alk P	56.76	60.72	33.66	54.45	55.99	58.41
Glucose	70.30	67.32	67.37	76.72	74.88	74.08
Na^+	138	135	125	140	140	142
K^+	4.3	6.3	17.7	4.3	4.6	4.9

on cell disruption as if absent from wbcs and the disparity between CPK-Bg and CPK-Bk (without AKI subtraction) in 2 may again be due to the action of glutathione reductase. After the same procedures with the rbc suspension large changes were noted in Na^+ and K^+, greater than previously found where cells are protected by incorporation in a clot, and indicating considerable cell disruption. The very large increases of LDH and PK, chiefly the liver form as in wbcs, likewise found in males 2 and 3 were not so marked in female 1. Both CPK-Bg and CPK-Bk showed marked elevations, again in males, but most of this was caused by AKI (subject 3), confirming the work of Todd et al. (1964) who found that rbcs had 30 times more AK than leucocytes, even if the far less numerous platelets did contain moderate amounts. Nevertheless, although true CPK was expected to fall slightly by its absence from rbcs, as did Alk P, glucose and Na^+, there was instead a 20% rise on haemolysis. In whole blood, therefore, changes in serum CPK (nett CPK-Bk), AKI, electrolytes and most of those in LDH are demonstrated to arise from rbcs, not wbcs, though both cell types can affect serum PK values.

This formal determination of the origins of these changes with subtraction of AKI to give true CPK, permitted in vivo examination of enzyme behaviour in whole blood by increasing the serum content after severe exercise. Several reports that serum enzymes are not elevated after exercise seem to conflict with established evidence (Swaiman and Awad, 1964; Forssell et al., 1975; Galteau et al., 1976), but these workers probably allowed either too little time for efflux to occur or else exercised their subjects only submaximally. More substantial studies, however, show that efflux does occur after prolonged exercise (Griffiths, 1966a; Shapiro et al.,

1973), after a time-lapse from the end of a short period of exercise (Nuttall and Jones, 1968; King et al., 1976), and after indications of marked depletion of ATP (Thomson et al., 1975a). Table XVIII shows that serum separated forthwith - procedure (a) - from blood taken on successive days after a severe evening training session showed little change in electrolytes, glucose or AKI. There was a small transient rise in LDH and a much larger prolonged elevation in true CPK, the enzyme most sensitive to the effects of exercise according to Sanders and Bloor (1975). These increases returned to pre-exercise values by day 4 or 5, a decline both Ahlborg and Brohult (1966) and King et al. (1976) confirmed and found exponential. Simultaneous chilling of clotted blood aliquots for 24 hours at 1.5°C without - procedure (b) - or with - procedure (c) - mechanical haemolysis disclosed the underlying enzyme movements. Both these procedures caused very similar changes in Na^+, K^+ and glucose on successive days, indicating a similar degree of efflux and of cell disruption. Like changes in AKI occurred but far less precisely. The sudden 2 fold increase of rbc LDH, shown by chilling and haemolysis on day 1, which returned to normal on day 2, suggests rapid incorporation, then release, of effluent muscle LDH. This LDH is then presumably cleared from serum by the tissues - Sweetin and Thomson (1973b) demonstrated that enzyme clearance may be a property of the normal cell - with rbcs in effect acting to buffer the rise in serum LDH, therefore reducing the magnitude of these elevations.

After day 1 serum CPK showed small oscillatory changes of (c) compared with (b), as if there was continuous movement of CPK across the cell membrane in an attempt to attain an equilibrium between cells and serum. It seems unlikely that simple membrane

Table XVIII opposite

Enzyme activities (in i.u./l at 25°C), and concentration of glucose (in mg/100 ml) and electrolytes (in mequiv/l), in serum separated from the clotted blood of a healthy male before, and on the 5 consecutive days after, a 2 hour period of severe exercise. Each specimen of whole blood was divided into 3 aliquots which were treated as follows

(a) allowed to clot at room temperature for 1 hour before the serum was separated and analysed

(b) stored at 1.5°C for 24 hours before the serum was separated and analysed

(c) stored at 1.5°C for 24 hours and subjected to 1 minute of vortex mixing to disrupt the blood cells before the serum was separated and analysed

Each CPK or AKI activity given is the mean of two estimations.

TABLE XVIII

EFFECT OF EXERCISE ON SERUM AND BLOOD CELL ENZYMES IN A HEALTHY MALE (AGED 23.27 YEARS)

Procedures: (a) Serum separated forthwith

(b) Clotted blood incubated 24 hours 1.5°C, serum separated

(c) Clotted blood incubated 24 hours 1.5°C, 1 min. in vortex mixer to haemolyse, serum separated

Days after exercise	Procedure	CPK-Bk	AKI	Nett CPK-Bk	LDH	Gluc.	Na^+	K^+
0	(a)	28.63	0.62	28.00	111.9	83.13	143	4.3
	(b)	30.34	1.73	28.61	221.8	81.07	137	8.9
	(c)	44.28	14.80	29.48	454.7	70.53	134	11.7
1	(a)	80.29	0	80.29	125.1	88.85	145	3.9
	(b)	97.06	1.60	95.46	517.0	80.15	138	8.8
	(c)	125.4	36.75	88.68	886.2	73.97	133	12.2
2	(a)	76.71	0.37	76.34	130.7	86.56	142	4.8
	(b)	73.51	2.59	70.92	238.0	79.69	139	8.6
	(c)	84.48	11.84	72.64	451.7	70.07	135	11.4
3	(a)	40.33	0.25	40.08	117.0	89.54	142	4.4
	(b)	44.28	0.74	43.54	263.3	82.44	138	8.8
	(c)	69.31	28.74	40.58	518.0	77.86	134	11.6
4	(a)	35.64	2.10	33.55	109.4	93.66	141	4.5
	(b)	37.99	6.04	31.94	227.4	86.10	137	8.5
	(c)	54.39	19.73	34.66	462.3	79.00	134	11.6
5	(a)	29.23	1.85	27.38	105.8	90.68	140	4.4
	(b)	30.59	2.71	27.87	185.3	90.00	136	8.0
	(c)	42.80	15.91	26.89	392.5	73.51	132	11.4

Each CPK and AKI value given is the mean of two estimations.

attachment could explain these observations. On day 1, a marked CPK efflux occurred from erythrocytes to serum (b), and simultaneously, (c) showed that these rbcs, now disrupted, contained less CPK than the (b) serum into which the CPK was being discharged, though still with a higher content than the (a) serum. This implies that during chilling CPK had been actively expelled from rbcs tolerant only of equilibrium, and also suggests that serum CPK values had been higher previously, a fact that accords well with reports that maximum values of CPK in serum are reached some 11 hours after physical exertion (Nuttall and Jones, 1968; King et al., 1976). Thus rbc CPK content responds as delayed but similar changes to those in serum; likewise an explanation is found for the prompt emphatic response of serum CPK compared with the smaller and slower changes of LDH in the same circumstances.

In carrier detection therefore, if serum CPK is assayed in precisely defined conditions (Thomson, 1969b; 1971), with the further refinements proposed here, i.e. the use of dithiothreitol as CPK activator and the subtraction of AKI from true CPK, then the enzyme is undoubtedly far more sensitive and dependable than the less stable and more inconvenient PK. This superiority of CPK is further enhanced by its relative stability at room temperature in serum and clotted blood allowing overnight posting, which is a considerable practical convenience in e.g. population studies. The AKI deduction removes false elevations due to haemolysis, so irredeemable in PK, ALD and LDH. The apparent equivalence of CPK in serum and erythrocytes likewise discounts the effect of efflux even without visible haemolysis, an ever present but often unsuspected risk in these other enzymes present in blood cells in very large amounts.

No doubt still further improvements might be made to the CPK assay. AMP, apart from its effect on AK, is said to exert some inhibition on CPK itself (Hess et al., 1968), thereby reducing the assay sensitivity. Diadenosine pentaphosphate (Lienhard and Secemski, 1973; Feldhaus et al., 1975) and fluoride (Rosano et al., 1976) have been suggested as alternative AK inhibitors but, although further work on the effect of diadenosine pentaphosphate on CPK appears necessary, fluoride inhibits CPK by some 6% (Szasz et al., 1976a). Szasz et al. (1976b) report that a compromise between inhibition of CPK and AK by using 3 mM AMP seems superior, but use of this may be restricted after storage or haemolysis, or for research purposes. Rosano et al. (1976) state that CPK could be accurately measured by the use of a blank correction method, equivilant to AKI subtraction but with no AK inhibitor added, and this does, in fact, appear to be the only worthwhile suggestion so far.

CHAPTER IV

DMD AND PURINE METABOLISM

A double-blind trial : Results

A 16-point scale of physical improvements (Table XIX) emerged during the double-blind trials of allopurinol, PA3 and PA5. From Table XX changes in the clinical status over the three 6 week periods can be followed in each patient (the numbering used to identify each individual was allocated after the trials were completed). In period B restoration of departed functions was clearly noted; thus of 8 patients (1-8) taking allopurinol daily 7 improved clinically and 1 did not, while in the placebo group (9-16) 2 improved and 6 did not. Using Fisher's Exact Probability Test this marked difference of effect was found to be significant* ($P = 0.0406$). The mean values of enzymes (Table XXI) in the 2 improved placebo patients, 9 and 11, were significantly increased by over 50% (Table XXIII, GOT* and CPK** respectively) and these, along with the small increase of GOT* in 16, were the only enzyme elevations increased in period B. CPK* and ALD** did, however, fall by about 20% in allopurinol patient 4. In patients 1-8 the values of γ-GT, which occurs in liver but not in muscle (Orlowski and Szewczuk, 1960) and is a sensitive monitor of liver health (Zein and Discombe, 1970), were quite normal indicating specificity of gene action and absence of liver damage by allopurinol.

Table XXII gives the mean values of other parameters tested. Only patients receiving allopurinol showed decreased serum and

Table XIX opposite

This table was used to determine the clinical status of the 16 DMD patients in the double-blind trials of allopurinol and procaine adenylates.

TABLE XIX

CLINICAL STATUS OF PATIENTS DURING TRIAL

Wheelchair - (1) Slumps in wheelchair, unable to hold head up.
(2) Slumps in wheelchair, holds head up.
(3) Sits up unsteadily, kicks feebly with dependent leg.
(4) Sits upright confidently, lifts knee from chair but cannot raise arms.
(5) Raises both arms above head.
(6) Raises both feet up to seat level of wheelchair.
(7) Can climb up from floor to sit on kitchen chair.
(8) Can climb from kitchen chair to standing up unsteadily, both hands gripping nearby furniture.

Ambulant - (9) Frequent collapse due to knees giving way, prefers being carried to walking.
(10) Walks slowly with rolling gait and very marked lordosis, unable to rise from chair unaided.
(11) Rises unaided from chair, but cannot attain standing from supine position without help.
(12) Walks less slowly, can stand up from supine position only by climbing up furniture.
(13) Stands up from supine position by climbing up own legs (Gowers' sign), still walks slowly with marked waddle and some lordosis, tires quickly and falls often, has sore legs and frequent muscle cramps after exertion.
(14) Can keep up with others walking quickly, minimal roll and lordosis, fewer falls and muscle cramps after exertion.
(15) Shares prolonged vigorous adventures with friends without usual rapid fatigue and sore legs, no more falls, minimal Gowers' sign.
(16) Gowers' sign and waddle disappear, normal activity, no more muscle cramps after exertion.

Table XX opposite

Changes in clinical status of the 16 DMD patients described during the double-blind trials of allopurinol and procaine adenylates.

TABLE XX

EFFECTS OF ALLOPURINOL & ADENYLATE

A = ambulant W = wheelchair

No.	Age (yrs)	TABLET ALLOCATIONS Periods on allopurinol	Adenylate in Period C	CLINICAL STATUS at end of Period A	B	C	CLINICAL IMPROVEMENT B v A	C v B
1 (A)	5.06	BC	Nil	13	15	16	+	+
2 (A)	6.67	BC	Nil	13	14	15	+	+
3 (W)	8.37	BC	Nil	2	3	6	+	+
4 (W)	11.36	BC	Nil	1	4	5	+	+
5 (A)	5.28	BC	PA5	12	12	12	0	0
6 (A)	7.74	BC	PA5	13	15	16	+	+
7 (W)	8.31	BC	PA5	2	5	5	+	0
8 (W)	13.39	BC	PA5	5	7	8	+	+
9 (A)	3.39	C	Nil	13	15	15	+	0
10 (A)	7.42	C	Nil	11	11	12	0	+
11 (A)	8.38	C	Nil	10	11	11	+	0
12 (W)	14.29	C	Nil	5	5	6	0	+
13 (A)	8.31	C	PA3	13	13	15	0	+
14 (A)	8.44	C	PA3	10	9	12	0	+
15 (W)	10.36	C	PA3	2	2	5	0	+
16 (W)	11.99	C	PA3	3	3	5	0	+

Table XXI

Serum enzyme activities (in i.u./l at 25°C) for each of the DMD patients shown in Table XX. Each figure is the mean of 6 estimations made at weekly intervals throughout each period.

TABLE XXI

MEAN VALUES OF ENZYMES FOR EACH 6 WEEK PERIOD

Patient	Period	Nett CPK-Bk	ALD	GOT	γ-GT
1	A	6955	85.39	164.1	3.60
	B	7491	97.01	210.1	3.67
	C	6471	86.99	159.8	4.26
2	A	3609	49.28	118.8	2.24
	B	4082	54.04	143.4	2.34
	C	3422	52.64	149.8	2.91
3	A	1677	14.57	27.13	2.77
	B	1795	16.43	31.91	2.05
	C	1660	17.35	36.79	2.34
4	A	1340	10.11	39.01	5.33
	B	1044	7.37	35.52	4.39
	C	1173	7.71	39.37	3.99
5	A	4547	47.92	142.0	1.45
	B	4598	51.82	157.8	2.56
	C	3670	39.37	132.7	3.12
6	A	2864	33.42	77.79	3.15
	B	3453	36.28	106.6	2.72
	C	2817	30.02	89.65	2.72
7	A	1985	17.29	72.19	10.64
	B	1754	17.68	73.13	10.77
	C	1440	14.59	70.05	9.18
8	A	1348	14.92	45.93	3.45
	B	1322	13.96	49.25	3.15
	C	1228	14.63	44.33	3.09
9	A	2279	47.91	92.38	3.77
	B	2875	60.25	146.8	2.32
	C	2857	64.83	142.1	2.07
10	A	1785	14.98	52.76	2.72
	B	2155	18.95	63.92	2.86
	C	1966	17.01	52.56	3.66
11	A	1805	18.90	44.78	3.49
	B	2713	24.87	58.29	2.64
	C	2369	29.76	74.71	2.80
12	A	1647	15.03	49.76	2.96
	B	1400	15.75	46.37	3.29
	C	1689	17.03	54.78	3.18

Continued ...

Table XXII opposite

Mean values of serum urate concentration (in mg/100ml), urinary urate and creatinine outputs (in mg/24 hours), manometric performance (height in cm of mercury, see P. 33) and time taken to climb the same flight of 10 stairs (in seconds) for each DMD patient shown in Table XX. Each figure is the mean of 6 estimations made at weekly intervals throughout each period.

TABLE XXI (Continued)

Patient	Period	Nett CPK-Bk	ALD	GOT	γ-GT
13	A	1907	24.90	53.47	1.46
	B	2096	24.63	60.08	2.56
	C	1934	22.66	59.30	3.07
14	A	2065	17.80	66.87	2.56
	B	1827	17.00	61.94	2.67
	C	2073	20.05	82.09	2.48
15	A	1604	15.72	52.86	3.77
	B	1357	14.05	45.56	4.07
	C	1323	14.71	46.83	4.15
16	A	1008	9.14	42.05	9.05
	B	1201	9.62	51.84	11.31
	C	1076	8.51	46.97	7.35

TABLE XXII

MEAN VALUES OF OTHER PARAMETERS FOR EACH 6 WEEK PERIOD

Patient	Period	Serum urate	24 hr output		Manometry		Stairs
			Urate	Creat-inine	1 hand	Both hands	
1	A	3.83	180.7	221.7		16.7	9.8
	B	2.58	135.7	234.1		19.9	8.8
	C	2.79	112.3	200.2		23.0	8.3
2	A	3.88	119.8	135.7		12.3	14.8
	B	2.90	63.97	118.8		16.2	11.8
	C	2.92	77.85	126.7		19.7	9.8
3	A	3.29	236.5	196.8	9.0	18.8	
	B	2.46	192.2	234.1	12.3	24.2	
	C	2.33	162.1	218.3	14.6	24.4	
4	A	2.89	261.1	157.2	11.4	20.9	
	B	2.03	194.2	177.6	11.3	22.8	
	C	2.21	250.2	219.4	13.3	22.5	

Continued ...

TABLE XXII (Continued)

Patient	Period	Serum urate	24 hr output		Manometry		Stairs
			Urate	Creat-inine	1 hand	Both hands	
5	A	3.05	293.1	261.3		9.7	37.8
	B	2.83	245.8	270.3		11.7	34.3
	C	3.33	296.6	287.3		13.4	36.7
6	A	3.21	388.9	371.0	18.6	31.2	8.3
	B	2.74	310.1	436.6	22.1	33.0	9.2
	C	3.11	453.6	437.7	29.4	37.1	8.8
7	A	3.57	386.9	212.6	4.3	12.9	
	B	2.92	263.3	227.3	6.1	13.9	
	C	3.25	277.6	250.0	14.2	20.3	
8	A	3.62	319.5	307.6	14.3	25.0	
	B	2.57	205.6	359.7	19.0	27.9	
	C	3.46	275.7	338.2	27.6	34.3	
9	A	3.56	200.9	221.7		7.8	16.0
	B	3.69	139.5	174.2		15.1	12.7
	C	2.34	128.5	188.9		19.1	9.1
10	A	3.76	262.7	202.4		11.7	71.2
	B	3.40	256.2	192.3		13.5	101.5
	C	3.20	230.0	231.9		16.6	104.8
11	A	3.70	319.6	260.1		15.1	53.6
	B	3.15	258.0	214.9		18.3	42.5
	C	2.22	185.9	256.7		21.4	36.8
12	A	4.03	309.7	295.2	18.4	26.2	
	B	4.60	369.3	365.3	24.3	27.7	
	C	3.70	287.4	376.6	26.7	33.4	
13	A	3.31	277.6	322.4		21.6	11.5
	B	3.28	327.5	381.1		23.8	10.3
	C	2.81	236.3	365.3		26.8	9.2
14	A	3.67	293.6	252.2	15.1	28.8	
	B	3.88	280.4	285.0	18.7	29.9	
	C	4.09	292.4	269.4	20.3	30.6	
15	A	3.01	378.8	313.3	11.2	22.9	
	B	3.43	306.9	224.9	12.7	25.8	
	C	3.34	330.2	253.3	13.7	24.7	
16	A	3.58	166.1	109.7	8.3	20.1	
	B	3.15	189.8	118.8	9.9	21.3	
	C	3.33	153.2	112.0	10.2	24.6	

urinary output of urate, significant only in some (Table XXIII). Interestingly, patient 5, who alone showed no clinical improvement on allopurinol, had the smallest drop both in 24 hour urinary and in serum urate. As nearly all patients in period B improved their performance on manometry, using 1 and both hands, with most of the ambulant boys ascending the stairs more quickly, little information was given by these measurements on an individual basis. However, although no significant differences were found between the group means of enzyme values from period A to B, this comparison proved useful when applied elsewhere (Table XXIV). Thus the fall of serum*** and urinary*** urate, and the rise of creatinine output*, became significant in 1-8 after allopurinol (1-8A v 1-8B), whereas no significant changes in these values occurred in the placebo group (9-16). Manometry using both hands revealed significant mean increases of physical performance in period B, in both allopurinol (18.43 up to 21.19***) and placebo (19.27 up to 21.92***) groups, with the improvement of the treated group marginally higher. Using 1 hand however, the increase did become significant only with allopurinol*. As expected, there were no differences between the 2 groups in period A (1-8A v 9-16A), while in period B only the serum urate*** was significantly different.

In Table XXV the mean percentage differences between periods are listed, in order to illustrate better the changes in means. These differences are compared to zero (no change occurring) and to the mean percentage difference of the other group (1-8 v 9-16). In the allopurinol group, serum and 24 hour urinary urate fell** by 26.58% and 27.49% respectively, while creatinine output rose by 8.73%*, moreover the percentage manual improvement (both hands, 16.75%) was likewise significantly greater** than zero. Manometry

Table XXIII opposite

Percentage differences, and their significance, between the means in periods A and B of each parameter measured in the DMD patients shown in Tables XX, XXI and XXII.

TABLE XXIII

SIGNIFICANCE AND PERCENTAGE DIFFERENCE BETWEEN INDIVIDUAL MEANS OF PERIODS A & B - ENZYMES

Patient	Nett CPK-Bk	ALD	GOT	ɣ-GT
1	+ 7.70	+13.60	+28.05	+ 1.69
2	+13.11	+ 9.67	+20.73	+ 4.83
3	+ 7.00	+12.74	+17.62	-26.21
4	-22.05 *	-27.12 **	- 8.95	-17.57
5	+ 1.12	+ 8.28	+11.11	+76.00
6	+20.58	+ 8.56	+37.01	-13.68
7	-11.65	+ 2.28	+ 1.29	+ 1.26
8	- 1.92	- 6.42	+ 7.21	- 8.61
9	+26.20	+25.76	+58.91 *	-38.58
10	+20.74	+26.50	+21.17	+ 4.96
11	+50.29 **	+31.60	+30.16	-24.30 **
12	-14.99	+ 4.81	- 6.82	+10.90
13	+ 9.94	- 1.08	+12.35	+75.76
14	-11.53	- 4.49	- 7.37	+ 4.22
15	-15.43	-10.64	-13.81	+ 7.85
16	+19.14	+ 5.30	+23.29 *	+24.99 *

- OTHER PARAMETERS

	Serum	24 hr Output		Manometry		
Patient	Urate	Urate	Cr/nine	1 Hand	Both	Stairs
1	-32.63*	-24.91	+ 5.61		+19.26	-10.17*
2	-55.86**	-46.62*	-12.50		+31.09**	-20.23**
3	-25.14***	-18.74	+18.79	+37.03*	+28.89**	
4	-29.88	-25.61	+12.95	- 0.74	+ 8.76*	
5	- 7.28	-16.17	+ 3.46		+20.69*	- 1.32
6	-14.61	-20.28	+17.68	+18.83*	+ 5.88	+10.01*
7	-18.28*	-31.94	+ 6.91	+43.13*	+ 7.74	
8	-28.92***	-35.65**	+16.91	+33.33***	+11.67	
9	+ 3.50	-30.57	-21.43		+92.56**	-20.83**
10	- 9.78	- 2.48	- 5.03		+15.38	+42.56**
11	-14.85	-19.29	-17.39	+74.67***	+21.00**	-20.71**
13	- 0.76	+17.99	+18.25	+51.01*	+10.04	-10.15*
14	+ 5.69	- 4.51	+13.00		+22.68**	+ 3.91
15	+14.16	-18.98*	-28.52**	+13.02	+12.81*	
16	-12.15	+14.25	+ 8.25	+19.01	+ 6.22	
12	+14.14	+19.26	+23.75	+31.67**	+ 5.60	

Table XXIV opposite

Mean values, calculated from Tables XXI and XXII, of

serum enzyme activities (in i.u./l at 25°C)

serum urate concentration (in mg/100ml)

urinary urate and creatinine outputs (in mg/24 hours)

manometric performance (height in cm of mercury)

time taken to climb the same 10 stairs (in seconds)

for the group of DMD patients who received allopurinol in period B (nos. 1-8) and for the group who received a placebo in period B (nos. 9-16) and the significance of the differences between them.

TABLE XXIV

GROUP MEANS FOR PERIODS A & B WITH SIGNIFICANCE OF DIFFERENCES

MEANS	Nos. 1-8 Period A	Nos. 9-16 Period A	Nos. 1-8 Period B	Nos. 9-16 Period B
Nett CPK-Bk	3042	1764	3194	1955
ALD	34.11	20.55	36.82	23.14
GOT	85.87	56.87	101.0	66.85
Serum urate	3.42	3.58	2.63	3.57
24 hr output				
Urate	273.3	276.1	201.3	265.9
Creatinine	232.0	247.1	257.3	244.4
Manometry				
1 hand	11.50	13.26	14.17	16.38
Both hands	18.43	19.27	21.19	21.92
Stairs	17.12	38.08	15.61	41.75

SIGNIFICANCES	1-8(A) v 1-8(B)	9-16(A) v 9-16(B)	1-8(A) v 9-16(A)	1-8(B) v 9-16(B)
Nett CPK-Bk	NS	NS	NS	NS
ALD	NS	NS	NS	NS
GOT	NS	NS	NS	NS
Serum urate	P<0.001	NS	NS	P<0.001
24 hr output				
Urate	P<0.001	NS	NS	NS
Creatinine	P<0.05	NS	NS	NS
Manometry				
1 hand	P<0.05	NS	NS	NS
Both hands	P<0.001	P<0.01	NS	NS
Stairs	NS	NS	NS	NS

Table XXV opposite

Mean changes, calculated from Tables XXIII and XXVI, in

serum enzyme activities (in i.u./l at 25°C)

serum urate concentration (in mg/100ml)

urinary urate and creatinine outputs (in mg/24 hours)

manometric performance (height in cm of mercury)

time taken to climb the same 10 stairs (in seconds)

in the successive periods of the experiment for each group of the DMD patients shown in Table XX. The change between periods is expressed in percentage terms for each parameter measured. The extreme right hand column (b) indicates whether the change is significantly different between groups, column (a) indicates whether the percentage change shown is significantly different from zero.

TABLE XXV

MEAN PERCENTAGE DIFFERENCE BETWEEN PERIODS A&B AND B&C

(a) - Significance of difference from 0
(b) - Significance of difference between ...

BETWEEN A&B	Nos. 1-8	(a)	Nos. 9-16	(a)	(b)... (1-8)&(9-16)
Nett CPK-Bk	+ 1.74	NS	+10.55	NS	NS
ALD	+ 2.97	NS	+ 9.72	NS	NS
GOT	+14.26	P<0.05	+14.74	NS	NS
Serum urate	-26.58	P<0.01	- 0.01	NS	P<0.01
24 hr output					
Urate	-27.49	P<0.001	- 3.04	NS	P<0.01
Creatinine	+ 8.73	P<0.05	- 1.14	NS	NS
Manometry					
1 hand	+26.32	P<0.01	+27.48	P<0.01	NS
Both hands	+16.75	P<0.01	+20.94	NS	NS
Stairs	- 5.43	NS	- 2.28	NS	NS

BETWEEN B&C	Nos. 5-8	(a)	Nos. 1-4	(a)	(b)... (1-4)&(5-8)
Nett CPK-Bk	-15.90	P<0.05	- 6.25	NS	NS
ALD	-13.50	NS	- 0.68	NS	NS
GOT	-11.50	P<0.05	+ 1.66	NS	NS
Serum urate	+19.20	P<0.05	+ 3.07	NS	P<0.05
24 hr output					
Urate	+26.63	NS	+ 4.40	NS	NS
Creatinine	+ 2.63	NS	+ 2.25	NS	NS
Manometry					
1 hand	+70.43	NS	+17.58	-	-
Both hands	+24.11	NS	+ 9.27	NS	NS
Stairs	+ 1.13	-	-11.75	-	-

BETWEEN B&C	Nos. 13-16	(a)	Nos. 9-12	(a)	(b)... (9-12)&(13-16)
Nett CPK-Bk	- 1.79	NS	- 0.36	NS	NS
ALD	+ 0.77	NS	+ 6.29	NS	NS
GOT	+ 6.16	NS	+ 6.34	NS	NS
Serum urate	- 1.51	NS	-19.45	NS	NS
24 hr output					
Urate	- 8.82	NS	-17.06	P<0.05	NS
Creatinine	- 0.54	NS	+12.90	NS	NS
Manometry					
1 hand	+19.73	NS	+10.01	-	-
Both hands	+ 6.40	NS	+21.87	P<0.01	P<0.05
Stairs	-11.28	-	-12.79	NS	-

performance with 1 hand was increased to the same extent in both groups, this being the only placebo parameter differing significantly from zero. A 14.26%* rise of GOT in the allopurinol group was the sole significant enzyme change.

It is difficult to determine if PA3 or PA5 had any perceptible clinical effect (Table XX), especially since improvement on allopurinol alone continued in period C (1-4). Only 5 showed no clinical improvement at all, whereas the rest maintained and even improved their progress of period B. The mean values of parameters tested in period C are given in Tables XXI and XXII. There was no significant percentage difference of individual enzyme means between periods B and C in any patient receiving either a PA5 or a PA3 placebo (Table XXVI, 1-4 and 9-12), however in those receiving PA5 (5-8), CPK* and ALD* in 5, and ALD* alone in 7, decreased by about 20%; while PA3 (13-16), GOT* rose in 14. Those on PA5 had also increased urate values, whereas PA3 patients showed little change, the effects of allopurinol and PA3 presumably being equivalent but opposite. All patients taking PA5, but only 2 placebo patients bettered their manometry performance, however.

Comparisons of the PA5 group means (Table XXVII) showed few significant changes; with the urate values of 5-8 in period C increasing* (B v C) and the creatinine excretion of those receiving PA5 being significantly greater* than the placebo group in period C, but not in B. The PA5, but not the placebo group, improved their performance on manometry using 1** and both* hands. Of the PA3 group means (Table XXVIII), in patients receiving allopurinol for the first time, only those taking placebo PA3 tablets as well showed a fall in serum urate*, with a rise in creatinine output*

Table XXVI opposite

Percentage differences, and their significance, between the means in periods B and C of each parameter measured in the DMD patients shown in Tables XX, XXI and XXII.

TABLE XXVI

SIGNIFICANCE AND PERCENTAGE DIFFERENCE BETWEEN INDIVIDUAL MEANS OF PERIODS B & C - ENZYMES

Patient	Nett CPK-Bk	ALD	GOT	ɣ-GT
1	-13.62	-10.33	-23.92	+29.52
2	-16.17	- 2.60	+ 4.44	+24.16
3	- 7.52	+ 5.64	+15.29	+14.46
4	+12.32	+ 4.57	+10.83	- 9.20
5	-20.18 *	-24.03 *	-15.90	+22.08
6	-18.41	-17.25	-15.89	0
7	-17.85	-17.48 *	- 4.21	-14.75
8	- 7.11	+ 4.77	- 9.99	- 1.97
9	- 0.64	+ 7.60	- 3.18	-10.45
10	- 8.74	-10.20	-17.78	+28.30
11	-12.66	+19.65	+28.17	+ 6.14
12	+20.61	+ 8.11	+18.14	- 3.29
13	- 7.75	- 8.00	- 1.29	+19.97
14	+13.48	+17.92	+32.53 *	- 7.09
15	- 2.46	+ 4.71	+ 2.79	+ 1.99
16	-10.43	-11.56	- 9.41	-35.00 *

- OTHER PARAMETERS

Patient	Serum Urate	24 hr Output Urate	24 hr Output Cr/nine	Manometry 1 Hand	Manometry Both	Manometry Stairs
1	+ 8.01	-17.26	-14.49		+15.48**	- 6.60
2	+ 0.62	+21.69	+ 6.67		+21.65***	-16.90*
3	- 5.49	-15.68	- 6.76	+18.24	+ 1.03	
4	+ 9.14	+28.86	+23.57	+16.92	- 1.10	
5	+17.69**	+20.69	+ 6.28		+15.00**	+ 6.80
6	+13.46	+46.30**	+ 0.26	+33.21***	+12.37**	- 4.55
7	+11.32	+ 5.42	+ 9.95	+132.9***	+46.10***	
8	+34.32***	+34.09	- 5.97	+45.17***	+22.98**	
9	-36.54*	- 7.86	+ 8.44		+26.52*	-28.29***
10	- 5.77	-10.24	+20.59		+22.84*	+ 3.25
11	-15.95***	-27.93	+19.47	+10.05	+17.35***	-13.33**
12	-19.54***	-22.19	+ 3.10	+ 9.97	+20.78**	
13	-14.47*	-27.86**	- 4.15	+62.17***	+12.63***	-11.28**
14	+ 5.49	+ 4.28	- 5.56	+ 8.92**	+ 2.23	
15	- 2.80	+ 7.59	+13.13	+ 5.29	- 4.51	
16	+ 5.72	-19.29	- 5.71	+ 2.52	+15.23*	

Table XXVII opposite

Mean values, calculated from Tables XXI and XXII, of

serum enzyme activities (in i.u./l at 25°C)

serum urate concentration (in mg/100 ml)

urinary urate and creatinine outputs (in mg/24 hours)

manometric performance (height in cm of mercury)

time taken to climb the same 10 stairs (in seconds)

for the group of DMD patients who received allopurinol and PA5 in period C (nos. 5-8) and for the group who received allopurinol and a placebo in period C (nos. 1-4) and the significance of the differences between them.

TABLE XXVII

PA5 GROUP MEANS FOR PERIODS B & C WITH SIGNIFICANCE OF DIFFERENCES

MEANS	Nos. 1-4 Period B	Nos. 5-8 Period B	Nos. 1-4 Period C	Nos. 5-8 Period C
Nett CPK-Bk	3603	2782	3182	2289
ALD	43.71	29.94	41.17	24.06
GOT	105.2	96.70	96.44	84.18
Serum urate	2.49	2.77	2.56	3.29
24 hr output				
Urate	146.5	256.2	150.6	325.9
Creatinine	191.2	323.5	191.2	328.3
Manometry				
1 hand		15.73		23.73
Both hands	19.98	21.63	22.4	26.28
Stairs				

SIGNIFICANCES	1-4(B) v 1-4(C)	5-8(B) v 5-8(C)	1-4(B) v 5-8(B)	1-4(C) v 5-8(C)
Nett CPK-Bk	NS	NS	NS	NS
ALD	NS	NS	NS	NS
GOT	NS	NS	NS	NS
Serum urate	NS	P<0.05	NS	P<0.05
24 hr output				
Urate	NS	NS	P<0.05	P<0.05
Creatinine	NS	NS	NS	P<0.05
Manometry				
1 hand		P<0.01		
Both hands	NS	P<0.05	NS	NS
Stairs				

Table XXVIII opposite

Mean values, calculated from Tables XXI and XXII, of

serum enzyme activities (in i.u./l at 25°C)

serum urate concentration (in mg/100 ml)

urinary urate and creatinine outputs (in mg/24 hours)

manometric performance (height in cm of mercury)

time taken to climb the same 10 stairs (in seconds)

for the group of DMD patients who received allopurinol and PA3 in period C (nos. 13-16) and for the group who received allopurinol and a placebo in period C (nos. 9-12) and the significance of the differences between them.

TABLE XXVIII

PA3 GROUP MEANS FOR PERIODS B & C WITH SIGNIFICANCE OF DIFFERENCES

MEANS	Nos. 9-12 Period B	Nos. 13-16 Period B	Nos. 9-12 Period C	Nos. 13-16 Period C
Nett CPK-Bk	2286	1620	2220	1602
ALD	29.96	16.33	32.16	16.48
GOT	78.60	54.86	81.04	58.80
Serum urate	3.71	3.44	2.87	3.39
24 hr output				
Urate	255.8	239.1	208.0	253.0
Creatinine	236.7	252.5	263.5	250.0
Manometry				
1 hand		13.8		14.7
Both hands	18.7	25.2	22.6	26.7
Stairs	52.2		50.2	

SIGNIFICANCES	9-12(B)v 9-12(C)	13-16(B)v 13-16(C)	9-12(B)v 13-16(B)	9-12(C)v 13-16(C)
Nett CPK-Bk	NS	NS	NS	NS
ALD	NS	NS	NS	NS
GOT	NS	NS	NS	NS
Serum urate	P<0.05	NS	NS	NS
24 hr output				
Urate	NS	NS	NS	NS
Creatinine	P<0.05	NS	NS	NS
Manometry				
1 hand		NS		
Both hands	P<0.05	NS	NS	NS
Stairs	NS			

and manometry performance* (both hands). The percentage differences between periods B and C (Table XXV), of CPK* and GOT* in patients taking PA5 (5-8) were lower than zero, with the increase in serum urate* greater than in the placebos (1-4). With the PA3 groups those receiving the placebo performed better with both hands* than did those on PA3.

A final comparison of period A with C can give little specific information on allopurinol, PA3 or PA5, but it does indicate, clinically and biochemically, a general effect of intervention in purine metabolism. Thus, in 9 and 11 (Table XXIX), the 2 patients receiving the placebo in period B who improved clinically, but did have increased enzyme elevations, maintained these new values, whereas 4 and 7 had decreases of ALD** and CPK* respectively. 2 other patients more than doubled their original γ-GT values, but these elevations were still below the adult normal range (Table III). Of the other parameters, many patients showed reduced urate values and 4 had a 40% increase* in creatinine output. All patients improved their manometry performance, in many instances markedly, though in period B several taking only placebo tablets did likewise.

The standard deviations and coefficients of variation, calculated for all parameters in every patient at the end of each period, showed completely random non-significant changes. Likewise the correlation coefficients of CPK/GOT and ALD, and GOT/ALD were not significant.

12 of the 16 patients, compared with a reported 80% (Emery, 1972), had values higher than expected by age for the algebraic

Table XXIX opposite

Percentage differences, and their significance, between the means in periods A and C of each parameter measured in the DMD patients shown in Tables XX, XXI and XXII.

TABLE XX IX

SIGNIFICANCE AND PERCENTAGE DIFFERENCE BETWEEN INDIVIDUAL MEANS OF PERIODS A & C - ENZYMES

Patient	Nett CPK-Bk	ALD	GOT	ɣ-GT
3			+35.60***	
4		-23.79**		
5				+114.9**
7	-27.45**			
9	+25.39*	+35.31*	+53.85*	
11			+66.83*	
13				+110.9*

- OTHER PARAMETERS

Patient	Serum Urate	24 hr Output Urate	24 hr Output Cr/nine	Manometry 1 Hand	Manometry Both	Manometry Stairs
1		-37.87*			+37.72**	-16.10**
2	-24.77**				+59.47***	-33.71***
3	-29.25***	-31.48**		+62.03***	+30.22***	
4			+39.57*	+16.06*	+ 7.57*	
5					+38.79***	
6				+58.30***	+18.98***	
7				+223.3***	+57.41***	
8				+93.56***	+37.33***	
9					+143.6***	-43.23***
10	-14.98*				+41.75***	+45.35**
11	-40.02***			+92.23**	+41.99***	-31.28***
12				+44.80***	+27.55**	
13	-15.12*	-14.88*		+144.9***	+23.94***	-20.29***
14				+34.81***	+ 6.22*	
15				+21.94*		
16					+22.41***	

sum of the R and S waves in lead V1 of the ECG. During periods B and C, however, these changed only slightly and at random to give no information, in accord with an origin anatomic rather than metabolic (Perloff et al., 1966).

Discussion

In DMD muscle the full effects of the dystrophic X-chromosome performing alone appear as the classically rapid progressive wasting towards a fatal issue. Serum CPK elevations in DMD specifically measure active dystrophic muscle mass and decline exponentially at the same rate in both ambulant and wheelchair cases with a half-life, estimated by Thomson et al. (1974), of 6.63 years. This value was originally obtained using CPK-Bg but, as has already been shown, nett CPK-Bk is much more accurate and when assayed in 22 ambulant patients of different ages gave a revised linear regression of log (CPK) on age ($y = 3.9436 - 5.2529 \times 10^{-3}.x$; $P<0.01$, 20 degrees of freedom) with a half-life of 4.7751 years. From serum ALD, likewise elevated in DMD due to muscle efflux (Schmidt and Schmidt, 1967), log (ALD) on age ($y = 1.9940 - 5.2516 \times 10^{-3}.x$; $P<0.001$, 38 degrees of freedom) gave a similar slope and half-life ($t_{\frac{1}{2}} = 4.7763$ years). In contrast, log (GOT) on age ($y = 2.3446 - 4.6742 \times 10^{-3}.x$; $P<0.001$, 24 degrees of freedom) gave a larger half-life of 5.3663 years probably due to the smaller contribution of muscle GOT compared to that of liver and heart. The close identity of the true CPK and ALD half-life indicates they may in fact measure the half-life of the decaying muscle mass whence these enzyme elevations arise, so that although a DMD patient ambulant at 4.8 years has lost 50% of his muscle, he reaches the wheelchair at 9½ years with some 25% still left.

That functioning muscle survives as long suggests that modest metabolic support in the appropriate area could enable indefinite survival.

In female DMD carriers, however, random fusion of myoblasts from clones with one or other X-chromosome already randomly inactivated (Lyon, 1962), gives a mature multinucleated muscle cell with a dual population of nuclei regulated by either a normal or dystrophic X-chromosome. Different proportions in different individuals gives a uniform X-chromosomal mosaic (Emery, 1965) with a range of carrier manifestation from the undetectable to grossly elevated serum enzymes and minor symptoms, but non-progressive and with normal life expectancy in all but rare instances (Thomson et al., 1975b). The carrier state therefore resembles a supported DMD, protected from progression by even a small proportion of normal muscle nuclei.

Thus in the affected muscle cell some limit is implied, beyond which, as in DMD, lack of normal nuclei causes eventual cell death with clinical progression, and within which, as in the carrier, their presence ensures survival by providing essential support the DMD nuclei cannot. In DMD patients such provision by other means would bring them within this limit to resemble carriers, still with marked serum enzyme elevations, but no longer clinically progressive.

The serious depletion of total muscle adenine nucleotides (Stengel-Rutkowski and Barthelmai, 1973), which may in fact wholly explain very many other features of DMD (Thomson et al., 1975b), suggests some defect in muscle purine metabolism, either by

insufficient synthesis or excessive irreversible catabolism to urate. In a comparison of 16 DMD patients (period A, mean age 8.67 years) and 20 normal boys (mean age 9.52 years), without significant difference in age ($t = 1.0268$; NS, 34 degrees of freedom), the urinary urate excretion (274.7 and 366.4 mg/24 hours respectively) was significantly** lower in DMD. Although no similar comparison is possible with serum urate, the same 16 DMD patients had a range of 2.6 - 4.7 (mean 3.6) mg/100 ml, whereas Harkness and Nicol (1969) found, using plasma from 113 normal boys, a range of 1.2 - 6.8 (mean 4.0) mg/100 ml. Further, since DMD muscle disapears rapidly with increasing age, the lack of significance in linear regressions of 24 hour urate excretion on age both in 20 normal boys ($y = 359.73 + 0.1049.x$; NS, 18 degrees of freedom) and 16 DMD patients ($y = 208.63 + 7.6178.x$; NS, 14 degrees of freedom), suggests that urinary urate output is independent of body muscle mass. Thus, these subnormal urate values suggest that the DMD gene may exert its effect by insufficient provision of essential muscle purines; and this study tests this hypothesis by using allopurinol to promote salvage and recycling of what purines may be available. Stevens et al. (1975) found an optimal concentration of serum urate for human learning and the abnormally low values in DMD may partly explain the mental retardation sometimes associated with this disease (Dubowitz, 1965). This symptom is invariably present in the Lesch-Nyhan syndrome (Lesch and Nyhan, 1964) characterised by gross overproduction of urate.

Allopurinol markedly increases salvage of hypoxanthine and there are many reports of its administration causing a substantial rise of intracellular ATP (Cunningham et al., 1974; Hopkins et al., 1975; Keaveny et al., 1975; Lindsay et al., 1975; Manze and

Dörner, 1975). In DMD muscle any increase of adenine nucleotide content after allopurinol should be accompanied by better function of the remaining muscle with appropriate clinical improvement, which if maintained must then resemble the non-progressive manifesting carrier state. Further clinical progress can only be expected if there is regeneration of muscle to replace that previously lost, or if the progressive restrictions of shrinking fibrous tissue replacement can be dealt with. In health, marked serum elevations of muscle enzymes during prolonged severe exertion (Griffiths, 1966a) suggest that muscle function, not enzyme retention (Thomson et al., 1975a) has prior claims on ATP. Evidence of increased muscle purine content in DMD, independently verifiable at biopsy, should therefore appear primarily as improved function with any diminution of enzyme elevations quite secondary to this; conversely, over-exertion without increased ATP will result in even higher elevations.

This study thus comprises 2 independent parts : periods A and B testing allopurinol alone using double-blind techniques, then period C likewise testing sublingual PA3 and PA5 superimposed on allopurinol. The duration of the study was deliberately brief to exclude any effect of increased growth or fibrous contraction with age, and because effective metabolic intervention should give measurable results within 6 weeks.

A small daily dose of allopurinol did produce a clear and significant clinical improvement in DMD patients, further enhanced by increased enzyme elevations implying over-exertion in the 2 placebo patients showing false clinical progress. Though most other individual serum enzyme elevations changed little, improvement

in 4 was confirmed by a decline in enzyme values after allopurinol. The expected fall of urate occurred as a direct effect of allopurinol, and a concomitant rise in hypoxanthine, xanthine and probably in muscle ATP, is thereby implied. The increased creatinine output, from typically low values, is important as it implies increased activity in a muscle mass likely to have changed little over such a short period. Diet seems to have little effect on urinary creatinine output (Van Pilsum and Seljeskog, 1958) which normally correlates closely with muscle mass (Ryan et al., 1957). Although virtually all patients did improve their performance on hand-bulb manometry, as if by practice, the allopurinol patients as a group improved most.

PA3 superimposed on allopurinol did not offer any obvious additional benefit, though the administration of allopurinol for the first time to this group, and the small number of patients tested makes assessment difficult. However, even though improvement on allopurinol continued in period C, PA5 did show an added effect, biochemically and physically. Its clinical effect could not, however, be measured with refinement using the comparatively large steps of Table XIX.

During the final 12 weeks of the trial, 15 of the 16 patients showed definite clinical improvement. This assumes particular interest since, in a condition characterised by rapidly progressive muscular weakness, all improvements attained by period C, which occurred in patients at every stage of the disease, have been maintained for well over 6 months on the same daily dose of allopurinol. In gout, where overproduction of urate occurs, allopurinol often, but not invariably (Kelley et al., 1968), reduces

the total amount of hypoxanthine, xanthine and urate excreted (Wyngaarden et al., 1965; Rundles et al., 1966). This is presumably due to hypoxanthine-guanine phosphoribosyl transferase (inosine monophosphate (IMP) : pyrophosphate phosphoribosyltransferase, E.C. 2.4.2.8.; HGPRT) salvaging the elevated hypoxanthine (Krenitsky et al., 1969) by its reaction with PRPP to form IMP. Thus, the concentration of PRPP, a substrate for glutamine phosphoribosyl-pyrophosphate amidotransferase in the initial rate limiting step of de novo synthesis (Caskey et al., 1964), is lowered (Kelley and Wyngaarden, 1970) thereby reducing de novo purine production. This feedback inhibition is most noticable in patients with excessive urate excretion who received very high doses of allopurinol (Rundles et al., 1966). However, there is no evidence that allopurinol has this effect in DMD, since its results are constant and persistent, thus raising the question of how much, if any, de novo synthesis does in fact occur in this disease.

These findings support the view that DMD may have some basis in defective purine metabolism, perhaps by failure of synthetic pathways. Further, if the abnormally low adenine nucleotides found in DMD muscle (Stengel-Rutkowski and Barthelmai, 1973) were shown to increase after allopurinol then this view would be greatly substantiated. In addition, a simple and clinically effective circumvention of the prospective defect seems possible using allopurinol, though only further experience, with more exact studies of allopurinol dosage and even of additional preformed purines, can determine its value.

Effect of allopurinol in DMD muscle : Results and discussion

Muscle biopsy specimens were assayed, for ATP, ADP, AMP, CP and G-6-P, in healthy male controls undergoing surgical repair and in ambulant DMD patients before and after 6 months and 1 year on allopurinol. Their clinical status had been initially, with differences in degree, that of 13 in Table XIX; after 4 to 8 weeks on allopurinol this improved in all but one to 15 and even 16, where it remained (Table XXX). Thus allopurinol appeared to prevent the steady progression characteristic of DMD.

For assay of normal muscle constituents, wet weight is the simplest and easiest reference available, though not for abnormal muscle, as DMD specimens may contain only a small amount of actual muscle with a very large amount of fat and fibrous tissue of quite different composition (Lilienthal et al., 1950). The use of NCN as a reference therefore eliminates errors arising from such abnormal composition.

Muscle content is reported here using both wet weight (Tables XXXI and XXXII) and NCN (Tables XXXIII and XXXIV) for reference. Wet weight as a standard was rejected as it leads to erroneous results due to the admixture of dystrophic muscle with fat and fibrous tissue. This gives a false apparent decrease in muscle content; for example, against wet weight group B has 27% of normal muscle ATP, and not 40% as with NCN. The NCN content of DMD muscle before allopurinol, expressed as wet weight (Table XXXI), was not found significantly different from that of normal muscle (Table XXXII, B/A), but NCN content in patients after allopurinol (E), although higher than in patients before allopurinol (B), was

Table XXX opposite

Details of all subjects who underwent a muscle biopsy.

All biopsies on DMD patients were performed on the vastus lateralis.

TABLE XXX

BIOPSY SUBJECTS

Subjects	No.	Age (yrs)	Clinical status Before	Clinical status After	Daily dose allopurinol (mg)
5 healthy males (A) **	1	12.51			
	2	20.80			
	3	38.91			
	4	48.65			
	5	56.32			
5 ambulant untreated DMD patients (B)	6	3.67			
	7	4.32			
	8	4.77			
	9	9.02			
	10	10.14			
4 ambulant DMD patients after 6 months allopurinol (C)	11	4.11	13	15	100
	12	4.81	13	15	100
	13	5.31	13	15	100
	14	9.52	13	13	150
5 ambulant DMD patients after 1 year allopurinol (D)	15	4.57	13	15	100
	16	6.23	13	16	100
	17	7.79	13	16	100
	18	8.83	13	15	150
	19	9.46	13	15	100
All 9 treated DMD patients (E)					

** Type of operation and muscle biopsied in normal males:-

1 Bilateral vesico-ureteric reflex (congenital) - pyramidalis
2 Remedial treatment for persistant shoulder dislocation - pectoralis major
3 Laparotomy for lumbar sympathectomy - internal abdominal oblique
4 Fractured femur repair - vastus lateralis
5 Artho plasty (Howe) - gluteus medius

Table XXXI opposite

Muscle content (in moles x 10^{-6}/g wet weight) of ATP, ADP, AMP, total adenine nucleotides, CP and G-6-P, with the amount of non-collagen nitrogen (in mg/g wet weight) in

(A) 5 healthy males

(B) 5 (male) DMD patients before treatment

(C) 4 (male) DMD patients after 6 months on allopurinol daily

(D) 5 (male) DMD patients after 1 year of allopurinol daily

(E) the 9 treated patients of (C) and (D) combined.

TABLE XXXI

MUSCLE CONTENT AS MOLES x 10^{-6}/g WET WEIGHT

	No.	ATP	ADP	AMP	Total Aden.	CP	G-6-P	NCN-mg/g Wet Wt.
A	1	4.75	0.73	0.10	5.59	17.46	0.73	25.99
	2	5.39	0.84	0.11	6.34	19.00	0.43	28.14
	3	4.16	0.69	0.03	4.88	13.59	0.36	23.68
	4	3.77	0.52	0.09	4.38	11.60	0.18	19.72
	5	3.84	0.59	0.24	4.67	11.67	0.81	22.26
Mean		4.38	0.67	0.11	5.17	14.67	0.50	23.96
SD		0.68	0.13	0.08	0.79	3.40	0.26	3.26
B	6	1.38	0.75	0.05	2.18	3.49	0.56	18.74
	7	1.46	0.54	0.09	2.09	2.02	0.49	15.66
	8	0.89	0.67	0.11	1.67	2.11	0.21	27.19
	9	0.71	0.30	0.13	1.13	1.68	0.20	14.91
	10	1.38	0.55	0.12	2.05	2.64	0.23	11.89
Mean		1.16	0.56	0.10	1.82	2.39	0.34	17.68
SD		0.34	0.17	0.03	0.43	0.71	0.17	5.85
C	11	2.38	1.05	0.06	3.49	6.88	0.11	20.79
	12	2.10	0.75	0.24	3.09	5.89	0.76	21.36
	13	2.82	0.94	0.35	4.11	6.46	0.64	22.51
	14	1.87	1.05	0.19	3.10	6.90	0.18	15.49
Mean		2.29	0.95	0.21	3.45	6.53	0.42	20.04
SD		0.41	0.14	0.12	0.48	0.47	0.33	3.12
D	15	3.52	0.66	0.15	4.32	8.55	0.33	23.82
	16	3.24	0.43	0.05	3.72	8.2	0.15	21.82
	17	2.21	0.32	0.22	2.75	5.11	0.19	18.79
	18	3.26	0.63	0.18	4.08	9.27	0.23	20.27
	19	2.29	0.18	0.22	2.68	6.73	0.08	13.55
Mean		2.90	0.44	0.16	3.51	7.57	0.20	19.65
SD		0.61	0.20	0.07	0.76	1.66	0.09	3.89
E								
Mean		2.63	0.67	0.19	3.48	7.11	0.30	19.82
SD		0.59	0.31	0.09	0.61	1.33	0.24	3.35

Table XXXII opposite

Ratios and significance of differences between the means of the muscle metabolite content, measured against wet weight and given in Table XXXI in

(A) 5 healthy males

(B) 5 (male) DMD patients before treatment

(C) 4 (male) DMD patients after 6 months on allopurinol daily

(D) 5 (male) DMD patients after 1 year on allopurinol daily

(E) the 9 treated patients of (C) and (D) combined.

TABLE XXXII

RATIOS AND SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS (WET WT.)

Comparisons of means	ATP	ADP	AMP	Total Aden.	CP	G-6-P	NCN
Ratio B/A	0.27	0.83	0.89	0.35	0.16	0.67	0.74
Difference	P<0.001	NS	NS	P<0.001	P<0.001	NS	NS
Ratio C/A	0.52	1.40	1.87	0.67	0.45	0.84	0.84
Difference	P<0.001	P<0.05	NS	P<0.01	P<0.01	NS	NS
Ratio D/A	0.66	0.66	1.44	0.68	0.52	0.39	0.82
Difference	P<0.01	NS	NS	P<0.01	P<0.01	P<0.05	NS
Ratio E/A	0.60	0.99	1.63	0.67	0.48	0.59	0.83
Difference	P<0.001	NS	NS	P<0.001	P<0.001	NS	P<0.05
Ratio C/B	1.97	1.69	2.11	1.89	2.74	1.25	1.13
Difference	P<0.01	P<0.01	NS	P<0.001	P<0.001	NS	NS
Ratio D/B	2.50	0.79	1.63	1.93	3.17	0.58	1.11
Difference	P<0.001	NS	NS	P<0.001	P<0.001	NS	NS
Ratio E/B	2.26	1.19	1.84	1.91	2.98	0.88	1.12
Difference	P<0.001	NS	NS	P<0.001	P<0.001	NS	NS
Ratio D/C	1.27	0.47	0.77	1.02	1.16	0.46	0.98
Difference	NS	P<0.01	NS	NS	NS	NS	NS

significantly lower* than normal.

In health, muscle adenylate content, against NCN, was remarkably constant, as shown by the small standard deviation of A in Table XXXIII, and quite unrelated to age; but CP showed a high negative correlation with age ($r = -0.9466$; $t = 5.0847$, $P<0.05$, 3 degrees of freedom) indicating some decline in energy reserves. In untreated DMD, ADP and AMP values were the same as normal, conflicting with the findings of Stengel-Rutkowski and Barthelmai (1973), who reported reductions of over 70% in DMD, but supporting the observations of Vignos and Warner (1963). Total adenylate was only 52%, ATP 40% and CP a mere 24% of normal (Table XXXIV). These gross reductions were all highly significant even in these small numbers.

6 months of allopurinol (group C) then more than doubled CP** and increased total adenylate by half**, with a similar but not significant rise in ATP (Table XXXIV, C/B). After 1 year on allopurinol, however, though CP and total adenylate increased only slightly, ATP had risen to 81% of normal (D/A), mainly at the expense of ADP, which was reduced by half** from values significantly* higher than normal reached after 6 months treatment. The main improvements in DMD after allopurinol thus clearly occur in ATP and CP with increases of 205 and 270% respectively, compared with untreated patients (D/B). A basic adequacy of both oxidative phosphorylation and CPK activity in DMD muscle is therefore implied, and the indifference throughout of G-6-P suggests a sufficient glycolysis.

These results indicate that the increased adenylate content

Table XXXIII opposite

Muscle content (in moles x 10^{-5}/g non-collagen nitrogen) of ATP, ADP, AMP, total adenine nucleotides, CP and G-6-P in

(A) 5 healthy males

(B) 5 (male) DMD patients before treatment

(C) 4 (male) DMD patients after 6 months on allopurinol daily

(D) 5 (male) DMD patients after 1 year on allopurinol daily

(E) the 9 patients of (C) and (D) combined.

TABLE XXXIII

MUSCLE CONTENT AS MOLES x 10^{-5}/g NCN

	No.	ATP	ADP	AMP	Total Aden.	CP	G-6-P
A	1	18.29	2.82	0.38	21.50	67.19	2.79
	2	19.15	2.98	0.40	22.53	67.52	1.5
	3	17.55	2.93	0.12	20.60	57.40	1.53
	4	19.14	2.62	0.44	22.20	58.81	0.91
	5	17.25	2.65	1.09	20.99	54.44	3.64
Mean		18.28	2.80	0.49	21.56	61.07	2.08
SD		0.88	0.16	0.36	0.81	5.95	1.11
B	6	7.38	3.98	0.26	11.62	18.62	3.01
	7	9.32	3.44	0.58	13.34	12.91	3.12
	8	3.28	2.46	0.41	6.16	7.75	0.77
	9	4.73	1.98	0.85	7.57	11.24	1.32
	10	11.57	4.60	1.04	17.22	22.23	1.92
Mean		7.26	3.29	0.63	11.18	14.55	2.03
SD		3.36	1.07	0.32	4.46	5.82	1.03
C	11	11.44	5.06	0.30	16.80	33.08	0.54
	12	9.81	3.51	1.14	14.47	27.57	3.55
	13	12.52	4.16	1.58	18.26	28.71	2.86
	14	12.06	6.76	1.22	20.04	44.54	1.14
Mean		11.46	4.87	1.06	17.39	33.47	2.02
SD		1.18	1.41	0.54	2.36	7.75	1.42
D	15	14.76	2.76	0.63	18.15	35.88	1.40
	16	14.87	1.96	0.24	17.07	37.61	0.69
	17	11.74	1.71	1.16	14.62	27.19	1.01
	18	16.09	3.13	0.91	20.12	45.72	1.12
	19	16.86	1.32	1.62	19.80	49.69	0.60
Mean		14.87	2.18	0.91	17.95	39.22	0.96
SD		1.95	0.75	0.52	2.24	8.81	0.33
E							
Mean		13.35	3.37	0.98	17.70	36.59	1.43
SD		2.38	1.74	0.50	2.16	8.40	1.06

Table XXXIV opposite

Ratios and significance of differences between the means of the muscle metabolite content, measured against non-collagen nitrogen and given in Table XXXIII in

(A) 5 healthy males

(B) 5 (male) DMD patients before treatment

(C) 4 (male) DMD patients after 6 months on allopurinol daily

(D) 5 (male) DMD patients after 1 year on allopurinol daily

(E) the 9 treated patients of (C) and (D) combined.

TABLE XXXIV

RATIOS AND SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS (NCN)

Comparisons of means	ATP	ADP	AMP	Total Aden.	CP	G-6-P
Ratio B/A	0.40	1.18	1.30	0.52	0.24	0.97
Difference	P<0.001	NS	NS	P<0.001	P<0.001	NS
Ratio C/A	0.63	1.74	2.18	0.81	0.55	0.97
Difference	P<0.001	P<0.05	NS	P<0.01	P<0.001	NS
Ratio D/A	0.81	0.78	0.49	0.83	0.64	0.46
Difference	P<0.001	NS	NS	P<0.001	P<0.001	NS
Ratio E/A	0.73	1.21	2.01	0.82	0.60	0.69
Difference	P<0.001	NS	NS	P<0.01	P<0.001	NS
Ratio C/B	1.58	1.48	1.68	1.56	2.30	1.00
Difference	NS	NS	NS	P<0.05	P<0.01	NS
Ratio D/B	2.05	0.66	1.44	1.61	2.70	0.48
Difference	P<0.01	NS	NS	P<0.05	P<0.001	NS
Ratio E/B	1.84	1.02	1.55	1.58	2.52	0.71
Difference	P<0.01	NS	NS	P<0.01	P<0.001	NS
Ratio D/C	1.30	0.45	0.86	1.03	1.17	0.48
Difference	P<0.05	P<0.01	NS	NS	NS	NS

after allopurinol may offer a similar support to that from normal nuclei in muscle of manifesting carriers, thus ensuring preservation of the DMD muscle fibre population. If some prospect of sustained clinical arrest may now seem possible, studies of purine synthesis in DMD should prove rewarding. Moreover, neonatal diagnosis (Zellweger and Antonik, 1975; Beckmann, 1977), with early intervention, become important and in older children some means, whether biochemically or by advanced orthopaedic procedures, to prevent the shrinking fibrous tissue progressively impeding residual muscle action.

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