

CHEMOTHERAPEUTIC AND ISOENZYME STUDIES ON CHICKENS
WITH HEREDITARY MUSCULAR DYSTROPHY

by

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ABSTRACT

The properties of blood plasma pyruvate kinase (PK) and phosphoglyceromutase (PGM) activities were investigated in chickens with a genetically determined form of myopathy. Data from these studies were used to assess the chemotherapeutic value of potential antidystrophic drugs in these dystrophic animals. The isoenzyme changes of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were also examined during the development of dystrophic chick embryonic muscle.

Very large increases in plasma PK and PGM activities were observed during the maturation of dystrophic chickens, reaching about 30-fold the normal value for PK and almost 20-fold the normal value for PGM. A high correlation exists between plasma PK and CPK activities in the development of dystrophy. A similar association also exists between plasma PK and PGM activities. Chickens heterozygous for muscular dystrophy also had PK elevations that were significantly above normal. By an isoelectric focussing method, it was shown that normal muscle, dystrophic muscle and dystrophic plasma contain as their major PK component, the M_1 isoenzyme. The PGM isoenzyme content, however, was found to be abnormal in dystrophic muscle and further, these isoenzyme forms had pI values different from the major PGM form seen in dystrophic plasma. The transition of CPK isoenzymes was essentially complete in dystrophic muscle after two days ex ovo. Denervation studies on muscle LDH showed isoenzyme patterns similar to myopathic muscle. Dystrophic chickens treated with IAPP, DPH and a number of antiserotonin drugs showed both functional and biochemical improvements

All these observations indicate that plasma PK and PGM are markers of the dystrophic muscle and as such, are very useful for monitoring the effects of dystrophy-retarding drugs. The

possible modes of action of serotonin antagonists, IAPP and DPH are discussed. Finally, these studies do not support a genetic persistence of embryonic isoenzymes in adult myopathic muscle.

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The literature survey for this thesis was completed in 1981.

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ABBREVIATIONS

Absorbance	=	A
Acetylcholinesterase	=	AChE
Adenosine 5'-Diphosphate	=	ADP
Adenosine 5'-Triphosphate	=	ATP
Anterior Latissimus Dorsi	=	ALD
Correlation Coefficient	=	r
Creatine Phosphokinase	=	CPK
Degree Celcius	=	°C
Diphenylhydantoin	=	DPH
Distribution Rate Constant	=	α
Duchenne Muscular Dystrophy	=	DMD
Elimination Rate Constant	=	β
Flip Number	=	FN
Half-Life	=	$t_{1/2}$
Hour	=	h
International milliunits per millilitre	=	mU/ml
Intraperitoneal	=	I.P.
Intravenous	=	I.V.
Isoelectric Point	=	pI
Killogramme	=	Kg
Lactate Dehydrogenase	=	LDH
MicroCurie	=	uCi
Microlitre	=	ul
Minute	=	min
Molecular Weight	=	MW
Myoglobin	=	Mb
Nicotinamide Adenine Dinucleotide (Reduced)	=	NADH
Nicotinamide Adenine Dinucleotide (oxidised)	=	NAD ⁺
Phosphoenolpyruvate	=	PEP

Phosphoglyceromutase	=	PGM
Posterior Latissimus Dorsi	=	PLD
Probability	=	P
Pseudocholinesterase	=	Ψ ChE
Pyruvate Kinase	=	PK
Standard Error of the Mean	=	SEM
Succinic Dehydrogenase	=	SDH
Watt	=	W

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CHAPTER 1

GENERAL INTRODUCTION

PROPERTIES OF THE GENETICALLY DYSTROPHIC CHICKEN

Muscular dystrophy of the chicken was first described in 1956 by Asmundson and Julian in a commercial flock of New Hampshire chickens. Since then, several genetic lines have been bred at the University of California, Davis. The disease is characterized by the progressive weakness and wasting of the fast glycolytic skeletal muscles of the chicken and this physical disability becomes visually observable after about 20 days posthatching when these birds fail to flip properly. A review of the major characteristics of some of these dystrophic lines is provided by Asmundson et al. (1966) and more recently by Wilson et al. (1979) and Barnard (1980).

GENETICS

The abnormality in the dystrophic chicken is caused by a single gene defect. However, as Asmundson et al. (1966) reported, the expression of the dystrophic gene may be modified by other "background" genes resulting in some variability in the expression of the disease in the different strains. The major effect of the dystrophic gene is, as pointed out above, on the fast-twitch glycolytic muscle fibres, which are ultimately destroyed (i.e. become necrotic) and replaced with fat, adipose and connective tissues. The gene is not lethal in the sense that these chickens live a relatively normal life-span since neither the heart nor the respiratory muscles are seriously affected as in the case of humans affected with the Duchenne form of the disease.

Unlike Duchenne muscular dystrophy which is inherited as an X-linked recessive trait, dystrophy in the chicken has been attributed to a simple autosomal recessive gene. There is, however, a variable penetrance of the dystrophic gene in heterozygotes (carriers) and it is probably more accurate to consider it as a "codominant" trait (Asmundson et al. 1966, Wilson et al. 1970). Although the gene is not sex linked, its expression may vary slightly (e.g. enzyme levels in plasma or righting ability) between the two sexes (Wilson et al. 1979).

DERIVATION OF LINES 412 AND 413 CHICKENS

From the original dystrophic line 301 chickens, several other dystrophic lines were selected at Davis either for high fat content or early onset of the disease. Crossing dystrophic line 304 with normal New Hampshire birds produced two homozygous dystrophic lines (413 and 415) and two genetically related normal lines (412 and 454) respectively. The present study used the dystrophic line 413 and its closely related normal line 412 chickens obtained originally from the University of California, Davis, and subsequently bred and maintained at Imperial College, University of London.

RIGHTING ABILITY

A characteristic feature of all dystrophic chickens is their inability to rise when placed on their backs. One test that is commonly used to measure the functional ability of these chickens is the flip test (FN). This simply measures the number of successful rightings out of say, 5 attempts, and gives a quantitative measure of the degree

of muscle 'weakness' in these chickens. Using this test, groups of dystrophic chicks can be distinguished from normal chicks as early as 20 - 25 days ex ovo by statistical analysis. By the time they reach 60 - 80 days ex ovo, virtually all of the dystrophic birds fail to right themselves even once and the FN for these birds approaches zero. In contrast, normal birds when subjected to this test always scored the maximum FN (i.e. 5).

ELECTROMYOGRAPHY

The pectoral muscle of the dystrophic chicken is characterized by the repetitive and prolonged firing of electrical activity in response to mechanical stimulation (Wilson et al. 1979). Holiday et al. (1965) first reported this abnormal electromyographic pattern in the muscle of these birds and investigations of other dystrophic strains also showed this abnormal effect (Wilson et al. 1979). Application of the postsynaptic membrane blocker, d-tubocurarine, to block the binding of acetylcholine did not have any effect on the abnormal electromyographic activity found in the dystrophic muscle. This, together with the hyperexcitability of the dystrophic muscle led Holiday and associates to conclude that dystrophic chicken muscle fibres may be "myotonic". Entrikin and Bryant (1979) recently observed that phenytoin, a membrane stabilising drug, decreased the abnormal electrical activity seen in dystrophic chicken muscles. That this same drug improved the righting ability of dystrophic chickens as reported by Entrikin et al. (1977) suggests a possible correlation between abnormal electrical activity and muscle weakness.

HISTOLOGICAL AND ULTRASTRUCTURAL CHANGES

Early histopathological changes that are usually observed in dystrophic muscle include rounding of the normal polygonal-shaped muscle fibres and splitting of some large fibres. Hypertrophy is accompanied by the presence of some very small fibres due partly to the atrophic process and also to sites of attempted regeneration. Along with these changes, the muscle cell nuclei also increase in number as the disease progresses and changes in its position occur from a predominantly central to a predominantly peripheral position in the case of pectoral muscle (Barnard 1980, Wilson et al. 1979, Julian and Asmundson 1963). After about 4 - 5 weeks of age, foci of necrosis are often seen, along with fibre vacuolation and sites of phagocytosis of degenerating fibres. At about two months, fat and connective tissue are seen replacing the destroyed fibres. By two years, almost all of the muscle fibres are replaced thus.

At the ultrastructural level, changes include a disruption of the normal myofibrillar pattern and enlargements of the sarcoplasmic reticulum and transverse tubules (Wilson et al. 1979, Mendell et al. 1979, Barnard 1980). The mitochondria also appear enlarged. An increase in the number of vesicles are also seen beneath the sarcolemma and become more apparent when they contain the enzyme, peroxidase. Large vacuoles are also seen in some dystrophic fibres.

CYTOCHEMISTRY

Changes in fibre type are seen in dystrophic muscle whereby the affected fast-twitch glycolytic fibres become more oxidative in character. Examples of this are the fibres seen in the posterior latissimus dorsi (PLD) muscle of the chicken. In the normal PLD muscle, all fibres are uniformly stained by phosphorylase activity after about 4 days ex ovo. In the dystrophic muscle of the same age, however, an increasing number of muscle fibres showed less phosphorylase staining (Barnard, 1980). Changes in the fibre type due to ATPase activity has also been reported in the PLD muscle. In the pectoral muscle of the dystrophic chicken, distinctive increases in the fibre content of oxidative enzymes such as succinic dehydrogenase (SDH) and NADH diaphorase have been observed (Ashmore et al. 1971, Cardinet et al. 1972).

METABOLIC DIFFERENCES BETWEEN MUSCLES

In the genetically dystrophic chicken, predominantly fast-twitch skeletal muscles like the pectoralis major, PLD and the biceps are preferentially selected by the dystrophic gene for muscle weakening and wasting, leaving the much slower contracting muscles like the anterior latissimus dorsi (ALD) and adductor muscles relatively unaffected. The metabolic pattern, mode of innervation and contractile properties are very different between fast and slow muscles (Vrbova and Gordon 1975, Vrbova et al. 1978, Gordon et al. 1977). Fast-twitch muscles like the PLD are usually focally innervated, have a high capacity for anaerobic glycolysis and being capable of developing rapid and large tensions. The much slower contracting muscles like

the ALD are characterized by multiple innervation, much more vascularisation and have a higher oxidative enzyme capacity than fast-twitch muscles. Slow muscles cannot develop as large a tension as fast muscles but can maintain tension for long periods of time. Also, slow muscles often have a high storage capacity for oxygen in their myoglobin which gives them their characteristic red colour.

SERUM ENZYMES

Creatine phosphokinase (CPK) is the most elevated muscle enzyme found to date in the blood plasma of dystrophic chickens. In line 413 chickens, its activity rises from a mean of about 200 mU/ml shortly after hatching to a peak of around 25,000 mU/ml after about 80 - 100 days (Barnard, 1980). The mean CPK activities of normal line 412 chickens does not rise much above 300 mU/ml throughout their period of development. A difference in the mean plasma CPK levels could be observed between normal and dystrophic chickens as early as about two weeks ex ovo. By about 100 days ex ovo, there is a difference of about 80-fold in the mean plasma CPK activity between normal and dystrophic chickens. After this period, there is a decline in the mean CPK levels of dystrophic birds due to a decrease in the number of active fibres in the muscle. An inverse correlation between the rise in plasma CPK and muscle weakness in line 413 dystrophic chickens has also been observed (Barnard et al. 1976).

Pyruvate kinase (PK) is another enzyme that has been reported to be elevated in the blood plasma of dystrophic chickens. In a short communication, Weinstock et

al. (1977) observed a five-fold increase in serum PK activity in Connecticut dystrophic chickens. At this laboratory, we (Liu et al. 1980) observed a much larger increase in plasma PK activity in line 413 chickens (about 30-fold increase). The properties of this marker enzyme in the dystrophic chicken and dystrophic hamster have been studied in some detail and will be presented in chapter 2.

Another muscle enzyme that is highly elevated in the blood plasma of dystrophic chickens is phosphoglyceromutase (PGM). This enzyme was investigated by the author and Lucy Rodrigues (final year undergraduate project student) because it is believed to be affected in human dystrophy (Professor E.A. Barnard, personal communications). As far as is known, no studies have previously been reported on this enzyme in either the human or animal dystrophies. The results on PGM will be presented in chapter 3.

Acetylcholinesterase (AChE), an enzyme found predominantly in the muscle and brain of the chicken, has also been reported to be elevated in the blood plasma of dystrophic chickens (Wilson et al. 1973, Lyles et al. 1980). The rise in blood plasma AChE activity is relatively slower than that of either CPK or PK, starting with an initial decline in activity just after hatching and then slowly increasing its activity relative to normal until at about 30 - 40 days ex ovo, there is a very steep rise in AChE activity. The plasma AChE activity reaches a plateau by about 100 days ex ovo and subsequently thereafter, the activity declines and reaches very low levels after about

a year. Lyles et al. (1980) provide evidence to suggest that plasma AChE is derived from muscle AChE (i.e. precursor relationship).

When the total muscle AChE content was investigated, a great over-synthesis of the enzyme in the dystrophic chicken muscle was observed when compared to normal muscle of the same age (Lyles et al. 1980). The over-production of AChE in dystrophic chicken muscle has been attributed to an increase in the synthesis of the lighter forms of AChE.

Another related enzyme, pseudocholinesterase (ψ ChE), also shows a concomitant increase in the dystrophic muscle when compared to normal muscle, although in this case, the rise is much smaller than for AChE. The enzyme is not, however, affected in the plasma of dystrophic chickens at any age. Why the levels of this enzyme is not elevated in the plasma of dystrophic chickens is yet unclear, but rapid chemical modification of this enzyme may play a large part due to its different chemical structure. The three-dimensional structure of this molecule may render it more impermeable through the sarcolemma membrane of the diseased muscle. Intracellular binding may also play a part.

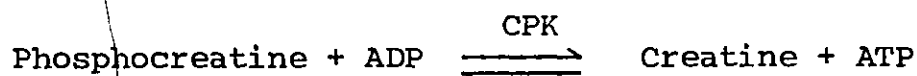
ENZYME MARKERS IN PROGRESSIVE MUSCULAR DYSTROPHY (DMD)

The use of serum enzymes in the detection of Duchenne and other related forms of muscular dystrophy in humans became possible when Sibley and Lehninger (1949) observed increased serum aldolase activities in two patients with muscular dystrophy. Since then, a number of other muscle enzymes have been found in increased amounts in the serum

of dystrophic humans. In 1959, Ebashi et al. were the first group of investigators to report the elevation of serum CPK activity in dystrophic humans and since then, this enzyme has proved itself to be the most sensitive and reliable indicator of muscle pathology up to the present time. Lactate dehydrogenase isoenzyme 5 (LDH-5) has also been used with success in the detection of Duchenne carriers (Roses et al. 1977) but two recent reports failed to confirm the sensitivity of this test (Somer et al. 1980, Burt and Emery 1979). Harano et al. (1973) recently observed greatly elevated levels of another sarcoplasmic enzyme, PK, in the serum of Duchenne patients and a number of subsequent reports indicate that this new enzyme marker may offer some advantages over CPK in the detection of Duchenne carriers (Alberts and Samaha 1974, Percy et al. 1979, Zatz et al. 1980). Other molecular markers have yet to be identified but the ultimate aim of all these diagnostic tests would be the identification of known carriers of the disease.

PROPERTIES OF SERUM CPK IN DUCHENNE HUMANS

This enzyme catalyses a very important reaction in skeletal muscle, namely, the formation of ATP and creatine from ADP and phosphocreatine according to the equation



The ATP that is formed provides an immediate source of energy for muscle contraction in the absence of energy derived via glycolysis or oxidative phosphorylation. There are several methods available for measuring CPK activity but the most widely used method involves coupling the

products of the CPK reaction (ATP) with two other enzymatic reactions and then spectrophotometrically measuring the increase in absorbance at 340 nm due to NADPH formation. The rate of NADPH formation is directly proportional to the amount of enzyme present.

Serum samples containing high levels of CPK activity may be stored for up to 24 hours at room temperature or for up to a few days at 4°C without significant loss of enzyme activity (Pennington, 1980). In this laboratory, all our plasma samples are analysed fresh for consistency, although we have found that plasma samples containing high CPK activity may be frozen in the presence of glutathione at -20°C for a few months without any significant loss of enzyme activity. However, plasma samples containing low CPK activity (e.g. normal serum or serum from Duchenne carriers) should not be stored this way and should always be analysed fresh.

Elevated levels of serum CPK activity is characteristic of many muscle diseases, but the largest rise is seen in the Duchenne form of muscular dystrophy. In Duchenne patients, there is a gradual increase in serum CPK activity after birth and the level reaches a peak after one or two years. At the peak level, the serum CPK activity in Duchenne patients reaches about 30 times the normal upper limit (about 89 IU/l serum, Percy et al. 1979), but in some cases, could exceed 200 times the normal value (Pennington, 1980). After this age, there is a gradual decline in CPK activity due possibly to a cessation in walking and also to a decrease in the number of active fibres present in the muscle

until at advanced stages of the disease (after about 10 years of age), very low or near normal levels are reached (Pennington, 1980).

Recently, a lot of interest has been focussed on the possibility of using foetal blood CPK for the prenatal diagnosis of muscular dystrophy. By the method of placental puncture aided by fetoscopy, Mahoney et al. (1977) and Dubowitz et al. (1978) were able to show an increase in CPK activity in foetal blood of affected (DMD) fetuses. Subsequent reports by Ionasescu et al. (1978) and Golbus et al. (1979), however, have indicated that this method of prenatal diagnosis of Duchenne muscular dystrophy is not reliable as normal CPK levels have been observed in the blood of affected fetuses. The unreliability of this test may be attributed to contamination of the pure foetal blood by the surrounding amniotic fluid during foetal blood sampling. Until pure foetal blood can be obtained and the upper normal limit for foetal blood CPK activity established, the value of this test should be treated cautiously.

Undoubtedly, one of the most important applications of serum CPK measurements in human muscular dystrophy is in the detection of Duchenne carriers. It has been generally found that approximately two-thirds of all known Duchenne carriers have an elevated serum CPK activity that is significantly higher than the normal upper limit (153). There remains, however, the remaining one-third that escapes this test and some of these women will eventually give birth to an affected son with DMD.

The activity of CPK in the serum of both dystrophic humans (DMD) and female carriers of the disease has recently been shown to be age-dependent. Percy et al. (1979) reported decreasing levels of serum CPK activity in both Duchenne patients and carriers with increasing age. This is particularly important in the identification of female carriers and this point should be taken into account when the serum CPK value of carriers is near normal.

A considerable amount of interest has been focussed lately on the activities of the individual molecular forms of CPK present in both the serum and muscle of patients with Duchenne and related forms of muscular dystrophy (Tzvetanova 1971, Cao et al. 1971, Somer et al. 1973, and Kuby et al. 1977). Unlike normal serum where only the MM isoenzyme (muscle form) is normally detected, serum from Duchenne and other muscular dystrophy patients often show the presence of the MB isoenzyme (foetal hybrid form) and in some instances, the BB isoenzyme (brain form) could also be detected. This abnormal CPK isoenzyme pattern also occurs in the dystrophic muscle where the CPK isoenzymes are presumed to have originated. Although the MB isoenzyme is present in heart muscle, the appearance of this isoenzyme in dystrophic serum does not appear to correlate with cardiac involvement in the disease (Silverman et al. 1976). The appearance of these foetal forms of CPK in the serum of dystrophic humans may possibly be used in the detection of female carriers of the disease. It is quite possible that in female carriers of DMD, there is an increase in the synthesis of the MB and BB isoenzymes whilst synthesis

of the MM isoenzyme decreases. Thus, if only the total serum CPK activity is measured in female carriers as is usually the case, then this value might not differ significantly from control values. However, if the individual isoenzyme activities of CPK are measured, then even small differences in the activities of one or more of these isoenzymes may be indicative of whether a suspected carrier is identified or not.

For isoenzyme fractionation and quantitation, the method of isoelectric focussing on an Ultrodex gel bed followed by spectrophotometric analysis of the active species as described below (Materials and Methods, chapter 2) could be employed. Other methods for quantitating isoenzymes are available but these are generally much cruder and therefore less reliable.

PROPERTIES OF SERUM PK IN DUCHENNE HUMANS (DMD)

For many years, CPK has been the enzyme of choice in the diagnoses of muscle diseases due to its superior sensitivity over other serum enzymes. It was, therefore, of no great surprise that when Harano et al. (1973) first reported the abnormal appearance of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) in the serum of patients with Duchenne and limb-girdle dystrophy, some reservations were voiced concerning the reliability of the PK test. In their study, Harano et al. (1973) found that serum PK was increased 28-fold compared with that of normal controls and these PK elevations were elevated in far-advanced diseases when both serum CPK and aldolase levels were normal. They concluded from this study that serum PK

may be a more sensitive indicator of muscle diseases than serum CPK. Shortly following this report, Alberts and Samaha (1974) provided further evidence to suggest that serum PK is more sensitive than serum CPK when they found that the incidence of raised PK levels in dystrophic humans is 50% higher than the incidence of elevated CPK levels. In their study, they also observed a much higher detection rate among Duchenne carriers using the PK test. Contrary to these findings, however, a study by Hardy et al. (1977) found a much higher detection rate among Duchenne carriers using serum CPK than serum PK. Recent studies by Weinstock et al. (1977), Sage et al. (1979), Percy et al. (1979) and Zatz et al. (1980) have all indicated that the combined use of these two serum enzymes may increase the detection rate in Duchenne carriers than if either were used alone.

Possibly one of the main factors concerning the sensitivity of the PK test is that some laboratories have been using frozen serum samples in their studies whilst others have used fresh serum. Harano et al. (1973) reported that the M_1 form of PK (the predominant isoenzyme that is presumably present in dystrophic serum) can be stored frozen at -20°C for some length of time without noticeable loss of enzyme activity. There is now growing evidence (Percy et al. 1979, Zatz et al. 1978 and 1980) to suggest that serum PK cannot be stored in this way without significant loss of enzyme activity and some of the conflicting reports (Hardy et al. 1977) concerning the sensitivity of the PK test may not have resulted if this and other factors

were taken into consideration.

Another property that serum PK activity exhibits in Duchenne patients and carriers is its correlation with serum CPK activity. Percy et al. (1979) observed significant correlations between these two serum enzyme activities in both DMD patients ($r = +0.67$) and carriers ($r = +0.68$). There was, however, no significant correlation between CPK and PK in the serum of normal individuals ($r = +0.24$). Earlier studies by Harano et al. (1973) which showed no association between serum CPK and PK activities in Duchenne patients were probably due to the fact that they were using frozen serum samples for their PK measurements. The association between serum CPK and PK activity also exists in the genetically dystrophic chicken (see chapter 2). The fact that there is a correlation between these two enzyme activities in serum suggests that the mechanism of their release from the diseased muscle may be similar.

The activity of serum PK, like that observed for CPK, has been shown recently to be age-dependent in Duchenne and other muscular dystrophy patients. Zatz et al. (1978) first reported that serum PK activity decreases with age in patients with Duchenne and Becker-type muscular dystrophy. Percy et al. (1979) also showed an inverse correlation between serum PK activity and age in both DMD patients and carriers. It is, therefore, very important that this factor should be taken into account when serum PK is used in the identification of Duchenne carriers.

Normal mammalian tissues contain at least three different forms of PK and these have been designated as M_1 , M_2 and L (Imamura and Tanaka 1972, Zatz et al. 1978, Harano et al. 1973). M_1 is the predominant isoenzyme present in normal adult skeletal muscle and is also the major isoenzyme found in the heart and the brain. L is the major isoenzyme found in the liver and kidney. M_2 is present in all tissues except possibly the skeletal muscle and erythrocytes. In dystrophic humans, Zatz et al. (1978) have recently shown that the greatly elevated levels of PK activity found in the serum of patients with Duchenne and other related forms of muscular dystrophy is of muscle origin. By electrophoretic evidence, they showed that the isoenzyme present in the serum of patients with various forms of muscular dystrophy (including DMD) is of the M_1 type. This does not, of course, eliminate the possibility that the PK found in the serum of dystrophic humans may have originated from either the brain or the heart but it does rule out many other possibilities. Since elevated PK levels occur in the serum of Duchenne patients long before any signs of cardiac involvement in the disease, this possibility can be ruled out. It is unlikely that the PK could have originated from the brain since apart from the possible connection between mental retardation and the disease itself, no other major abnormality has been reported in the brain.

It is interesting that unlike CPK, no other additional isoenzymes of PK were observed by Zatz et al. (1978) in the serum of dystrophic humans. It seems, therefore, that the presence of foetal muscle enzymes in the pathological

muscle is not a generalised feature of the dystrophic process.

LACTATE DEHYDROGENASE ISOENZYMES IN DUCHENNE DYSTROPHY

The activities of the individual isoenzymes of lactate dehydrogenase (LDH) have also been investigated in some detail in both the serum and muscle of patients with Duchenne and related forms of muscular dystrophy. In normal adult human muscle, five isoenzymatic forms of LDH (LDH₁₋₅) have been recognised (Schapira et al. 1977 and Emery 1964), with LDH₅ being the major form. LDH from myopathic muscle, however, shows mainly the presence of only the LDH₁₋₄ isoenzymes, with LDH₅ apparently missing (Schapira et al. 1977) or present in much reduced amounts (Emery, 1964). Schapira and associates (1977) further showed that the isoenzymatic pattern of LDH in adult myopathic muscle was identical to the pattern seen in myopathic foetal muscle.

In normal human serum, five isoenzymatic forms of LDH have likewise been identified, with LDH isoenzymes 1 and 2 being more prominent (Wroblewski et al. 1961, Wieme et al. 1961 and Vesell 1961). A well established observation is an alteration in the isoenzyme profile of serum LDH in many diseased states and which reflect the disease of the affected tissue. In liver tissue, for example, LDH₅ predominates and it has been observed that in hepatitis the pattern of LDH isoenzymes in serum changes so that LDH₅ becomes the most prominent band (Wroblewski et al. 1961 and Wieme et al. 1961). In human muscular dystrophy, however, growing evidence suggests that this is not the case and there is now much controversy concerning the value of serum

LDH and in particular, the LDH isoenzyme 5. The possible use of serum LDH in the diagnosis of muscle diseases began when Richterich et al. (1961), Wieme et al. (1962) and Somer et al. (1977) independently reported elevated levels of LDH activity in the serum of X-linked muscular dystrophy patients; the increase being mainly attributed to an increase in the activities of LDH₁₋₃. Roses et al. (1977), in contrast, reported increased LDH isoenzyme 5 activity in the serum of many carriers, including many genetically possible carriers with normal serum CPK activity. They further suggested that the combined use of serum CPK and LDH₅ will increase the detection rate in Duchenne carriers. Contrary to the findings of Roses et al. (1977), two earlier studies by Hooshmand et al. (1969) and Hausmanowa-Petrusewicz et al. (1977) showed LDH₅ to be decreased rather than increased in the serum of obligatory carriers. Two recent studies by Somer et al. (1980) and Lane et al. (1979) also failed to confirm the sensitivity of the LDH₅ isoenzyme test in the serum of Duchenne carriers.

Besides the possibility that the individual isoenzymes of LDH may not be significantly affected in the serum of Duchenne carriers, these conflicting reports on the value of serum LDH₅ may be attributed to the different methodology being employed. All of the estimations that have been done on LDH isoenzymes involve separating the different isoenzymes by gel electrophoresis and then scanning the intensity of the end-products of the various isoenzymatic reactions. This form of isoenzyme quantitation

is less reliable and could contribute significantly to the conflicting results that various laboratories obtain in the detection of Duchenne carriers. The more sensitive method of isoelectric focussing on an Ultrodex gel bed as described below (Materials and Methods, chapter 2) may help in the quantitation of LDH isoenzymes.

Another factor not been considered in previous studies on the identification of carriers is the age-dependancy of LDH activity. Like serum CPK and PK, the activity of LDH isoenzymes may decrease linearly with increasing age in both Duchenne patients and carriers. It is interesting to note that both serum CPK and PK enzyme activities show near normal values in Duchenne carriers at the age of between 30 - 40 years, about the average age that Somer et al. (1980) and Lane et al. (1979) used in their studies.

Since some of the reported studies above used frozen serum samples for their LDH isoenzyme 5 test (e.g. Somer et al. 1980), this may be another contributing factor in the confusion regarding the value of this test, as Yasmineh et al. (1978) have pointed out. Although serum containing high LDH isoenzyme activities may not be significantly affected after freezing and thawing, this may not be true in the case of carriers where the LDH isoenzyme activities are already low.

OTHER POSSIBLE ENZYME MARKERS OF MUSCULAR DYSTROPHY

A number of other enzymes that have been reported to be abnormally elevated in the serum of dystrophic humans (though the size of these elevations are much smaller than

those reported for either CPK or PK) includes aspartate aminotransferase (or glutamic oxaloacetic transaminase, GOT), malate dehydrogenase (MDH), isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase and aldolase (Hughes 1962, Giusti et al. 1967, and Heyck et al. 1966). Phosphoglyceromutase (PGM), another sarcoplasmic enzyme, is found to be greatly elevated in the plasma of dystrophic chickens and may also be elevated in the serum of young Duchenne boys. Abnormal isoenzymatic patterns of hexokinase have also been reported in the muscle of dystrophic humans (Ellis et al. 1976) and may be elevated in the serum of these patients. At the present moment, however, it appears that serum CPK and PK will feature dominantly in the detection of Duchenne patients and carriers until more detailed studies are carried out on some of the other enzymes that are mentioned above.

CHEMOTHERAPEUTIC TRIALS IN DYSTROPHIC ANIMALS

Due to the inaccessibility of dystrophic humans for chemotherapy trials, many investigators have recently turned their attention to animals with a hereditary form of muscular dystrophy. Most, if not all the drug therapy work done on animals have been carried out in the genetically dystrophic chicken, hamster and mouse.

In the genetically dystrophic chicken, diphenylhydantoin (DPH) has been reported by Entrikin et al. (1977) to improve significantly the righting ability of these birds. DPH is an anticonvulsant drug and is believed to work by stabilizing the membrane against depolarising influences. The serotonin antagonists, cyproheptadine and methysergide,

have likewise been reported to improve the righting ability of dystrophic chickens and of simultaneously lowering the plasma CPK levels (Barnard et al. 1976). Serotonin may be involved in the pathogenesis of the disease by affecting the microcirculation in the dystrophic chicken muscle or may act directly on the muscle in a deleterious way. D-Penicillamine, a sulfhydryl compound which protects essential thiol groups of enzymes and structural proteins against oxidation, has also been reported to improve the righting ability of dystrophic chickens and at the same time, suppressed their plasma CPK levels (Park et al. 1979). Protease inhibitors (e.g. pepstatin, leupeptin and antipain) injected directly into the pectoral muscles of dystrophic chickens were observed to improve the histological appearance of the diseased fibres (Stracher and Mc Gowan, 1978).

RELEVANCE OF ANIMAL MODELS OF DYSTROPHY TO THE HUMAN DISEASE

The hereditary forms of muscular dystrophy in animals provides us with an experimental system to investigate the effects of many potential antidystrophic drugs that may not be practicable for one reason or another in man. Up to date, muscular dystrophy has been observed in a variety of animals (e.g. chicken, mouse, hamster, mink, turkey, dog, sheep and also possibly in the duck and cow), but only the dystrophic chicken, dystrophic mouse and the dystrophic hamster have been studied in any detail (Harris 1979, Cardinet and Holiday 1979). Controversy regarding

the relevance of these animal models to the study of the human disease, especially the Duchenne form of muscular dystrophy, still continues.

In the dystrophic mice, for example, the disease is associated with a major abnormality of the central nervous system, cranial and somatic nerve amyelination, denervation and physiologic abnormalities involving the motor axons (Bradley and Jaros 1979, Aguayo et al. 1979). In addition to these abnormalities, the mode of inheritance of the disease is different from DMD as are some of its histopathologic characteristics.

The dystrophic hamster, on the other hand, shows a myopathy of the heart in addition to the skeletal muscles (Homburger 1979, Mendell et al. 1979). In Duchenne muscular dystrophy, the heart is not affected until at a very late stage of the disease. Like the dystrophic mouse, the gene is inherited as an autosomal recessive trait and certain histopathological features are also different from DMD. The dystrophic hamster is, therefore, more a model of a cardiomyopathy, than a primary myopathy of the muscle.

Dystrophy in the chicken, however, shows no signs of any of the abnormalities seen in either the dystrophic mouse or the dystrophic hamster and probably forms one of the closest animal models of a primary myopathy in the skeletal muscles of dystrophic humans (Barnard, 1980). Like DMD, dystrophy in the chicken is characterized by a progressive weakening and wasting of certain pre-selected skeletal muscles. Many of the histopathological changes

that are seen in DMD such as fibre necrosis, hypertrophy, vacuolation, fibre splitting, increases in muscle nuclei content, and fat and connective tissue infiltration are also seen in the dystrophic chicken line that are used in the present study. In addition to these histopathological changes, elevations of CPK and a number of other muscle enzymes in serum are characteristic of both the human (DMD) and animal dystrophies. Although the mode of inheritance in the chicken (autosomal recessive) is different from that of DMD (X-linked recessive), this does not necessarily imply that the disease is expressed differently in the two species.

Although the dystrophic chicken may provide us with an animal model that closely resembles all the characteristic features of DMD, the primary biochemical lesion may not be the same in each case. Thus, if we found a drug that will completely arrest the progression of the disease in the dystrophic chicken, this same drug may have no effect on the course of the dystrophic process in humans. Another point that one should consider is that even if the primary biochemical defect in both the dystrophic chicken and DMD is identical, a drug found to be effective in one may not necessarily be effective in the other. Differences in physiological processes between man and animals may render the drug to be inactivated much quickly in man than in the chicken, or vice versa.

Aims of the Present Study

The genetically dystrophic chicken provides us with an animal model of dystrophy that closely resembles all the characteristic features of Duchenne muscular dystrophy. Unlike dystrophic humans, it is readily accessible and provides a convenient test system for investigating the actions of various drugs on the disease process.

Using this animal model, the first objective will be to characterise the properties of PK and PGM in both the muscle and the blood plasma of dystrophic chickens. This will involve examining the changes in plasma PK and PGM levels during the development of the dystrophic chicken. By employing the method of isoelectric focussing, it should be possible to obtain the complete isoenzyme complement of PK and PGM in normal muscle, dystrophic muscle and dystrophic plasma. From these studies, it will be possible to know whether dystrophy affects the expression of these isoenzymes in the muscle and also, the origin of any isoenzyme forms present in the dystrophic plasma.

If it is found that both plasma PK and PGM can serve as markers of the dystrophic muscle, then the next objective would be to use one of these plasma enzymes in conjunction with other parameters to assess the chemotherapeutic value of a number of potential antidystrophic drugs in these dystrophic animals. To complement these chemotherapy trials, the pharmacokinetic properties of two anti-serotonin drugs will also be examined in these birds.

In addition, the isoenzyme changes of CPK, LDH and PK will be examined during the maturation of normal

and dystrophic chick embryonic muscle. It is hoped that these studies will help to answer the following questions concerning the pathogenesis of dystrophic muscle:

(1) Are there manifestations of the pathogenesis in the embryonic muscle?

(2) Is there a genetic persistence of the embryonic isoenzymes present in mature dystrophic muscle because the differentiation of embryonic muscle is incomplete?

Finally, to complement these studies, the isoenzyme changes of LDH and PK will also be examined in normal chicken muscle following denervation. It is hoped that this study will help to answer whether the presence of embryonic or foetal isoenzyme forms in adult dystrophic muscle is due to a defect in the mechanism controlling differentiation in muscle or results from the partial regeneration of atrophic muscle fibres occurring in diseased muscle.

CHAPTER 2

BLOOD PLASMA PYRUVATE KINASE AS A MARKER OF THE DYSTROPHIC CHICKEN MUSCLE

INTRODUCTION

In Duchenne and closely related forms of muscular dystrophy in man, elevations of a number of skeletal muscle enzymes in the blood plasma are well known (Rowland 1976, Pennington 1980). Of these, creatine phosphokinase (CPK) was found to exhibit by far the largest and most consistent serum elevation in such myopathies, and this occurred in isoenzymatic forms characteristic of the dystrophic muscle CPK (Schapira et al. 1968, Somer et al. 1973). When animals with hereditary muscular dystrophy were also examined, similar increases in plasma CPK occurred, particularly in the dystrophic chicken and the dystrophic hamster as the disease developed (Barnard et al. 1976, Chou et al. 1975, Chow 1975). The exact nature by which these muscle enzymes are released into the blood in both the human and animal forms of muscular dystrophy is at present unknown but a generalized membrane defect involving the sarcolemma may be the cause of these elevations.

In the last decade, many new diagnostic methods have been developed for muscular dystrophy patients and carriers in Duchenne and related forms of muscular dystrophy (Lane et al. 1979). Some of these methods include serum CPK measurements (Walton and Gardner-Medwin, 1974), K^+ efflux rate in erythrocytes (Lane et al., 1979), sensitivity of erythrocyte ghost membrane ATPase to ouabain (Pearson, 1978), membrane phosphorylation studies (Roses et al., 1976), increased protein synthesis in polyribosomes (Ionacescu et al., 1976), increased serum haemopexin levels

(Danieli and Angelini, 1976), histological and histochemical studies (Dubowitz and Brooke, 1973) and the use of LDH isoenzyme 5 from serum and muscle biopsies (Roses et al., 1977). Of these, the most widely used and probably the most reliable test for carrier detection up to date is serum CPK. However, even this test can only detect up to two-thirds of all known carriers. In the search for a more sensitive marker of dystrophy and one that could also be used for monitoring the chemotherapeutic effectiveness of potential antidystrophic drugs in dystrophic chickens, it was decided to investigate another potential enzyme marker of dystrophy, namely pyruvate kinase (PK).

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) is an enzyme found abundantly in the skeletal muscle and catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP in the glycolytic pathway to yield a molecule of ATP and pyruvate. This is a very important step in glycolysis as it represents one of two stages in the glycolytic pathway where there is a net synthesis of ATP.

Recently, a number of reports have shown that this enzyme is also greatly elevated in the serum of dystrophic humans. In three studies, the mean PK value for Duchenne patients was found to be 19 to 28 times the normal mean (Alberts and Samaha 1974, Harano et al. 1973, Weinstock et al. 1977), and in another recent study, 39 times (Zatz et al. 1978). In carriers for this gene, detectable increases in serum PK were commonly present (Alberts and Samaha 1974, Hardy et al. 1974, Weinstock et al. 1977).

In addition, three very recent reports have indicated that the combined use of serum PK and CPK increased the overall detection rate in carriers than would otherwise be if either enzyme were used alone (Zatz et al. 1978, Percy et al. 1979, Weinstock et al. 1977).

The object of the present study is to assess the potential value of plasma PK as a marker of dystrophy in both the chicken and the hamster with a genetically determined form of muscular dystrophy. Very large increases in plasma PK levels were observed during the development of the dystrophic chicken and the dystrophic hamster. Heterozygous chickens (carriers) also showed significant elevations when compared to normal chickens. Further, it was shown that the PK isoenzyme form found in the dystrophic plasma was of muscle origin. All these observations confirm that plasma PK is a marker of the dystrophic muscle and hence is likely to be of value in the chemotherapeutic assessment of potential antidystrophic drugs. A report of this study has recently been published (Liu et al., 1980).

MATERIALS AND METHODS

Animals. Eggs of strain 413 (homozygous, dystrophic) and the corresponding normal control strain 412 chickens were either purchased directly from the Department of Avian Sciences, University of California, Davis, or were produced by breeding within those strains at Imperial College, or (for heterozygotes) between them. After incubation and hatching, the birds were maintained from the 2nd day ex

ovo in environmental chambers (about 12 chicks per cage) with a constant temperature (25°C) and relative humidity (40 - 50%), and a 12-h light cycle. All had free access to food and water.

Blood Specimens. Blood was taken from the jugular vein (without anaesthesia) at weekly intervals in the case of hatched chicks, into heparinized Eppendorf tubes normally by the Animal Unit Staff. In the case of embryonic blood, the embryo was carefully removed from the egg shell without breaking the cord attached to the yolk, dried quickly all round with a paper towel, and then a cut was made across the cord. The uncontaminated blood that flows out from the remaining end of the cord attached to the embryo was collected into heparinized Eppendorfs. The plasma in each case was at once separated, stored at 0°C, and used within a few hours for PK and CPK determinations

The dystrophic hamsters were bred at Imperial College from the AA strain (which originated in the BIO - 14.6 line) and were all homozygous for dystrophy. Blood was drawn by heart puncture under light general anaesthesia [50mg/Kg Ketamine (Parke - Davies), intramuscularly] by the A.U. staff, clotted immediately, and the fresh serum used. It was essential to anaesthetize these animals and to take precautions to avoid agitating them, using an anaesthetic agent effective in about 1 min, as here, to avoid increase in their resting CPK and PK concentrations which can occur in both dystrophic and normal hamsters when disturbed or active. It was determined that the anaesthetic

agent used had no effect per se on the enzyme activities. Chemicals. All chemical agents were from Sigma Chemicals Ltd., except where specified.

Tissues. The tissues were removed at once after killing and homogenized in 0.1M KCl, 0.005M MgCl₂, 0.01M Tris-HCl buffer (pH 7.5). The following protease inhibitors were also present at this and subsequent stages, as noted: 10⁻⁴M ethylene glycol - bis (β-amino-ethylether)-N, N'-tetraacetic acid, 10⁻³M benzamidine, 5μg/ml soybean trypsin inhibitor, 100μg/ml bacitracin, and 10⁻⁴M phenylmethylsulphonyl fluoride (PMSF). (Separate trials showed that these inhibitors had no effect on the PK or CPK activity.) The homogenate was centrifuged in a Beckman L6 centrifuge at 140,000g, 30 min at 4°C. The supernatant was taken for isoelectric focussing.

For chicken erythrocyte PK, the red cells were pelleted from heparinized blood, washed twice in isotonic saline and haemolyzed in water at 4°C, using the minimum ratio giving complete lysis. The white cell layer was removed in the washing procedures. The red cell ghosts were removed at 10,000g (10 min) and the supernatant used at once for electrofocussing.

Assays. PK activity was measured by recording the decrease in A_{340nm} due to NADH, using an automated multisample analyzer, the LKB 8600 (Mark II) reaction rate analyzer, with a thermostated sample holder. Temperature of the sample prior to and during assay was 25°C, except where stated. The Boehringer-Mannheim kit for the PK assay was

used. The manual assay with this kit, as prescribed by the manufacturer, was adapted to the automated system by adding the triethanolamine buffer (pH 7.4), phosphoenolpyruvate, NADH, and LDH to the sample and allowing an initial reaction period of about 20 min at room temperature for all pyruvate present to be consumed. Addition of ADP then initiated the PK reaction, which was recorded directly.

CPK activity was measured similarly at 25°C by Dr. J. Lyles in the LKB analyzer using the CPK Automated Test Combination kit (Boehringer-Mannheim) in the standard manner prescribed for that kit. CPK standards from the manufacturer were used to confirm the absolute values obtained.

Enzyme activities are expressed in International milliunits per millilitre plasma or extract under these standard conditions.

Quantitative Isoelectric Focussing. The procedure for flat bed electrofocussing on an Ultrodex gel specified in LKB Application Note 198 were modified as indicated below to improve both the efficiency and economy of the method. A thin glass plate with rubber borders having dimensions 5cm (width) x 11cm (length) x 0.5cm (height) was made. Electrode strips, three layers high, were soaked in 2% pH 6-8 ampholine solution and placed at the anode end of the rectangular rubber block. Similarly, electrode strips soaked in 2% pH 8-9.5 ampholine solution were placed at the cathode end. This ensures that the appropriate pH is reached at either end of the gel on the completion of focussing. A thick slurry (30ml) containing 1.65g Ultrodex, 1% of both pH 6-8 and 8-9.5 ampholines and 10% sucrose was

made up and de-gassed for 5 min. The slurry was then poured onto the glass plate and the surface of the gel was smoothed over using a perspex rod. A hair dryer was used to force evaporate the excess water in the gel. The gel is ready to use when approximately 25% of its weight is lost due to evaporation. Further evaporation causes the gel to crack. This drying process usually only takes about 30 min. A further layer of electrode strip was placed at both ends and soaked in either 1M NaOH (cathode) or 1M H_3PO_4 (anode). The thin glass plate containing the gel was placed on the cooling bed of the LKB multiphor apparatus. Constant circulation of ice-cooled water around the bed kept the running temperature at 0°C and served to minimize both enzyme denaturation and focussing time because a higher power input could be applied. Samples (10 - 25ul) were applied; constant power (4W) was maintained, for a focussing time of about 6h. Myoglobin was used as an internal marker to indicate completion of focussing. The Ultrodex bed was fractionated into 3-mm-thick sections; the gel from each such section was placed in an Eppendorf tube, and 0.5ml 0.85% NaCl solution was added to the extract, agitated mechanically for 30 sec, and centrifuged for 2 min in a bench centrifuge. 125ul supernatant was taken for the PK assay. In each section prior to removal, the pH was measured by a micro-electrode (at room temperature), with buffer standardization also at that temperature. In the system used, horse muscle myoglobin (Sigma, type I, principally ferric) had a main component with $\text{pI} = 7.3$, and was used as an internal reference point.

Physical Testing of Chickens. All Flip Number measurements and data reported in the present study were kindly donated by Dr. P.J. Barnard. The Flip Number (FN) of the chickens was measured at weekly intervals. Chickens were placed on their backs on a flat surface under standardized conditions and allowed to right themselves. The FN is the number of successful rightings of five immediately consecutive attempts.

RESULTS

MEASUREMENT OF PK ACTIVITIES

Using the manual kit supplied by Boehringer-Mannheim and adapting it to the LKB automated kinetic analyzer, the PK assays of blood or tissue extracts became highly reproducible between duplicate assays. It was observed that the blood samples from dystrophic animals could be stored frozen for this assay, provided the freezing was done rapidly at -78°C . Table 1B shows that there is no significant difference between the mean PK activity of fresh and frozen plasma samples. If the blood samples were frozen slowly at -20°C , variable but significant losses occurred, this effect being greater in the hamster serum (A), and is as reported for PK in human dystrophic sera frozen in the same way. All measurements of PK reported subsequently in this thesis were made on fresh, unfrozen samples.

Because most values in the literature for PK activities are given for 30°C , and that the values in this report are for 25°C , a correction factor of 41% is needed for comparison. The data in Table 2 shows that chicken

TABLE 1

Effects of Freezing on Blood PK Activity

Species	No. of animals	Age (days)	Treatment ^b	Mean PK $\pm$ S.E.M. ^a		Change	<u>P</u>
				Fresh	After Freezing		
Chicken	5	49	A	1941 $\pm$ 52	1407 $\pm$ 241	-28%	<0.002
	14	27	A	736 $\pm$ 110	442 $\pm$ 82	-40%	<0.02
	10	67	B	3204 $\pm$ 271	3593 $\pm$ 301	+12%	N.S.
Hamster	15	45	A	4118 $\pm$ 257	1702 $\pm$ 342	-59%	<0.001

^aAll assays were at 25°C, after equilibration (15 min) at that temperature.

^bThe plasma (dystrophic chicken) or serum (dystrophic hamster) sample from each animal was (A) frozen slowly by placing in a freezer at -20°C, or (B) frozen rapidly at -78°C by placing on solid CO₂, with an assay at each stage. In each case, subsequent storage prior to thawing was at -20°C, for up to 8 weeks.

P = Significance of the difference between initial and final activity, by t test; N.S. = Not significant ($P > 0.05$).

TABLE 2

Effect of Temperature on Chicken Plasma PK Activity

Temp.	No. of Animals	Mean PK $\pm$ SEM (mU/ml)	Increase for 5°C rise
25°C	10 ^a	1228 $\pm$ 134	41%
30°C	10 ^a	1725 $\pm$ 184	

^aThe same plasma samples were used at the two temperatures.

plasma PK displayed a relatively large temperature coefficient so that efficient thermostating in the autoanalyzer and temperature pre-equilibration were required to obtain accurate and reproducible results.

PLASMA PK ACTIVITY IN DYSTROPHIC CHICK EMBRYOS

Following two recent reports (Mahoney et al. 1977 and Dubowitz et al. 1978) that showed plasma CPK to be of value in the detection of Duchenne muscular dystrophy at the level of the human foetus, it was naturally very interesting to observe whether PK is raised in the blood of dystrophic chick embryos. Table 3 shows the mean plasma PK levels in normal and dystrophic chick embryos as a function of their age in ovo and 2 days after hatching. As the data clearly shows, no significant differences between normal and dystrophic plasma PK activities were apparent throughout the period investigated. Contamination by embryonic fluid surrounding the embryo which could affect the results is minimised by the nature of the method outlined above when collecting the blood.

PLASMA PK ACTIVITY IN DYSTROPHIC CHICKENS

Plasma PK activities in normal chickens changed relatively little during the 1.5-year investigated (Fig. 1). Although there was an initial increase of about twofold in the PK activity during the first few weeks after hatching (e.g. mean values were about 80 mU/ml plasma at day 12 and about 140 mU/ml plasma at day 33), the enzyme content in blood plasma remained essentially constant thereafter. Variation within this normal population was very small and the standard error bars were too small to be depicted.

Table 3
Plasma Pyruvate Kinase (PK) Activity in Chick Embryos

Days <u>in ovo</u>	Dystrophic Plasma mU per ml plasma	Normal Plasma	Probability (P)
12	* 51 $\pm$ 7.4 (4)	* 48 $\pm$ (2)	>0.05 (N.S.)
13	21 $\pm$ 3.0 (4)	25 $\pm$ 4.8 (4)	>0.05 (N.S.)
14	34 $\pm$ 13 (4)	70 $\pm$ 21 (4)	>0.05 (N.S.)
15	51 $\pm$ 12.5 (4)	85 $\pm$ 24 (4)	>0.05 (N.S.)
16	60 $\pm$ 10 (4)	61 $\pm$ 10 (4)	>0.05 (N.S.)
18	91.5 $\pm$ 9 (4)	69 $\pm$ 12 (6)	>0.05 (N.S.)
2 days <u>ex ovo</u>	119 $\pm$ 6.4 (4)	122 $\pm$ 12.5 (2)	>0.05 (N.S.)

* Mean plasma PK activity $\pm$ standard error of the mean. Number of chick embryos used in parenthesis. Level of P accepted as being statistically significant $\leq$ 0.05.
 N.S. = Not Statistically Significant.

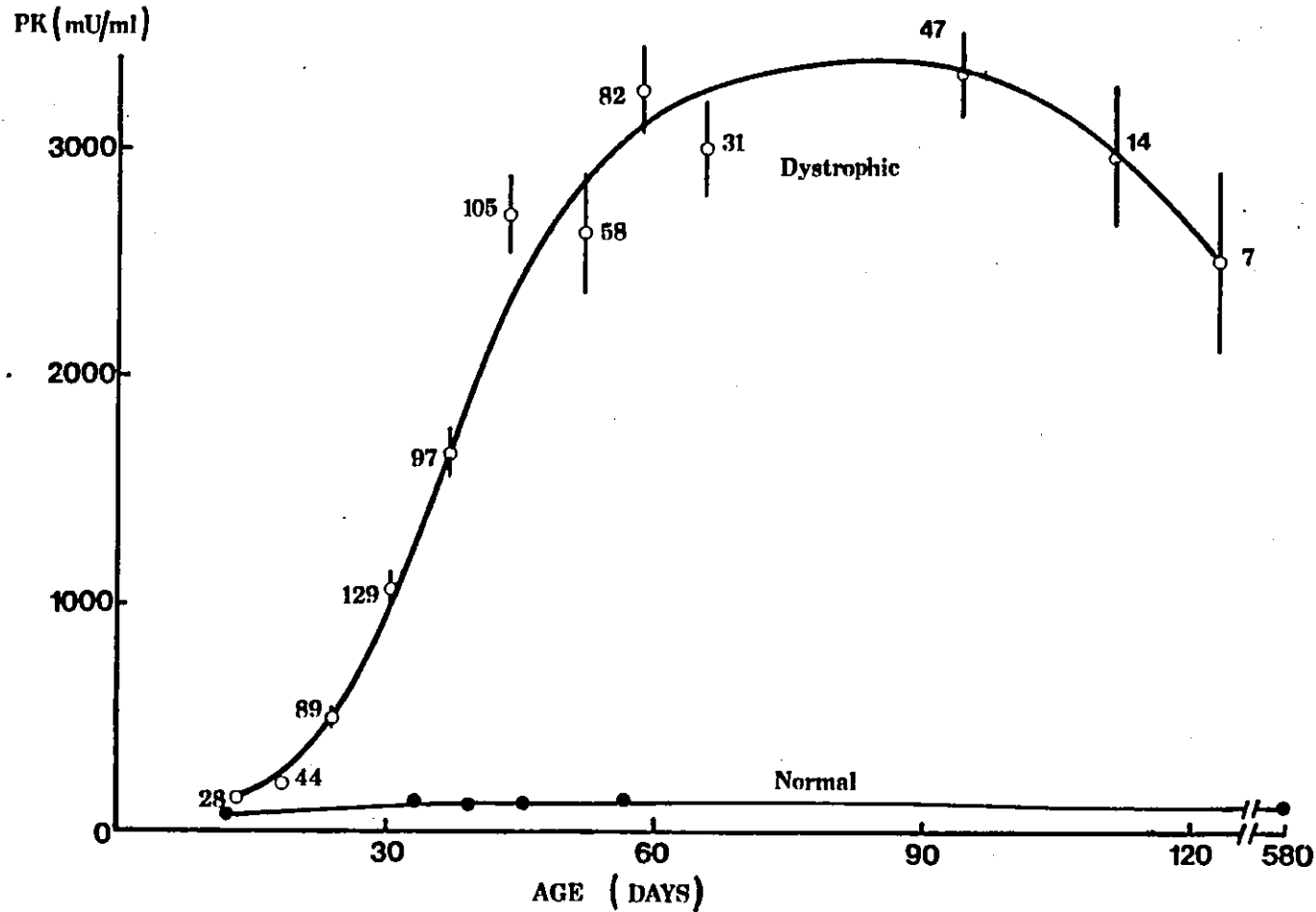


Fig. 1. Change of pyruvate kinase (PK) with age in the chicken. The mean PK activity in dystrophic chicken plasma (o), with its standard error, or in normal chicken plasma (●), is shown. For normal chickens, the standard error was too small to depict here. The number of dystrophic birds used for each mean is indicated next to each point.

Plasma PK activities in chickens homozygous for dystrophy, in contrast, increased markedly from about 140 mU/ml plasma after 12 - 15 days to 3000 - 4000 mU/ml plasma at 60 - 100 days. There was an average increase of about 30-fold in the PK activity during the maturation of these dystrophic chickens. After about 60 days, a plateau was reached and the PK activity remained fairly constant up to about 100 days ex ovo. After that period, the plasma PK activity started to decline, presumably due to replacement of necrotic muscle fibres by connective tissues and lipid deposits by that stage. Evidence for this was obtained (Dr. J. Pizzey, personal communications) from photograph sections of old dystrophic chicken muscle (over two years old) having very few muscle fibres and containing large quantities of fat and connective tissue present. In the plateau region of the curve, the individual variation in PK values among the dystrophic chickens became greater as the rate of development varied between individuals.

PK CONTENT IN NORMAL AND DYSTROPHIC MUSCLE

Having found very large increases in PK activity in the dystrophic chicken plasma and not normal plasma, it was of interest to see if there is any significant difference in the absolute amount of the enzyme present in normal and dystrophic chicken muscle. The results in Table 4 showed a very substantial decrease in muscle PK content in dystrophic muscle when compared to normal muscle of the same age, a decrease of about 65%. The difference was found to be highly significant ($P < 0.001$) by statistical analysis and are consistent with an earlier finding

Table 4

PK Content in Normal and Dystrophic Pectoral Muscle

<u>Age(days)</u>	<u>Normal Muscle</u>	<u>Dystrophic Muscle</u>
	(mU per g of wet tissue)	
61d	531,955	149846
	369,621	167,328
	464,524	162,334
	353,138	126,160

Mean $\pm$ SEM = 429,810 $\pm$ 41,972 151,417 $\pm$ 9187

% Difference relative to normal = -65%

Probability (P) < 0.001

Statistical Inference by Students 't' test. Level of (P) accepted as being significant $\leq$ 0.05.

(Weinstock et al. 1977) that showed a similar decrease in PK content in the muscle of Connecticut chickens.

CORRELATION BETWEEN PLASMA PK ACTIVITY AND WEIGHT

Because birds of a given age vary significantly in weight and their muscle mass does not increase linearly with age, a more reliable measure of development than age would be bodyweight. When plasma PK activity is plotted as a function of bodyweight and not of age, a linear correlation was seen in the dystrophic chicken (Fig. 2). Plasma PK activity in normal chickens, although not shown, remains constant with increase in bodyweight. The correlation between plasma PK and bodyweight seen in dystrophic chickens is applicable up to the plateau region of Fig. 1 and not in the later stages of development.

PLASMA PK ACTIVITY IN MALE AND FEMALE DYSTROPHIC CHICKENS

In the human Duchenne form of muscular dystrophy, the genetic defect is sex-linked and the abnormal gene is carried on the X-chromosome of the affected person whereas in the chicken, the abnormal gene is carried in one of its many autosomes. Nevertheless, secondary differences in dystrophic characteristics might occur between the sexes in the chicken, due to their somewhat different pattern of growth or to hormonal influences on muscle. This was investigated to the plateau region of Fig. 1 (Fig. 3), but no significant difference between males and females was found in their plasma PK levels at all ages ($P > 0.1$ for all pairs of these points in Fig. 3).

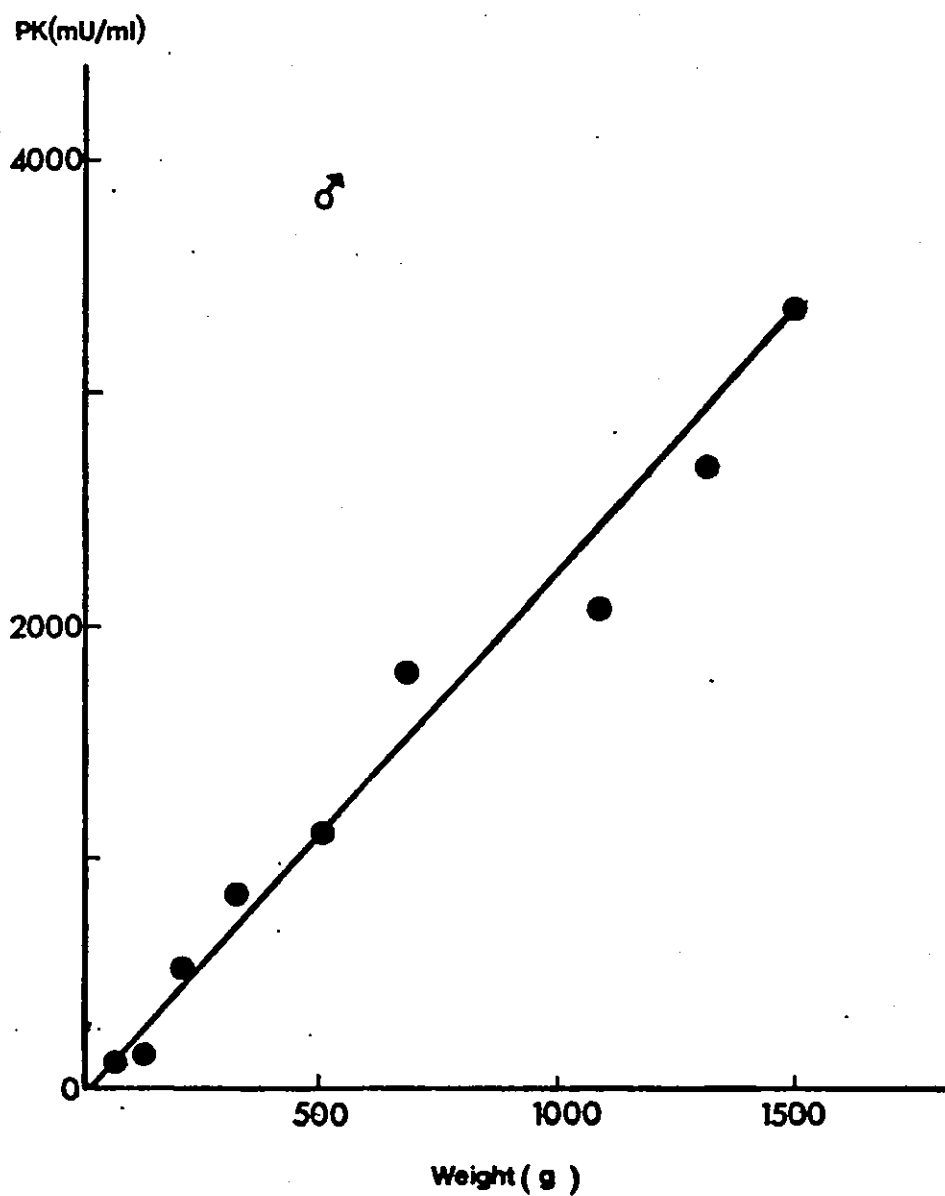


Fig.2 . Plasma pyruvate kinase (PK) activity in dystrophic chickens as a function of bodyweight. Each point represents the mean of 12 to 15 males; ages were 2 to 8 weeks.

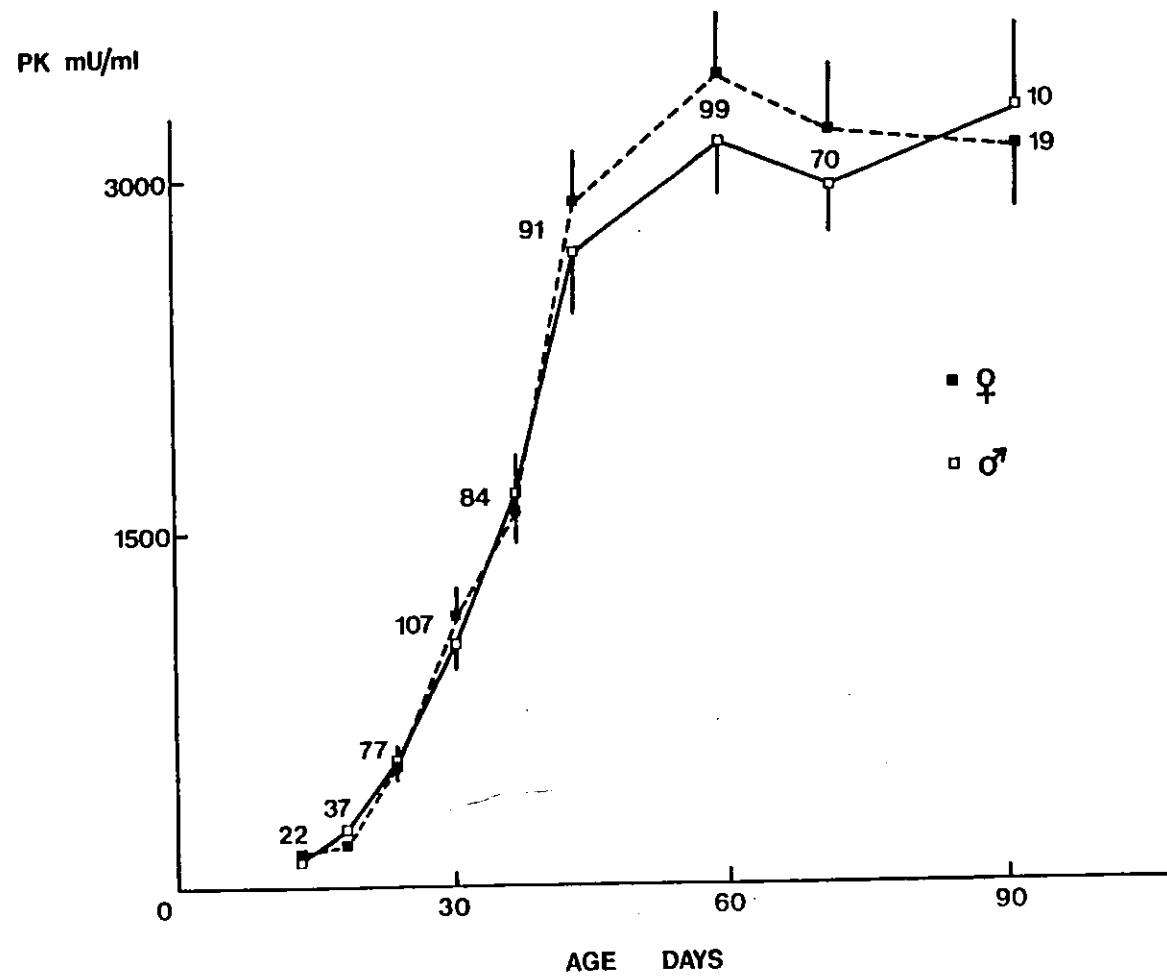


Fig. 3. Plasma PK activity in male ($\square$) and female ($\blacksquare$) dystrophic chickens as a function of age.

CORRELATION BETWEEN PK ACTIVITY AND FLIP NUMBER

Another useful and important parameter one can correlate PK with in the dystrophic chicken is the flip number (FN). This is a measure of the increasing muscular disability of the chicken as the disease develops. Reported in Fig. 4 is the change in FN for male and female dystrophic chickens as a function of age. A small difference between the righting ability of male and female dystrophic chickens was observed as the disease developed, though this was not reflected in a difference in their PK values (Fig. 3). When Figs. 1 and 4 are superimposed on top of each other (Fig. 4), an inverse correlation between the accumulation of PK in the blood plasma and the onset of muscular disability is seen. After about 60 days ex ovo, when the PK activity in the blood plasma reaches a maximum in the dystrophic birds, their righting ability declined to almost zero.

CORRELATION BETWEEN PLASMA CPK AND PK ACTIVITIES

A similar correlation was also observed between the appearance of plasma CPK and that of PK in the dystrophic chicken. Whenever examined, these two enzyme activities moved in parallel during the development of dystrophy. This is illustrated at two different ages (Figs 5a and 5b), showing a very high degree of association between plasma CPK and PK of the same dystrophic chicken. The ratio of the egress of CPK to PK varied between 2 and 3 depending on age. This ratio was lower at day 25 ex ovo than at day 46 ex ovo, indicating that the relative rate of PK accumulation in blood plasma initially was faster than that of CPK.

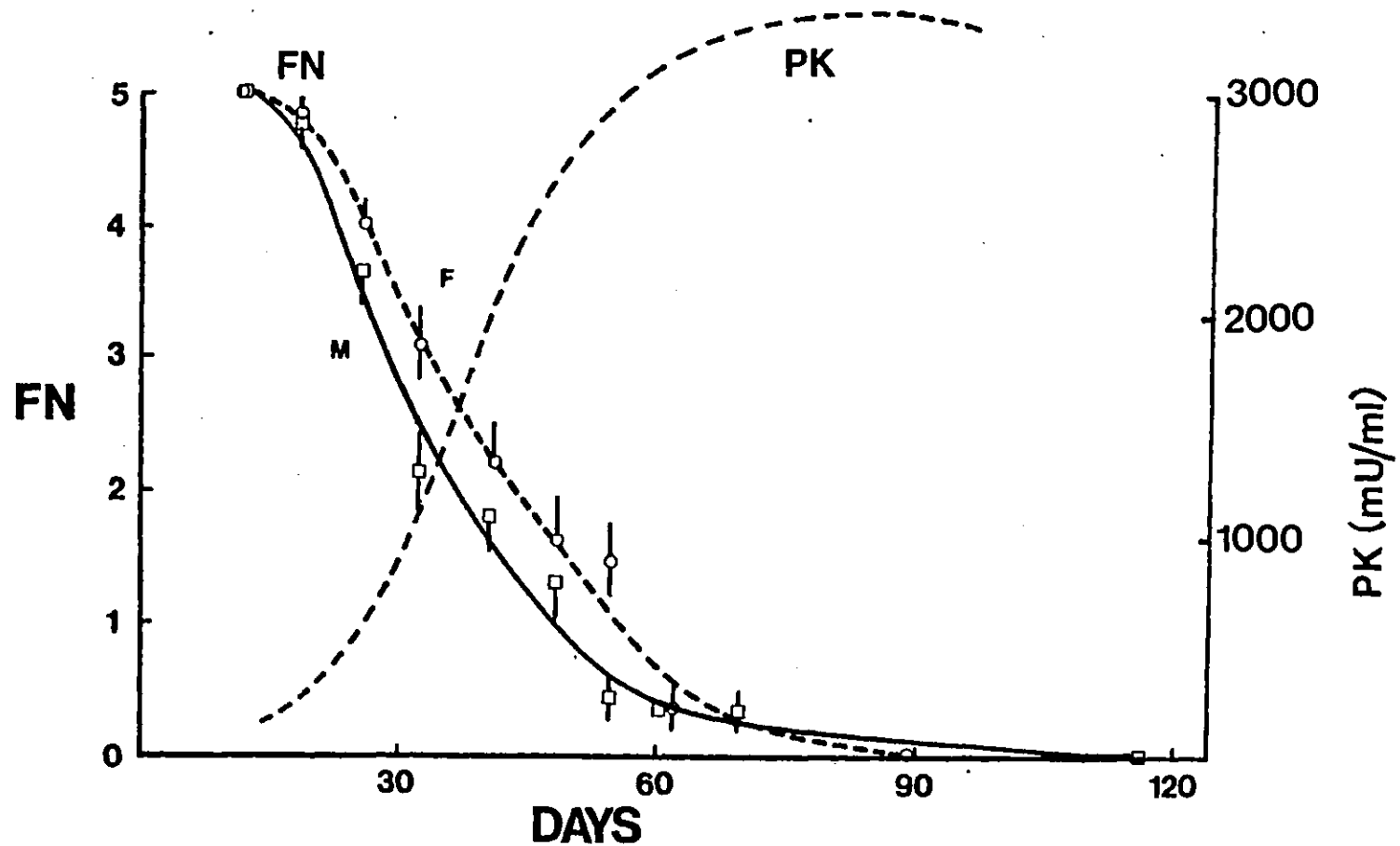


Figure 4. Correlation between plasma PK activity and muscle weakness. Mean flip numbers (FN) of male (M) and female (F) dystrophic chickens are indicated by solid lines. Mean PK activities of both male and female dystrophic chickens are represented by the dotted curve. Each point in the FN curves represent the mean values of between 20-60 dystrophic birds.

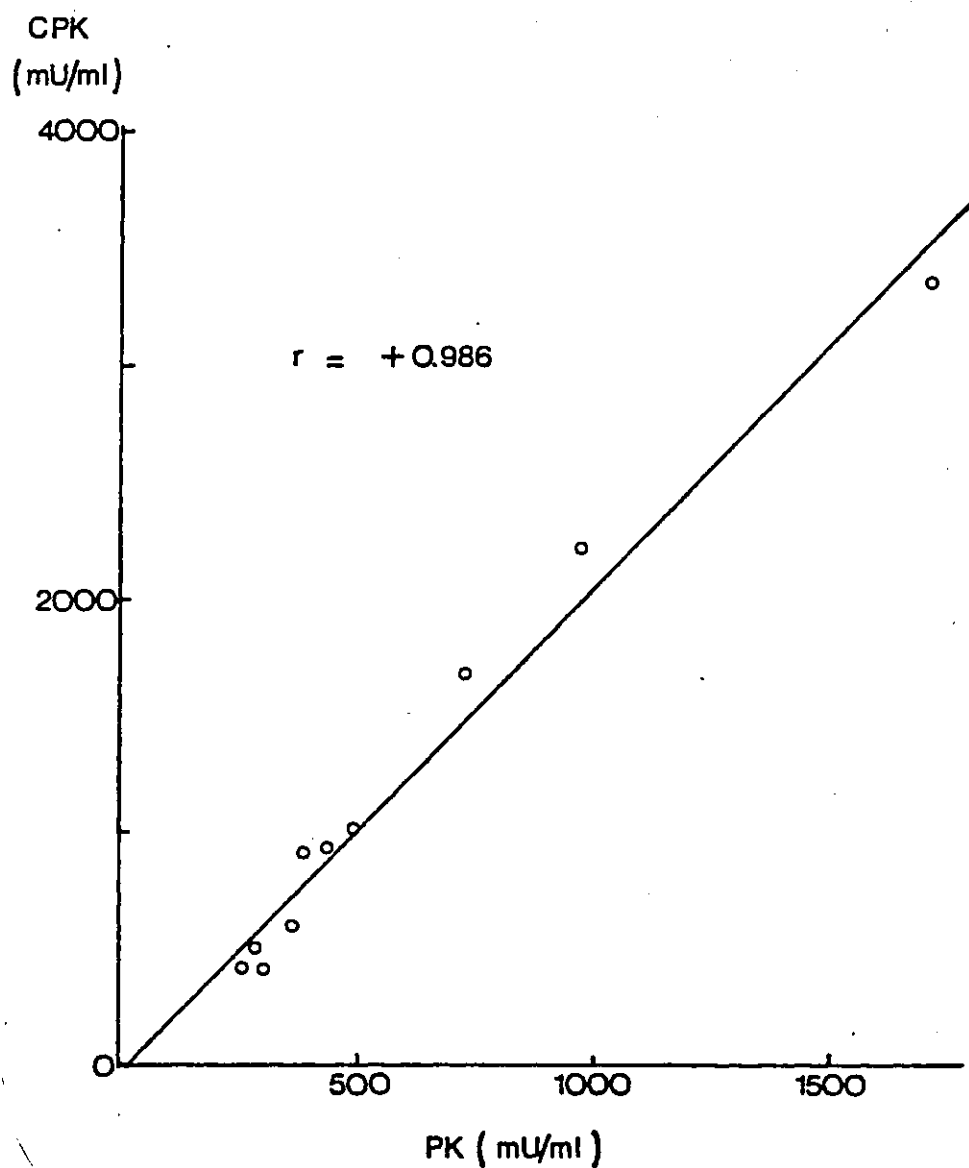


Fig.5 a. Association of creatine phosphokinase (CPK) and pyruvate kinase (PK) activities in plasma from dystrophic chickens. Points represents single birds of various weights and either sex at age 25 days. The correlation coefficient (r) was + 0.986.

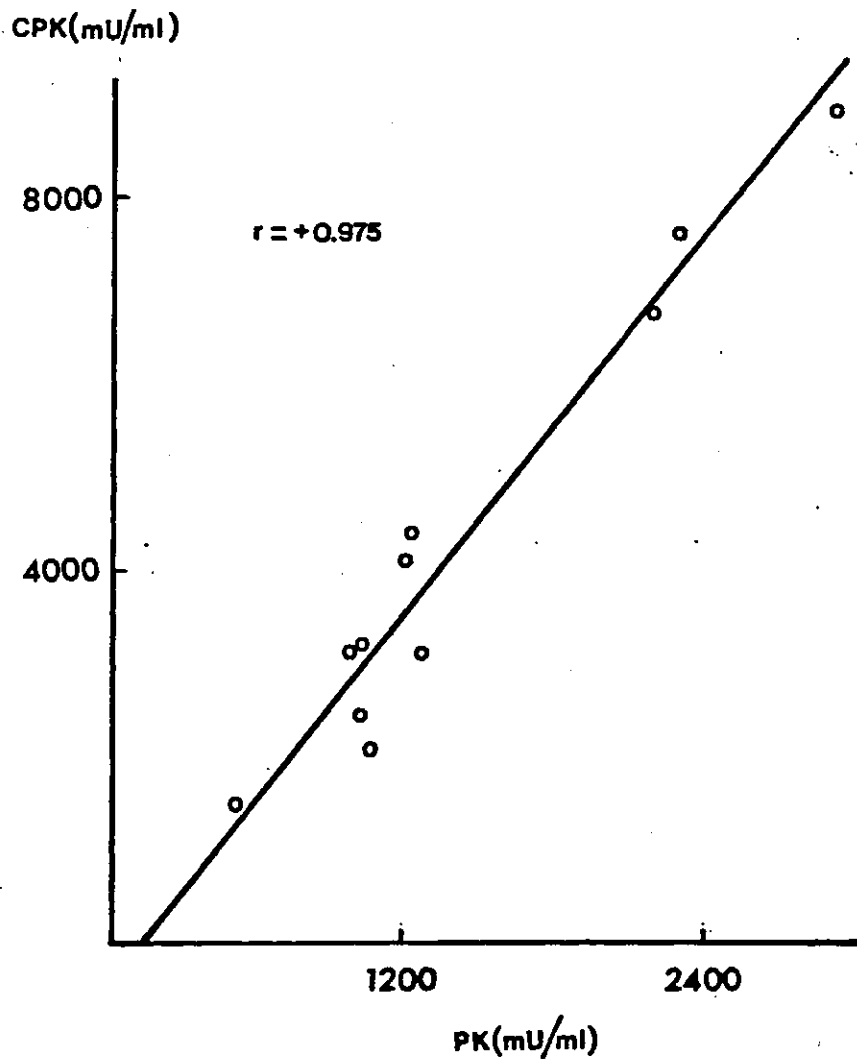


Fig.5 b. Association of creatine phosphokinase (CPK) and pyruvate kinase (PK) activities in plasma from dystrophic chickens. Points represents single birds of various weights and either sex at age 46 days. The correlation coefficient (r) was + 0.975.

PLASMA PK AND CPK ACTIVITIES IN NORMAL AND HETEROZYGOUS DYSTROPHIC CHICKENS

In addition to chickens homozygous for dystrophy, heterozygous chickens were also available for comparison with normal controls and are reported in Table 5. Their plasma PK activities were always higher than the corresponding normals, being about twice the normal value on average. When tested for significance at two different ages (day 28 and 40) using the Students 't' test, probability values of less than 0.1% that these differences could have arisen by some random event were obtained.

Alongside PK, plasma CPK values were also compared in normal and heterozygous dystrophic chickens. Here, the difference was less marked and the ranges did overlap to some extent at the two ages tested. When tested for significance (using Students 't' test) on this occasion, plasma CPK only became significantly elevated at day 40 and not at day 28 as did PK.

PLASMA PK ACTIVITY IN THE DYSTROPHIC HAMSTER

Another animal model which closely resembles the human form of Duchenne muscular dystrophy is the genetically dystrophic hamster. In this myopathy, both the skeletal and cardiac muscles are affected. Very high serum levels of CPK activity in this animal have been reported. In the present study, serum PK activities were also investigated during its development and the results are presented in Table 6. The early time course of its increase could not be reliably established, because samples could

Table 5

Plasma PK and CPK Activities in Normal and Heterozygous
Dystrophic Chickens

Enzyme	Age (days)	Normal ^a (mU/ml)	Heterozygotes ^a (mU/ml)	<u>P</u>
PK	28	135 $\pm$ 5 (11)	263 $\pm$ 20 (4)	<0.001
	40	113 $\pm$ 4 (13)	288 $\pm$ 7 (4)	<0.001
CPK	28	268 $\pm$ 14 (7)	305 $\pm$ 22 (4)	N.S.
	40	275 $\pm$ 10 (13)	395 $\pm$ 17 (4)	<0.001

^aMean enzyme activity, $\pm$ S.E.M., Number of animals tested given in parentheses.

P = Significance of the difference between normal and heterozygote by t test;

N.S. = Not significant ($P > 0.05$).

Table 6

Serum PK Activity in Hamsters

Age (Days)	mU/ml	
	Dystrophic ^a	Normal ^a
42-47	4179 _± 261 (25)	320 _± 39 (8)
59-61	4478 _± 285 (15)	260 _± 9 (7)
65-67	6187 _± 536 (12)	
74-75	4980 _± 598 (12)	
82-88	4079 _± 361 (25)	361 _± 68 (5)
102	3876 _± 402 (7)	
114-131	3671 _± 358 (15)	

^aMean activity _± S.E.M. Number of animals tested given in parentheses.

not be taken from hamsters less than 40 days old without too great a trauma and erratic values. By about 40 days the serum PK had already increased very greatly and remained in the same range for at least 130 days. Normal hamsters, however, did not change appreciably in their PK output and had a range of about 200 to 400 mU/ml serum during the period investigated. This range is relatively greater than normal chickens.

The association of serum CPK with PK was also investigated in the dystrophic hamster. The correlation between these two enzyme activities in blood were less than that observed in the dystrophic chicken, though the ratio of CPK to PK output in the blood were essentially the same.

ORIGIN OF PLASMA PK IN THE DYSTROPHIC CHICKEN

In the above studies so far done, it has been indicated that the increased PK activity in the blood of dystrophic chickens originates from the muscle. But what firm evidence do we have that it does indeed arise from the muscle and not say from the erythrocytes (since these cells are also affected in dystrophy) or liver, brain, kidney or other tissues? Evidence for the above hypothesis is provided by looking at the isoenzymes of PK in the dystrophic chicken.

In chickens, three distinct isoenzymes of PK have been identified by immunological, electrophoretic and chromatographic methods and these are M_1 , M_2 (or K) and L types. The M_1 isoenzyme is found exclusively in the muscle, heart and brain whereas the M_2 and L isozymes are present in most other tissues of the chicken.

Quantitative isoelectric focussing on an Ultrodex gel bed showed one major component of PK activity (M_1) in normal pectoral muscle with $pI = 8.45$ (8.40 and 8.50 in two determinations), and a minor peak with $pI = 8.1$ (Fig.6). Focussing of dystrophic pectoral muscle gave an identical profile (Fig. 7) with a major peak of activity (M_1) with $pI = 8.47$ (8.50, 8.45, 8.45, in three determinations) and also a minor peak at $pI = 8.12$. That the isoenzymes from dystrophic and normal pectoral muscle are really the same in their pI values was confirmed by focussing a mixture of the two extracts, when both the major and minor components of normal and dystrophic muscle coincided precisely (Fig. 8).

Unlike muscle, normal chicken liver (Fig. 9) showed a major component of PK activity at $pI = 7.97$ (7.95, 8.00, in two determinations) and a minor component at $pI = 7.36$ and 7.00. The major component corresponds to M_2 , and the minor components are probably heterogeneous L forms.

Electrofussing of the dystrophic chicken erythrocyte PK (Fig. 10) gave two active peaks of activity, a major peak at $pI = 7.75$ and a minor peak at $pI = 7.30$. The pI 7.75 isoenzyme is probably a distinct erythrocyte PK form not previously recognised in chickens. Erythrocytes from normal chicken gave an identical profile on focussing (not shown) with a major component at $pI = 7.7$ and a minor component at $pI = 7.3$.

The pI 7.3 component of erythrocyte PK is probably identical to one of the L forms seen in the liver. The main

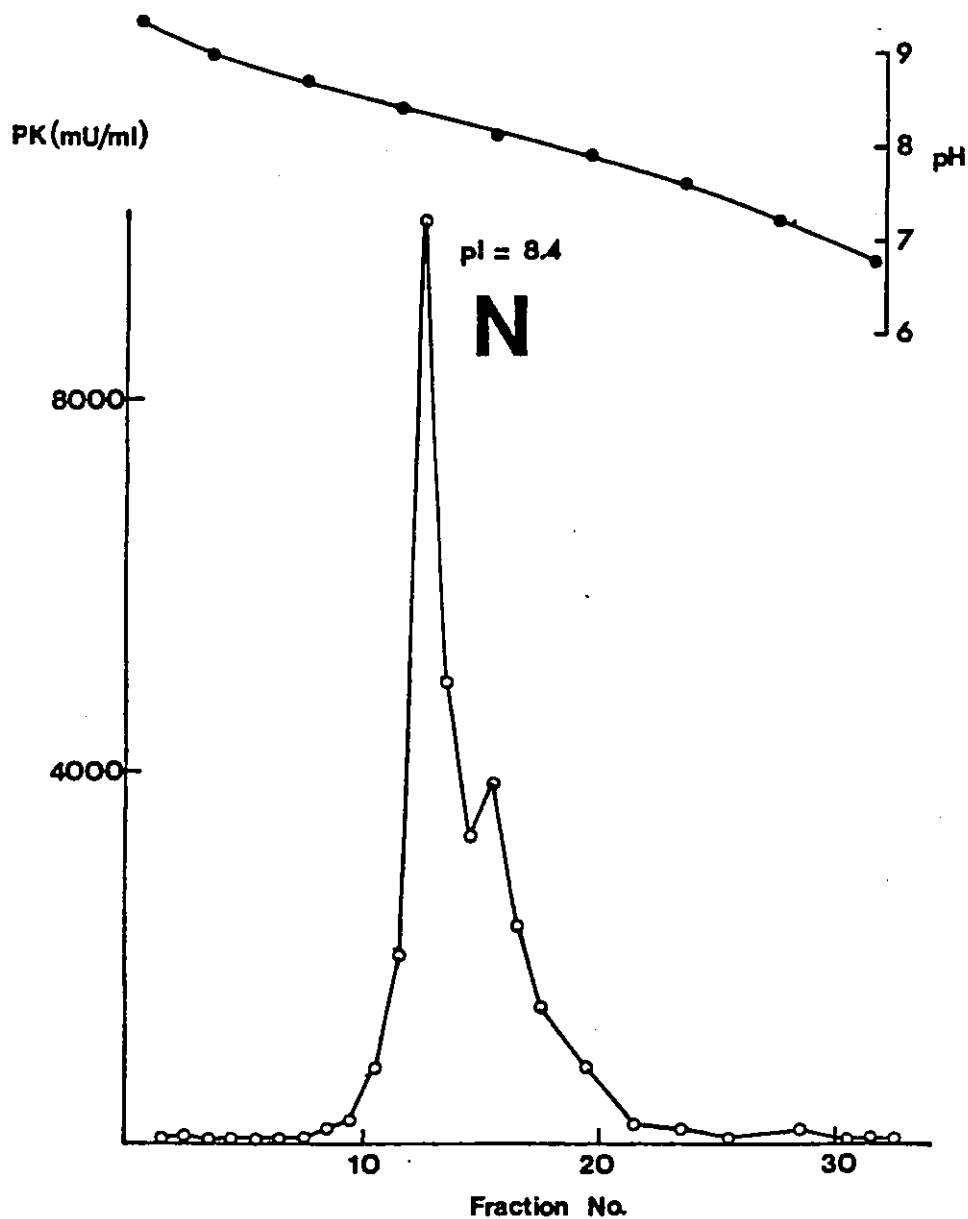


Fig. 6. Isoenzyme profile of pyruvate kinase (PK) from normal chicken pectoral muscle (40-day-old chicken). Isoelectric focussing, in the presence of protease inhibitors, was carried out as described under Materials and Methods, and the enzymatically active species measured (o). The pH gradient was measured after focussing (●).

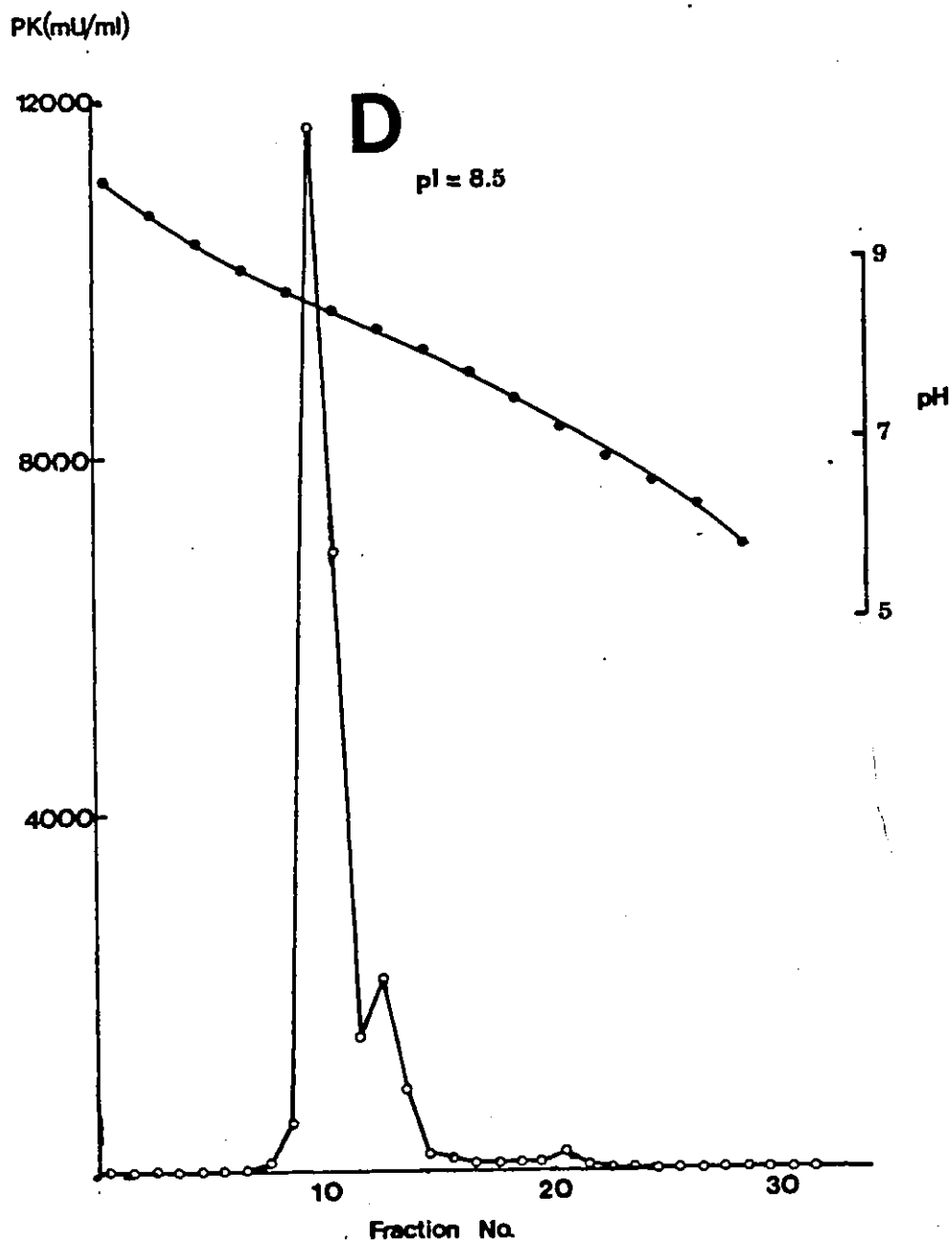


Fig. 7. Isoenzyme profile of pyruvate kinase (PK) from 40-day-old dystrophic chicken pectoral muscle. Isoelectric focussing, in the presence of protease inhibitors, was carried out as described under Materials and Methods, and the enzymatically active species measured (o). The pH gradient was measured after focussing (●).

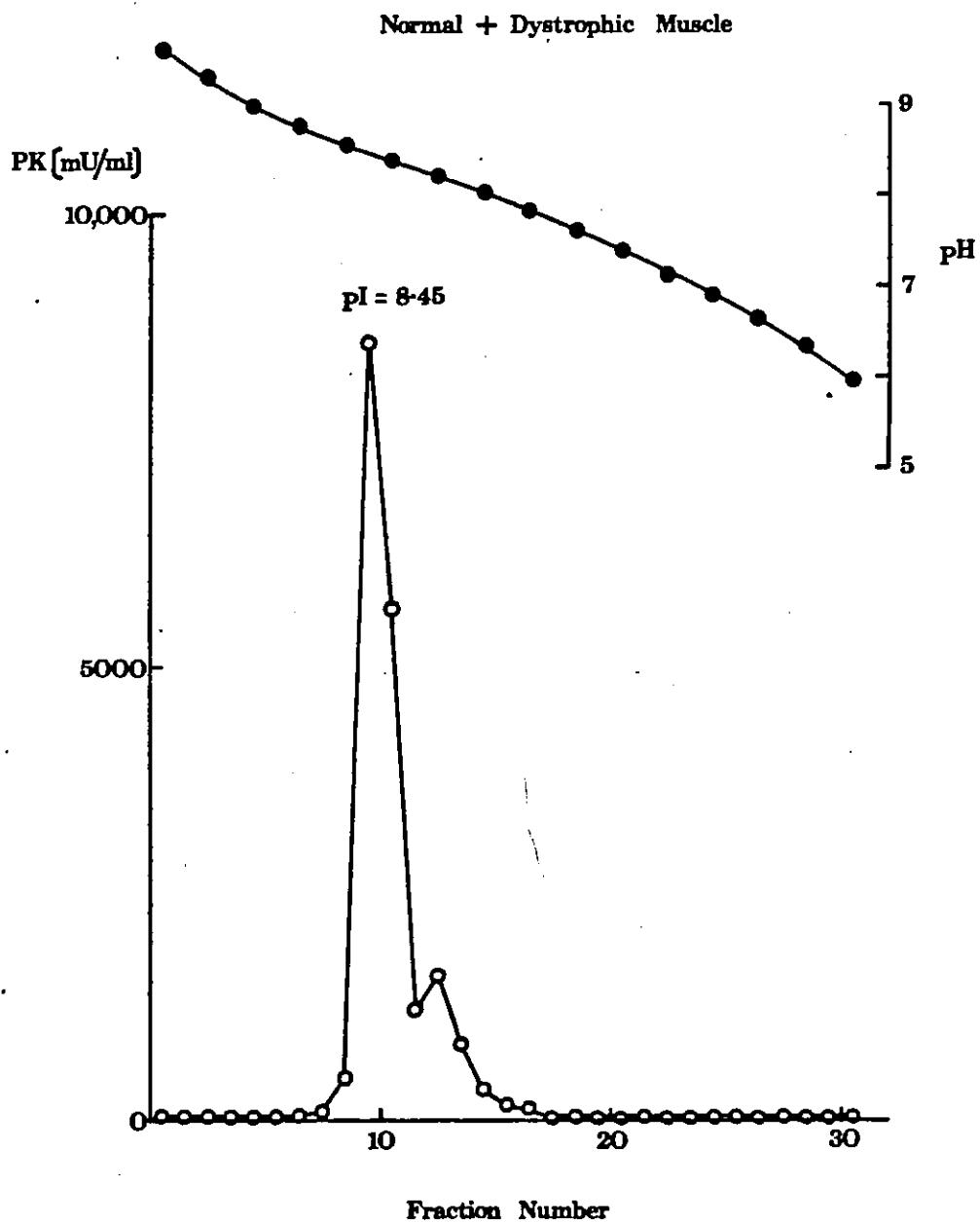


Fig. 8. Isoenzyme profile of pyruvate kinase (PK) from a mixed normal and dystrophic (60-day-old) pectoral muscle sample (in ratio 1:1 by total activity).

Isoelectric focussing, in the presence of protease inhibitors, was carried out as described under Materials and Methods, and the enzymatically active species measured (o). The pH gradient was measured after focussing (●). pI values at the peaks are shown.

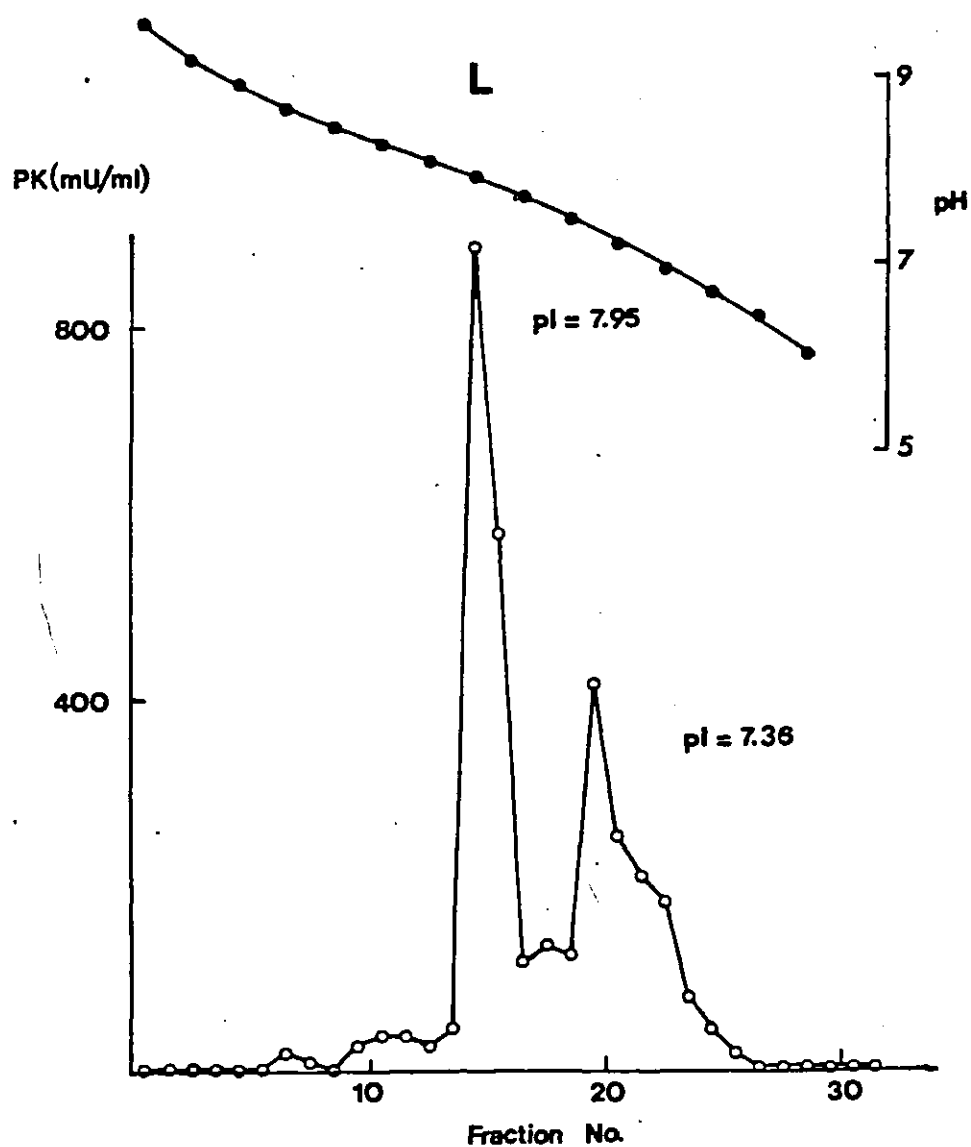


Fig. 9. Isoenzyme profile of normal liver (L) pyruvate kinase (PK) from chicken (60-day-old). The pI values of the two main peaks are shown.

Dystrophic Erythrocyte

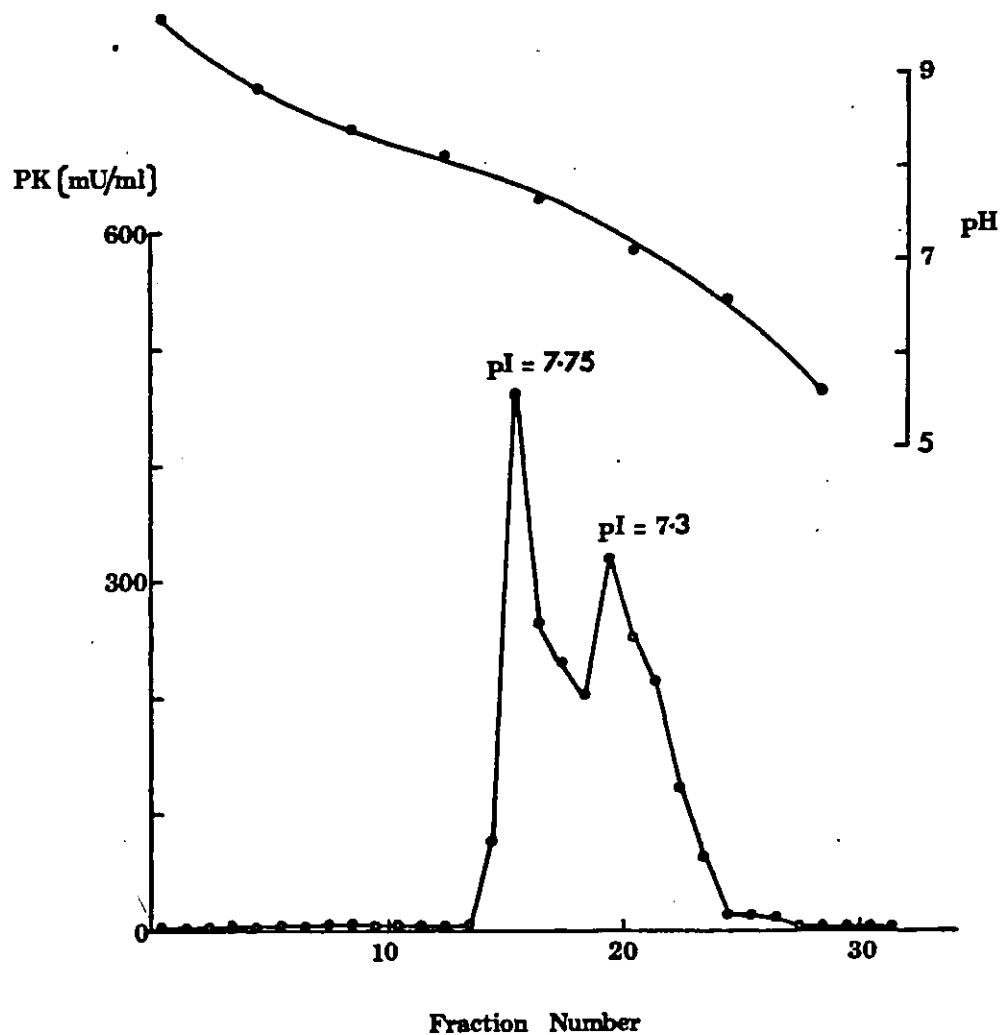


Fig. 10. Isoenzyme profile of erythrocyte pyruvate kinase (PK) from a dystrophic (35-day-old) chicken. The pI values of the two main peaks are shown.

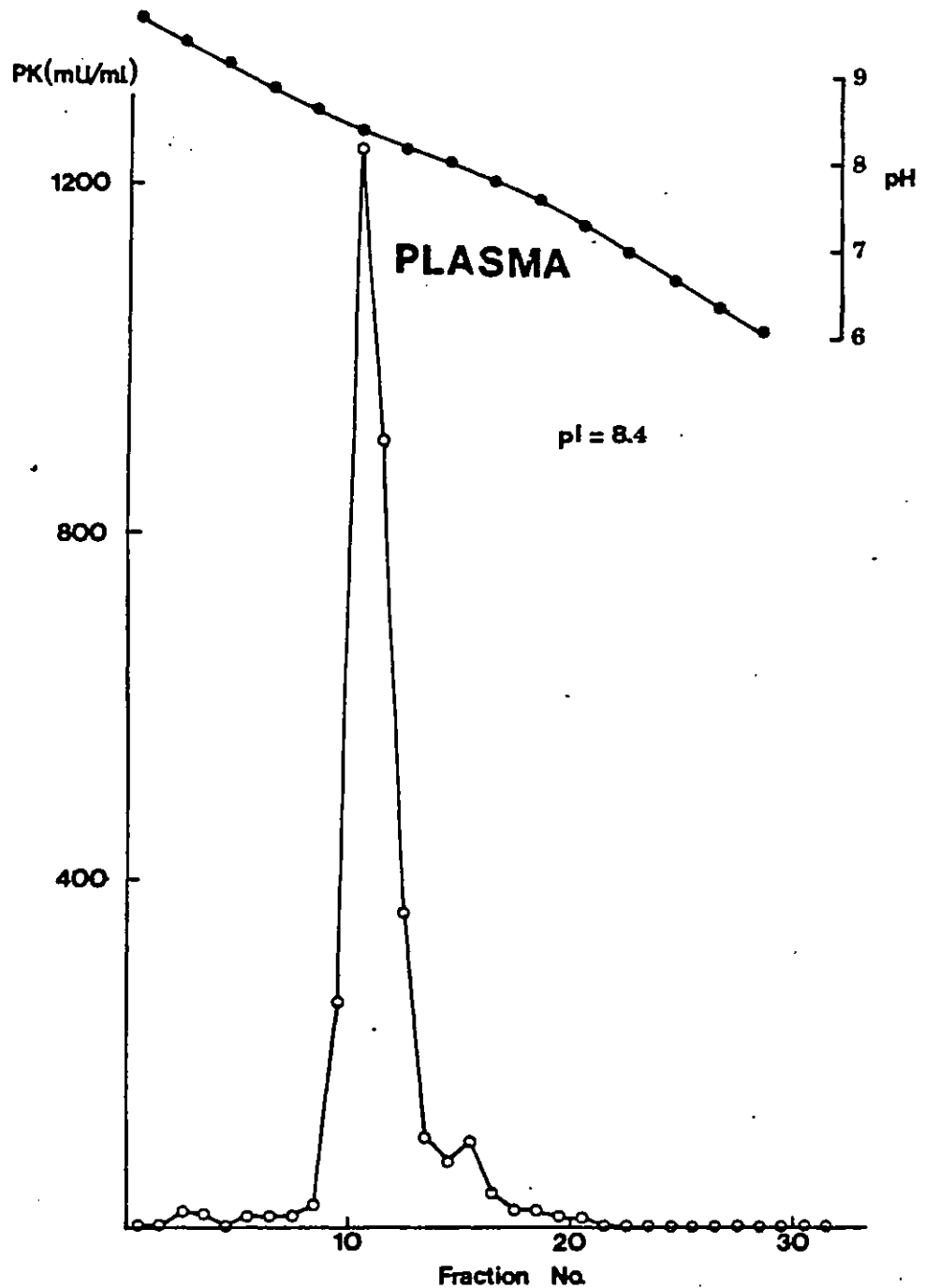


Fig. 11. Isoenzyme profile of plasma pyruvate kinase (PK) from a dystrophic (50-day-old) chicken. The peak has $pI = 8.40$. Another sample assayed similarly gave an identical profile, with $pI = 8.45$.

erythrocyte form is close in pI to the M_2 form but is a little more acidic, a situation recently recognised in mammals, too. The minor component of PK activity seen in chicken muscle may be the M_2 form or some other wild type form reported elsewhere (Strandholm et al. 1975).

When dystrophic chicken plasma was subjected to electrofocussing, a profile identical to that of muscle was obtained (Fig. 11), having a major peak of PK activity at pI = 8.4 (M_1) and a very minor peak at pI = 7.97 (M_2). From these studies, it is clear that the blood plasma and erythrocyte forms of PK are distinct (pI = 8.40 and 7.75), and contamination by PK originating in erythrocytes (which is low in activity anyway) did not occur in the plasma samples used in this study.

Discussion

Several interesting and important facts have emerged concerning the properties of blood plasma PK in both the dystrophic chicken and the dystrophic hamster. As the results show, very large increases in blood plasma PK activities were observed in both these dystrophic species, reaching about 30-fold the normal mean value in the chicken and 20-fold in the hamster. These increases are of the same order as the mean values found for humans affected with the Duchenne or Becker form of muscular dystrophy (2, 70, 219, 238). As in humans (238), the excess PK appearing in the chicken blood consists almost entirely of the M_1 type isoenzyme that is characteristic of skeletal muscle, with the M_2 isoenzyme found only in trace amounts. That the accumulation of this enzyme in chicken blood is associated with the onset of muscular disability is clearly shown in Fig. 4 where an inverse relationship between blood plasma PK activity and the flip number is demonstrated. In addition to these observations, the release of dystrophic muscle PK into the blood circulation parallel very closely the rate of release of another dystrophic marker, that of blood plasma CPK, as shown by the very high degree of association between the two enzyme activities in dystrophic chicken blood (Figs. 6a and 6b). Further, when the accumulation of PK in dystrophic chicken blood reaches a plateau (after about 60 days ex ovo in Line 413 birds), very marked histopathological changes can be seen in the dystrophic muscle (9). All these observations suggest, therefore, that blood plasma PK could be used as a marker of dystrophy in these animals and should also prove invaluable in the chemotherapeutic monitoring of

possible antidystrophic drugs (see chapter 6).

In contrast to the 30-fold increase in the activities of dystrophic plasma PK found in this study, a short communication by Weinstock et al. (220) showed a rise in serum PK in Connecticut-line dystrophic chickens to a maximum of only fivefold the normal mean value at 4 to 8 weeks. Moreover, the relative difference described in that report (220) between normal and dystrophic groups was greater for PK than for CPK in the serum, whereas in this study (Figs. 5a and 5b), even greater increases were observed for CPK. The very high dystrophic chicken plasma values observed in this study have now been found consistently in London line 413 birds (originated from Davis, California) for over three years (9, 10, 11, 120). It appears, therefore, that the Connecticut flock (New Hampshire/White Leghorns) of homozygous dystrophic chickens develops the enzyme increases above normal values much more slowly (and at a different ratio of CPK/PK) than does the Davis line 413 chickens. This and other such differences must be kept in mind when comparing other features in these birds. In addition, the PK values reported in the present study were measured at 25°C whereas those reported by Weinstock et al. (1977) and others were measured at 30°C, therefore, a correction factor of 41% (Table 2) would have to be added to the PK values measured in this study for comparison.

By evidence obtained from electrofocussing (Figs. 9 and 12), the PK isoenzyme found in the blood plasma of dystrophic chickens is of the M_1 type, the form that is characteristic of skeletal muscle. The liver and most

other tissues of the chicken, like mammals, contain other isoenzymatic forms such as M_2 and L (51) but in the heart muscle and brain, the M_1 isoenzyme also predominates (196). Therefore, the identification as M_1 does not in itself prove that the dystrophic blood PK comes from skeletal muscle, but does rule out many other tissues from consideration. Since there is no reason to implicate the brain or the heart as the source of PK release during the early stages of dystrophy, these two possibilities can also be ruled out. That the release of PK in chicken blood parallels the release of acetylcholinesterase (10, 226), an enzyme specifically elevated in the dystrophic skeletal muscles (226, 227), and also that of CPK, strongly suggests that the plasma PK does indeed arise from the dystrophic skeletal muscle. Further, the very substantial decrease in PK content in the dystrophic muscle on a per gram of wet tissue basis (Table 4) adds further support to the fact that plasma PK does arise from muscle and not from other tissues.

The findings of the present study also shows that the chicken red blood cell (both normal and dystrophic) possesses a distinct form of PK in addition to the M_2 form that is seen in other tissues (e.g. liver and kidney). This previously unidentified isoenzymatic form of PK, having a pI of around 7.7, corresponds to the R form of PK recognised in human (in man, an additional minor component is also observed) and rat erythrocytes (89, 103). In the human erythrocyte, Kahn et al. (1978) found the major R form has a 62,000 MW subunit replacing an 85,000

MW subunit of L PK from the liver, although there is some disagreement about the structure (89). In the chicken, therefore, at least three distinct forms of PK have been recognised previously (M_1 , M_2 and L), in addition to the new R form that is found in the present study. Their isoelectric points are 8.5 (M_1), 8.0 (M_2), 7.3 (L) and 7.7 (R). An earlier study by Eigenbrodt and Schoner (1977) recognised that the M_1 , M_2 and L forms of PK in the chicken had pI values of 8.5, 8.3 and 6.3 respectively. In the present study, protease inhibitors were used in the extraction of PK from solid tissues and may account for some of the differences that we see because in their absence (22, 51, 196) some modification of their primary structure might occur.

It is interesting that the dystrophic muscle in both the chicken (Figs. 7 and 8) and humans (Zatz et al. 1978) contains the same M_1 form of PK as normal muscle, and not the pattern of embryonic muscle (Strandholm et al. 1975). This contrasts with muscle CPK, where in human dystrophic muscle, additional isoenzymes such as the brain type (BB) and brain-muscle hybrid type (MB) which is normally only present in foetal muscle, were also found in addition to the skeletal muscle MM form (110, 172). This abnormal CPK isoenzyme pattern was also observed during the development of chick muscle (221, 194). However, in each case, the additional CPK isoenzymes in the dystrophic muscle were present in only minor proportions [e.g. approx. 3% of total in early chick breast muscle (Stewart et al. 1981) and 6-13% in mature dystrophic chicken breast muscle (194, 221)], and the normal MM type is still present as the

major CPK form in the dystrophic muscle (110, 188, 221, 194). This reversion towards the embryonic state in mature dystrophic chicken breast muscle also occurs with aldolase and lactate dehydrogenase (LDH) isoenzymes (Schapira and Dreyfus, 1977). These abnormal isoenzyme patterns might arise due to some limited form of regeneration that occurs in the diseased muscle fibres. Certainly, when normal muscle is provoked to regenerate either by injection of high concentrations of saline (Kitahara et al. 1977) or by denervation (see chapter 4), the normal isoenzyme pattern for some enzymes (e.g. CPK, LDH and glycogen phosphorylase) is disturbed and becomes accompanied by the appearance of minor amounts of embryonic or foetal-type isoenzymes. The PK isoenzyme pattern found in this study, together with reports on CPK (Stewart et al. 1981 and also chapter 4 of this thesis) and PK (see chapter 4) isoenzyme transitions during the development of chick embryonic breast muscle, argues strongly against the hypothesis proposed by some workers (Kuby et al. 1977, Schapira et al. 1978) that muscle differentiation during foetal or embryonic development may be impaired in some way in dystrophy.

The nature of the biochemical change in the muscle cell that results in the release of PK and certain other enzymes into the blood in both man and animals affected with muscular dystrophy is yet unclear. Certainly, a number of experimental conditions are known in which the efflux of certain enzymes into the blood can be mimicked or altered in some way. One well known example is when the energy content of a cell in the form of ATP is reduced

to a significant level and enzyme release results as a consequence (Thomson et al. 1975, Diederichs et al. 1979). The explanation put forward for the ATP-effect (Macknight and Leaf, 1979) is that the ion-flux set up across the cell membrane by the ATP-dependent Na^+ , K^+ -pump collapses on ATP starvation and the cell begins to accumulate mainly Na^+ , Cl^- and water. The cell swells until a critical point is reached where the cell membrane is stretched and sufficiently large lesions are produced through which enzymes and certain other molecules may diffuse along their concentration gradients. Evidence that muscle ATP in DMD is significantly reduced may add support for the above hypothesis on the mechanism of enzyme release from the pathological muscle (Vignos et al. 1963, Stengel-Rutkowski et al. 1973). Abnormal extracellular Ca^{2+} levels have also been shown to augment the release of enzymes from isolated mouse skeletal muscle (Soybel et al. 1978) but the way in which Ca^{2+} does this is unclear. Another situation that releases large quantities of enzymes (in this case, PK) into the blood is observed in vitamin E-deficient rats (Chow, 1975) which show some of the signs of dystrophy. Vitamin E is known to have antioxidant properties, thus protecting membrane lipids from peroxidation damage (Mc Cay et al. 1972). Serotonin may also have a role to play in the release of certain enzymes into the blood from the dystrophic or pathological muscle (see chapter 6). How all these differing aspects of enzyme release from muscle may be unified into a common underlying mechanism in dystrophy remains a challenging and exciting one and awaits further experimental evidence.

CHAPTER 3

BLOOD PLASMA PHOSPHOGLYCEROMUTASE AS A MARKER OF THE DYSTROPHIC CHICKEN MUSCLE

INTRODUCTION

In both humans and animals with a genetically determined form of muscular dystrophy, elevations of a number of muscle enzymes in the blood plasma are well known (Rowland 1976, Pennington 1980, Barnard et al. 1976, Liu et al. 1980, Hughes 1962, Giusti et al. 1967 and Heyck et al. 1966). Of these, only creatine phosphokinase (CPK) and pyruvate kinase (PK) are sensitive enough to serve as markers of dystrophic muscle. In the detection of female carriers of Duchenne muscular dystrophy, however, even the combined use of these two serum enzymes can only detect up to about 80% of all known carriers. In an effort to find a more sensitive marker for this disease and having received information regarding another possible enzyme marker of dystrophy (Professor E.A. Barnard, personal communications), my project student (Lucy Rodrigues) and I decided to investigate the enzyme phosphoglyceromutase (PGM) in chickens with hereditary muscular dystrophy.

Phosphoglyceromutase (2,3-diphospho-D-glycerate: 2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) is an enzyme found abundantly in skeletal muscle and catalyses the isomerization of 3-phosphoglyceric acid to 2-phosphoglyceric acid in the glycolytic pathway. PGM isolated from animal tissues also require 2,3-diphosphoglycerate as a cofactor. The importance of this enzyme is not limited only by its essential role in glycolysis but also to the part it has to play in controlling the level of 2,3-diphosphoglycerate which acts as a modulator for the binding of oxygen to haemoglobin in erythrocytes.

In the present study, changes in the activity of plasma PGM was investigated during the development of the dystrophic chicken and very large increases in its activity was observed. The isoenzyme pattern of dystrophic muscle PGM was also investigated and found to be abnormal when compared to normal muscle. Plasma PGM from young Duchenne boys showed only a modest elevation when compared to normal control values. These observations taken together, strongly indicate that plasma PGM is a marker of dystrophy in these animals although its use in the diagnosis of Duchenne muscular dystrophy needs to be further investigated.

MATERIALS AND METHODS

Animals. See Materials and Methods, chapter 2.

Blood Specimens. See Materials and Methods, chapter 2.

Fresh human plasma from Duchenne patients were kindly supplied by Professor V. Dubowitz, Jerry Lewis Muscle Research Centre, Hammersmith Hospital, London.

Tissues. In both embryos and adult chickens, the pectoralis major muscle was dissected at once after killing. The sample was weighed and homogenized in 0.1M $MgCl_2$, 0.001M EDTA, 0.01M Tris-HCl buffer (pH 7.4). After noting the total volume, the homogenate was centrifuged at 50,000 g for 20 min at 4°C in an MSE centrifuge. The supernatant was taken for enzyme assays and isoelectric focussing.

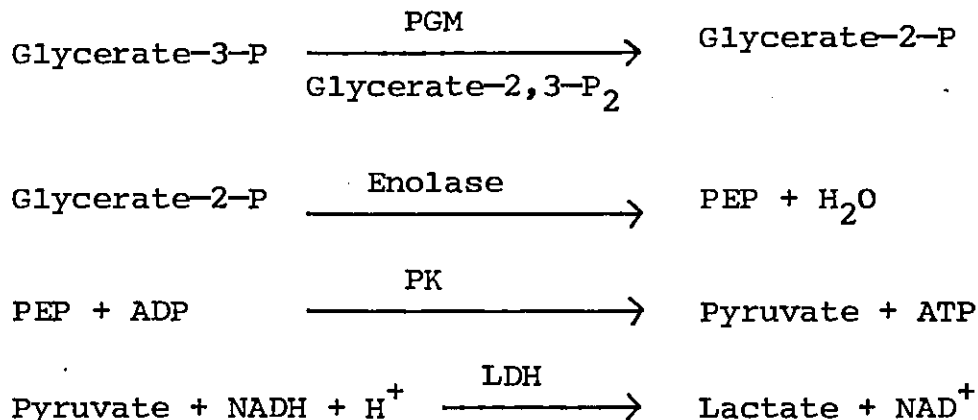
For chicken erythrocyte PGM, the red cells were pelleted from heparinized blood, washed twice in isotonic saline and left to haemolyse in cold deionized water of known volume for approximately 5 min. Both the leucocyte

layer and any remaining plasma were removed by the washing procedure. After centrifugation at 15,000 g for 5 min, the supernatant was used at once for PGM determination. More concentrated haemolysates were used for isoelectric focussing.

Chemicals. All chemical agents for the PGM assay and staining were from Sigma except the enzymes enolase and LDH which were obtained from Boehringer-Mannheim Corp. Enzyme kits for both PK and CPK assays were purchased from Boehringer-Mannheim Corp.

Assays. The Boehringer-mannheim modified version of the enolase-coupled assay of Grisolia (1957) was used for the U.V. spectrophotometric determination of PGM.

Principle of the assay:



PGM activity was measured by recording the decrease in $A_{340\text{nm}}$ due to NADH using the LKB 8600 (Mark II) kinetic rate analyzer, with a thermostated sample holder (pre-set at 25°C). The manual procedure as prescribed by Boehringer-Mannheim was adapted to the automated system by adding the Tris-HCl buffer (pH 7.4) containing Mg^{2+} ions, NADH, ADP, glycerate-2,3-P₂, LDH, PK and enolase to the sample and allowing the assay mixture to incubate at 25°C for

about 10 min. Addition of glycerate-3-P then initiated the PGM reaction, which was recorded directly.

Both PK and CPK activities were measured as described previously (see Materials and Methods, chapter 2).

Enzyme activities are expressed in International milliunits per millilitre plasma or extract under these standard conditions.

Staining for PGM Activity. The reaction mixture was of similar composition to that used in the assay procedure. It consisted of 15mls of 0.17M Tris-HCl buffer (pH 8) with the following additions: 15mg 3-phospho-D-glycerate, 8mg 2,3-diphospho-D-glycerate, 22.5 mg ADP (Na_2 salt), 15mg NADH (Na_2 salt), 60 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 30 μl enolase (400 U/ml), 25 μl PK (400 U/ml) and 100 μl LDH (200 U/ml). The staining mixture was applied over the entire gel surface in a final 1% agarose overlay and allowed to set. After incubation at 37°C for about 10 - 20 min, visualisation under long wave U.V. showed dark bands of PGM activity due to the loss of fluorescence as NADH is converted to NAD.

Isoelectric Focussing. The LKB 2117 Multiphor apparatus was used for this purpose. Electrofocussing was performed in a thin-layer polyacrylamide gel (8% acrylamide gel) containing 1% LKB Ampholine carrier ampholytes (pH 3.5 - 7.0). Sample volumes of between 5 to 25 μl were applied to the gel surface via filter paper strips. Horse myoglobin (pI 7.3 major, pI 7.0 minor) was used as an internal marker to indicate the completion of focussing. Constant circulation of ice-cooled water around the bed kept the running temperature at 0°C. Constant power (3W) was main-

tained for a focussing time of about 5h.

RESULTS

MEASUREMENT OF PGM ACTIVITIES

Using the Boehringer-Mannheim modified version of the enolase-coupled assay of Grisolia (1957) and adapting it to the LKB automated kinetic analyzer, the PGM assays of blood or tissue extracts became highly reproducible between duplicate assays. It was observed, however, that blood samples frozen overnight at -20°C showed a significant loss in enzyme activity. Addition of 9 mM glutathione to the blood samples had a variable effect on the enzyme activity and in view of this finding, all measurements on PGM activity reported subsequently in this study were made on fresh, unfrozen samples.

PLASMA PGM ACTIVITY IN DYSTROPHIC CHICK EMBRYOS

Reported in Table 7 are the mean plasma PGM levels in normal and dystrophic chick embryos from 14 days in ovo to 18 days in ovo. As one can observe, plasma PGM activities in normal and dystrophic chick embryos did not change appreciably during that period of time. Only at day 17 in ovo was there a significant difference in PGM activity between normal and dystrophic embryonic plasma. However, this difference may be due to contamination of the normal embryonic blood during sampling.

PLASMA PGM ACTIVITY IN DYSTROPHIC CHICKENS

Plasma PGM activities in normal chickens changed relatively little during the entire period investigated (Fig. 12). From day 11 to about day 40 ex ovo, there was an increase of about twofold in PGM activity (mean values

Table 7

Plasma PGM Activity in Chick Embryos expressed as mU/ml plasma

<u>Days in ovo</u>	<u>Normal</u>	<u>Dystrophic</u>	<u>Significance (P)</u>
14	* 183 $\pm$ 26(5)	* 232 $\pm$ 52(4)	N.S.
15	132 $\pm$ 16(4)	158 $\pm$ 20(5)	N.S.
16	205 $\pm$ 23(5)	208 $\pm$ 32(5)	N.S.
17	159 $\pm$ 34(5)	285 $\pm$ 37(7)	P < 0.05
18	211 $\pm$ 31(6)	269 $\pm$ 13(8)	P = 0.08

* Mean $\pm$ S.E.M., No. of embryos in parenthesis. Level of P accepted as being significant $\leq$ 0.05.

N.S. = NOT SIGNIFICANT

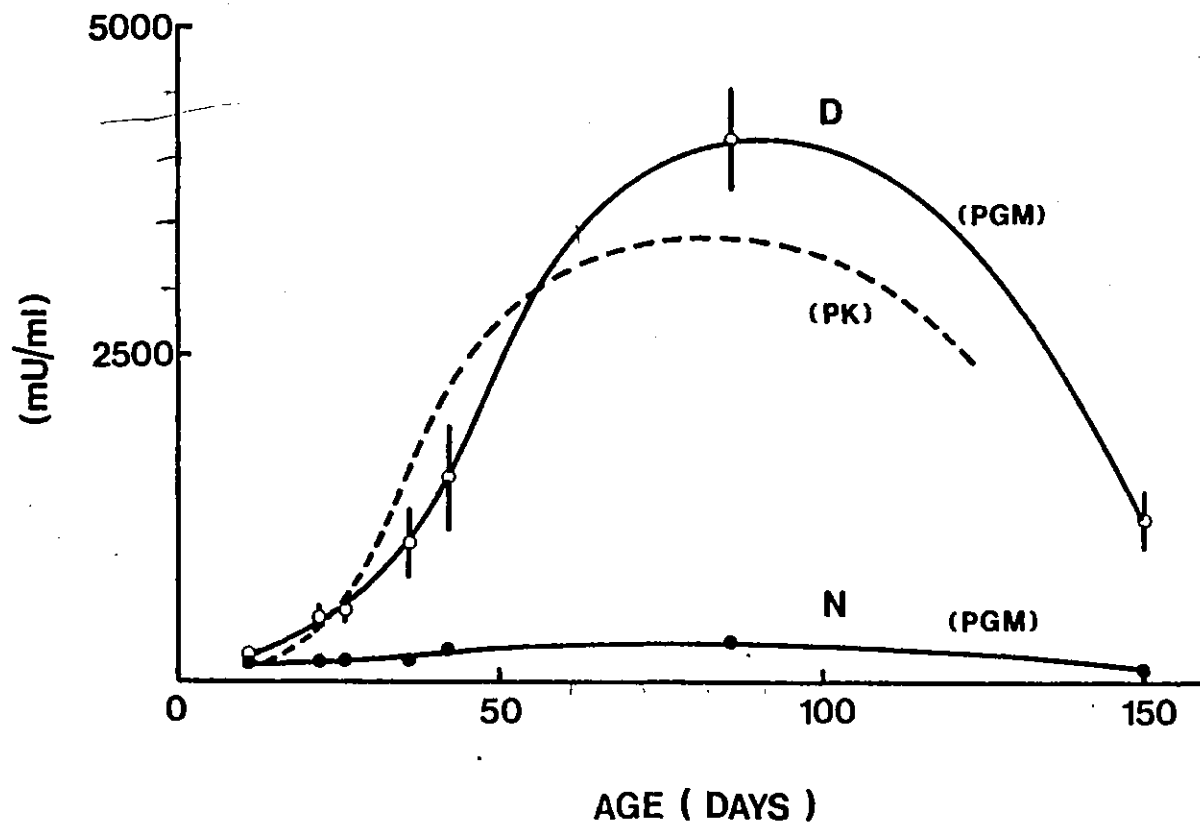


Fig. 12. Change of phosphoglyceromutase (PGM) activity with age in the chicken. The mean PGM activity in dystrophic chicken plasma (o), with its standard error, or in normal chicken plasma (●), is shown. The dotted curve line is the corresponding change in plasma PK activity in dystrophic chickens with age.

were 135 mU/ml plasma at day 11 to 257 mU/ml plasma at day 42) and remained at that level up to about 90 days ex ovo. After 150 days, the mean plasma PGM activity decreased to less than 100 mU/ml plasma. Variations within these normal chickens were so small that it was not possible to depict standard error bars to the points in Fig. 12.

Plasma PGM activities in dystrophic chickens, however, increased markedly from 208 mU/ml plasma after 11 days to around 4000 mU/ml plasma at 86 days (Fig. 12). This represents an increase of about 20-fold in the PGM activity during the maturation of these chickens. As shown in Fig. 12, a plateau was reached after about 80 days and then subsequently thereafter, the plasma PGM activity decreased dramatically and reached a mean value of about 1000 mU/ml plasma at 150 days. This decline in PGM activity presumably parallel the loss in muscle fibre content taking place at this stage and its subsequent replacement by fat and connective tissues.

MUSCLE PGM CONTENT IN NORMAL AND DYSTROPHIC CHICKENS

To find out whether the increased PGM activity observed in the dystrophic plasma is reflected in the PGM content of dystrophic muscle, the pectoralis major muscle of the chicken was chosen for this purpose. As reported in Table 8, muscle PGM activities in both normal and dystrophic chick embryos increased gradually from about 15,000 mU/g wet muscle at day 14 in ovo to about 100,000 mU/g wet muscle at day 4 ex ovo. Statistical analysis (Students 't' test) showed no difference in PGM content between normal

Table 8

Muscle PGM Content in Normal and Dystrophic Chickens

<u>Age(days)</u>	<u>Normal Pectoral</u>	<u>Dystrophic Pectoral</u>	<u>Probability(P)</u>
	<u>Muscle PGM Activity expressed as mU/g wet muscle</u>		
14d in ovo	* 15,002 $\pm$ 401 (5)	* 16,729 $\pm$ 819 (4)	N.S.
15d in ovo	21,214 $\pm$ 626 (5)	20,926 $\pm$ 1099 (5)	N.S.
16d in ovo	20,560 $\pm$ 427 (5)	22,250 $\pm$ 941 (5)	N.S.
17d in ovo	23,692 $\pm$ 713 (5)	23,982 $\pm$ 1158 (5)	N.S.
18d in ovo	31,580 $\pm$ 2995 (5)	34,487 $\pm$ 1680 (5)	N.S.
4d ex ovo	111,832 $\pm$ 21,363 (4)	89,934 $\pm$ 15,105 (4)	N.S.
61d ex ovo	652,677 $\pm$ 23,184 (4)	187,345 $\pm$ 28,009 (4)	P<0.001

* Mean $\pm$ standard error of the mean, no. of animals used in parenthesis.

N.S. = Not Statistically Significant (P>0.05)

and dystrophic chicken muscle during this period (i.e. $P > 0.05$ for all the ages tested). Muscle PGM content from a 61-day-old dystrophic chicken, however, showed a 71% decrease in its activity when compared to normal muscle of the same age (Table 8).

PGM ACTIVITY IN ERYTHROCYTES

In order to discount the possibility that erythrocytes are the source of PGM elevations in dystrophic plasma, the PGM activity was measured in both diseased and healthy erythrocytes. PGM activities in plasma were also measured for comparison. At the two ages investigated (Table 9), no significant difference in PGM activity was observed between normal and dystrophic erythrocytes (i.e. $P > 0.05$ for days 26 and 42). Table 9 also illustrates the fact that in the dystrophic birds there is no inverse correlation between the levels in plasma and those in erythrocytes. These findings suggest that the PGM in the dystrophic plasma does not arise from the erythrocytes present in the blood.

ISOENZYMES OF PGM IN NORMAL AND DYSTROPHIC CHICKENS

In humans, three isoenzymatic forms of PGM have been identified, each probably being determined by two gene loci, which have been designated PGM_M (muscle type) and PGM_B (brain type). The active form of the isozymes exist as dimers and polypeptide units derived from the two gene loci give rise to three possible combinations: BB, MB and MM. At pH 8.6, the BB isozyme migrates relatively more rapidly in gel electrophoresis towards the anode than the MM isoenzyme. The MB isozyme exhibits intermediate mobility. The isoenzymes are widely distributed and each displays a characteristic tissue specificity. In the development of

Table 9

Phosphoglyceromutase Activities in Chicken Erythrocytes

<u>Age(days)</u>	<u>Normal Erythrocytes</u>		<u>Dystrophic Erythrocytes</u>		<u>P</u>
	<u>PGM Activity expressed as mU/ml of whole blood</u>				
26d	2506	*(145)	1552	*(836)	
	1074	(116)	3581	(475)	
	931	(154)	1671	(406)	
	1050	(164)	1542	(475)	
	836	(145)	2029	(454)	
Mean $\pm$ SEM	1279 $\pm$ 309 (145 $\pm$ 8)		2077 $\pm$ 385 (530 $\pm$ 77)		P > 0.10
42d	4584	(241)	6685	(1074)	
	4016	(289)	5777	(2984)	
	4775	(270)	2865	(1194)	
	2627	(241)	2364	(716)	
	4298	(241)	3342	(2029)	
Mean $\pm$ SEM	4060 $\pm$ 380 (257 $\pm$ 10)		4207 $\pm$ 852 (1600 $\pm$ 406)		P > 0.10

* PGM activities in plasma (expressed as mU/ml plasma) are in parenthesis . Level of P accepted as being significant ≥ 0.05 .

human foetal muscle, there is a transition from the BB form via an MB hybrid to the MM adult form (Omenn and Cheung, 1974). In the chicken, however, the number and types of PGM isoenzyme present is very much less certain but in one report (James et al. 1972), as many as six isoenzymes have been observed in frozen breast muscle but with only two main types prevalent in fresh muscle.

To establish the origin of blood plasma PGM in the dystrophic chicken, isoenzymes of PGM in normal muscle, dystrophic muscle, dystrophic plasma and dystrophic erythrocytes were investigated by the method of isoelectric focussing. Using this high resolution system, at least six isoenzymatic forms of PGM were revealed in normal chicken pectoral muscle (Fig. 13) with three main forms focussing nearest the acidic end of the gel. For the ease of reporting, these three main forms have been designated as M1a, M1b and M1c as illustrated in Fig. 13. Of the remaining three minor forms, one coincided with the major form seen in brain extracts (Fig. 14) and is therefore presumed to be the brain type. Brain extracts as shown in Fig. 14 also contain small amounts of the muscle-type M1a. Focussing of dystrophic muscle, however, gave four and sometimes five different isoenzymes of PGM due to the variable appearance of the brain type (Figs. 13 and 14); they all coincided with the forms seen in normal muscle. The most acidic form (M1c) seen in dystrophic muscle seems to be present in much reduced amounts when compared to the same form in normal muscle (Figs. 13 and 14). To confirm that the different isoenzymes of PGM in muscle are truly in-

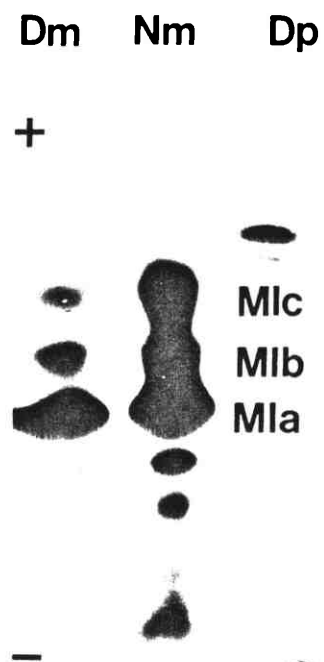


Fig.13 Isoenzymes of PGM from 8-week-old chickens. Dystrophic pectoral muscle (Dm), normal pectoral muscle (Nm) and dystrophic plasma (Dp) were electrofocussed for about 5-hrs in a pH 3.5 - 7.0 ampholine gradient.

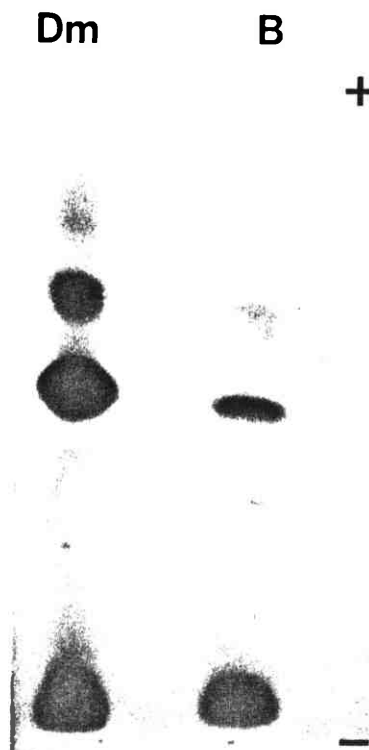


Fig.14 Isoenzymes of muscle and brain PGM from 30-day-old chickens. Dm - dystrophic pectoral muscle. B - brain.



Fig.15 Isoenzymes of blood PGM from dystrophic chickens. rbc - red blood cells from 31-day-old chickens. Dp - dystrophic plasma from a 6-week-old chicken.

trinsic to muscle and not due to contamination by erythrocytes, both perfused and unperfused normal muscle were subjected to isoelectric focussing. Although not included in this report, the two samples gave identical isoenzyme patterns.

Focussing of dystrophic plasma (Fig. 13) gave essentially one main form which is slightly more acidic than either of the muscle MIC forms. One of the three very weak bands that accompanied the main component of PGM activity did, however, coincided with the muscle MIC form. When this experiment was repeated, the same pattern emerged for both muscle and dystrophic plasma.

To further discount the possibility that PGM in dystrophic plasma may originate from the erythrocytes, both dystrophic plasma and an extract of red cell haemolysate were electrofocussed together in one gel and the findings are shown in Fig. 15. As illustrated, the isoenzymes present in both plasma and erythrocytes are clearly different and it is highly unlikely that the PGM circulating in the dystrophic chicken could have arisen from the erythrocytes. It is also of interest to note that erythrocytes contain as one of its main components of PGM activity the MIa form seen in the muscle.

PLASMA PGM ACTIVITY IN DUCHENNE HUMANS

Following the novel finding that dystrophic chicken plasma contains highly elevated levels of PGM activity, it was of interest to extend these investigations to the human Duchenne form of muscular dystrophy. Due to the limited

Table 10

Plasma PGM and PK Activities in Normal and Duchenne Humans

<u>Status</u>	<u>Age(Years)</u>	<u>PGM Activity(mU/ml)</u>	<u>PK Activity(mU/ml)</u>
D.M.D.	13	21	76
	14	26	258
	11	26	52
	8	128	252
	7	57	624
	9	19	118
	13	18	72
		<u>*42 ± 15</u>	<u>*207 ± 76</u>
Controls	11	25	15
	24	8	20
	26	10	—
	24	8	—
	23	6	—
		<u>*11.4 ± 3.4</u>	<u>17.5</u>

* Mean ± Standard Error. Using Students 't' test, no statistical difference between dystrophic and normal plasma PGM activity was observed ($P > 0.10$). Level of P accepted as being significant ≤ 0.05 .

supply of blood specimens from Duchenne patients, only a small group of dystrophic humans were investigated. When considered as a group, the mean plasma PGM activity in Duchenne patients was found to be elevated almost four-fold compared to the mean value observed in the normal control group (Table 10). On statistical analysis, however, no significant difference between the mean values of the two groups could be observed ($P > 0.05$). The negative statistical finding is most probably attributed to the large range in PGM values observed in the Duchenne group. On examining the individual PGM values in normal and dystrophic humans, there does appear to be an age-related decrease in its activity in the blood plasma of both groups. Further, the mean PGM activity observed in the normal control group would be expected to be higher if the average age of this group was the same as the Duchenne group.

For a comparison, plasma pyruvate kinase (PK) activities are also shown for the same group of Duchenne boys reported in Table 10. When the group as a whole was considered on this occasion, an increase in the mean plasma PK activity of about 12-fold was observed when compared to the mean value of the two normal controls. Like PGM, plasma PK in Duchenne humans also show an age-related decrease in its activity in blood as has been reported previously.

Discussion

Very large increases in the levels of blood plasma PGM activities have been observed to occur during the maturation of dystrophic chickens (Fig. 13). These increases reached about 20-fold the normal mean value after about 80 days ex ovo and are less than the 30-fold increase reported for plasma PK (Liu et al. 1980, see also chapter 2) and much less than the 100-fold increase reported for plasma CPK (Barnard, 1980). These findings show for the first time that plasma PGM activities are abnormally elevated in chickens with muscular dystrophy. No studies to date on blood plasma PGM levels have likewise been reported in dystrophic humans. The appearance of this enzyme in dystrophic chicken blood also parallel closely the increase in activity of plasma PK (Fig. 13), and hence that of plasma CPK also (chapter 2). Since the latter two plasma enzymes are markers of the dystrophic muscle (Liu et al. 1980, Barnard 1980), these findings on plasma PGM activity in the dystrophic chicken suggest it too can serve as a marker of dystrophy in these animals, although its sensitivity is less than that observed for either PK or CPK.

Several observations from this study suggest that the PGM activity appearing in dystrophic chicken blood is of muscle origin. First, the very high PGM activity found in the dystrophic plasma is associated with a concomitant decrease in activity of this enzyme in the dystrophic muscle (Table 8). This is a common observation for enzymes that are elevated in the blood plasma of both dystrophic humans and animals (Dreyfus and Schapira 1962, Weinstock et al. 1977, Stewart et al. 1981, see also chapter 2). Second, although the PGM activity

in erythrocytes are relatively high compared to normal plasma. No evidence was obtained to suggest that the enzyme was leaking out of the cells in any significant amount in the dystrophic animals (Table 9). Further, isoenzyme studies on dystrophic plasma PGM showed that these isoenzyme forms are very different from those seen in erythrocytes (Fig. 16) or, in fact, the brain (c.f. Figs. 14 and 15). Although the main band of PGM activity in dystrophic plasma does not correspond to any of the main muscle forms, one of its minor bands did, however, coincide with the muscle M1c form (Fig. 14). One likely explanation for this observation is that the PGM isoenzymes found in the dystrophic plasma were of muscle origin, but once released into the dystrophic blood, they undergo some form of proteolytic modification and become more acidic in character than the original muscle M1c form. The isoenzyme studies does not in itself reveal the tissue of origin of the plasma PGM, but does rule out the erythrocytes as a possible source of release. However, the concomitant appearance with PK and CPK leads one to conclude that it does indeed arise from the dystrophic muscle.

It is interesting that the dystrophic chicken pectoral muscle should contain less isoenzyme forms of PGM than the corresponding normal muscle as shown in Figs. 14 and 15. For a number of other sarcoplasmic enzymes like CPK, LDH and aldolase, the reverse pattern of isoenzyme composition holds true where additional isoenzyme forms are usually present in the dystrophic muscle (Schapira et al. 1977, Kuby et al. 1977, Cao et al. 1971, Tzvetanova 1971). Whether all the different isoenzyme forms that were observed in both normal and dystrophic pectoral muscle are true representatives of the native

PGM isoenzymes of the respective muscles is unclear at the present moment. In contrast to the results obtained here with fresh chicken pectoral muscle, an earlier study by James et al. (1972) showed that freshly killed chicken breast muscle contained only two isoenzyme forms of PGM. When they repeated their studies using frozen chicken breast muscle, however, they also found six isoenzyme forms present. To account for this rather unusual finding, they suggested that the extra forms seen in frozen muscle may be due to autolysis of the two native forms present in fresh muscle. However, a more likely explanation for the discrepancy in results between this study and that of James et al. (1972) is that some of the isoenzyme forms in muscle may be phosphorylated forms of the native isoenzymes. Evidence for the existence of phosphorylated forms of PGM have been observed in human erythrocytes (Hass and Miller, 1978). Thus, the observed difference in PGM isoenzyme composition between normal and dystrophic pectoral muscle reported earlier may be a reflection of the greater degree of phosphorylation of the native isoenzymes in the normal muscle.

The parallel release of PGM, PK and CPK from the dystrophic muscle into the blood plasma suggests that the mechanism of their release is similar. If the efflux of enzymes and other macromolecules from muscle depended on diffusion alone, then one would expect that amongst other things, the size and shape of the enzyme molecule in question would affect its rate of release. Although the enzyme activities of CPK (MW 70,000) and PGM (MW 60,000) in dystrophic chicken plasma are higher than PK (MW 215,000), their relative amounts in the blood do not appear to correlate with the size of these macromolecules. In addition, LDH which has a MW of 140,000 is considerably

smaller than the PK molecule but is only slightly elevated in dystrophic chicken blood. Further, low molecular weight molecules like amino acids which one might expect to find elevated in dystrophic blood does not normally occur. Thus, there appears to be no evidence to suggest that the rate of enzyme release from the dystrophic muscle is correlated to the molecular weight of the molecule. Apart from molecular weight, another important factor to consider is the concentration of these enzymes in muscle relative to plasma. For PGM, this ratio was calculated to be around 54 at 61 days ex ovo whereas that observed for PK was calculated to be about 43 at the same age. Thus, it appears that PGM is being released at a slightly greater rate than PK from the pathological muscle and this is consistent with the slightly higher activity seen for PGM in dystrophic chicken plasma. Unfortunately, the CPK content in dystrophic chicken muscle in Line 413 birds has not been measured and therefore the muscle to plasma activity ratio for this enzyme is not known. Other factors such as enzyme retention due to intracellular binding, their rate of synthesis in muscle, rate of enzyme inactivation or its rate of clearance from the dystrophic plasma may all affect the levels of a particular enzyme in the dystrophic blood.

Compared with CPK or PK, preliminary results showing plasma PGM activities in dystrophic humans are not very encouraging (Table 10). Although the mean plasma PGM activity in Duchenne boys was found to be almost four-fold elevated when compared to normal control values, the difference between the two groups was not found to be statistically significant. When the effect of age is taken into account, the difference

between normal and Duchenne is even less convincing. However, further and more extensive studies need to be performed in younger Duchenne boys using age-matched controls so that the value of this enzyme marker in the detection of DMD may be ascertained. In the dystrophic chicken at least, plasma PGM has been shown to be a sensitive marker of the dystrophic muscle.

CHAPTER 4

ISOENZYME CHANGES IN THE PATHOGENESIS OF MUSCULAR DYSTROPHY

INTRODUCTION

Besides the elevation of a number of skeletal muscle enzymes in the serum of Duchenne humans, several groups of investigators have also reported the appearance of foetal-like forms of some of these enzymes in both the muscle and serum of patients with Duchenne and related forms of muscular dystrophy. This is true for creatine phosphokinase (CPK) where the presence of either the BB or MB (or sometimes both) isoenzyme was detected in addition to the adult muscle form MM in both the serum and muscle of Duchenne patients (Kuby et al. 1977, Cao et al. 1971, Tzvetanova 1971, and Somer et al. 1973). However, when the isoenzymes of muscle lactate dehydrogenase (LDH) were investigated in dystrophic humans, the adult form that is normally present in healthy muscle (LDH₅) was found to be absent in the myopathic muscle (Schapira et al. 1977 and Emery 1964). In addition, the isoenzyme pattern of the myopathic muscle was found to be identical to that observed in foetal muscle.

When animals with hereditary muscular dystrophy were also examined, similar abnormal appearances of foetal or embryonic-like isoenzymes appeared in the muscle for a number of enzymes. In the pectoralis major muscle of dystrophic chickens, Schapira and associates (1977) observed the presence of embryonic isoenzymes of aldolase and LDH that were not present in normal muscle. In addition, Kitahara and associates (1977) detected the presence of non-muscle foetal type isoenzymes of CPK and phosphorylase in the muscle of dystrophic mice.

The presence of these foetal isoenzymes in the muscle of dystrophic humans like that observed in dystrophic animals has led to the suggestion by some investigators that the differentiation of foetal muscle in Duchenne patients may be impaired in some way. To test this hypothesis, a study was made of the isoenzyme changes of CPK, LDH and pyruvate kinase (PK) during the development of normal and dystrophic chicken muscle. It is hoped that this study will help to answer two important questions concerning the pathogenesis of dystrophic muscle both in the human and animal forms of muscular dystrophy and these are:

(1) Are there manifestations of the pathogenesis in the embryonic muscle?

(2) Is there a genetic persistence of the embryonic isoenzymes present in mature dystrophic muscle because the differentiation of embryonic muscle is incomplete?

In addition to the studies mentioned above, the isoenzymes of LDH and PK were investigated in both denervated and innervated muscles of a normal chicken. It is hoped that this latter part of the study will also help to answer the question regarding whether the presence of foetal-like forms of CPK, LDH and other enzymes in the muscle of dystrophic humans are due to a genetic defect in the mechanism of differentiation of human foetal muscle or result from the partial regeneration of atrophic muscle fibres occurring in the mature dystrophic muscle.

MATERIALS AND METHODS

Animals. As described above (Materials and Methods, chapter 2).

Blood Specimens. Blood samples were collected and processed as described previously.

Chemicals. Ultrodex powder (evaporation limit 35%) and ampholine carrier ampholytes were obtained from LKB chemicals Division, South Croydon, Surrey.

NN'-Methylenebisacrylamide, acrylamide, ammonium persulphate and TEMED (NNN'N'-Tetramethylethylenediamine) were obtained from BDH Laboratory Reagents Ltd., Poole, Dorset.

PEP (tricyclohexylammonium salt), ADP (Na_2 salt), NADH (Na_2 salt), LDH (200mU/ml), CPK Test Combination kits (U.V. method), PK Test Combination kits (U.V. method) and LDH Test Combination kits (U.V. method) were all obtained from Boehringer-Mannheim Corporation, Crawley, Sussex.

Sodium pyruvate, agarose powder (type 1), myoglobin from equine skeletal muscle (type I), benzamidine hydrochloride, bacitracin, soybean trypsin inhibitor, phenyl-methylsulphonyl fluoride (PMSF) and bovine catalase were all obtained from Sigma Chemicals, Poole, Dorset.

Tri-(hydroxymethyl)-methylamine Tri Buffer, KCl, NaCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, sucrose, and EGTA (ethyleneglycol-bis(β -amino-ethylether)-N,N'-tetraacetic acid) were all obtained from Fissions Ltd., Loughborough, Leics.

Tissues. Tissues were removed and processed as previously described for PK (Materials and Methods, chapter 2).

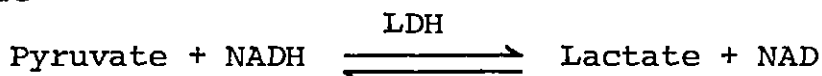
Assays. CPK activity was measured as described previously (Materials and Methods, chapter 2).

Quantitative Isoelectric Focussing. The procedure for flat-bed electrofocussing on an Ultrodex gel was as that described previously (Materials and Methods, chapter 2). For electrofocussing of CPK isoenzymes, ampholines having a pH range of between 3.5 - 7 or 4 - 8 were used. Horse myoglobin (Main component with pI = 7.3) and bovine catalase (pI = 5.5) were used as internal reference points.

Isoelectric Focussing on Polyacrylamide. Isoelectric focussing on polyacrylamide was as described in Materials and Methods, chapter 3. For both PK and LDH, an 8% polyacrylamide gel containing 1% LKB Ampholine carrier ampholytes (pH 5 - 9) was used. Horse muscle myoglobin was used as an internal marker to indicate the completion of focussing. Constant power (3W) was maintained for a focussing time of about 4 - 5h.

Staining for LDH Activity.

Principle:



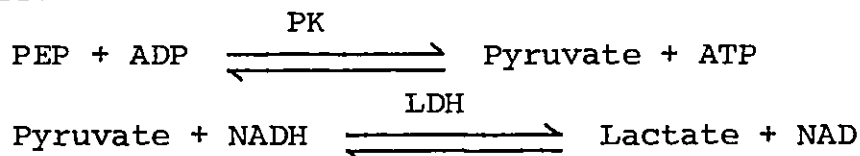
Under long wavelength U.V. light, NADH fluoresces and NAD does not.

The reaction mixture consisted of 30mls of 0.05M Tris-HCl buffer (pH 8.0) with the following additions: 20mg NADH (Na₂ salt) and 200mg sodium pyruvate. The staining mixture was applied over the entire gel surface in a final 1% agarose overlay and allowed to set. After incubation at 37°C for about 10 - 20 min, visualization under

long wave U.V. showed dark bands of LDH activity due to the loss of fluorescence as NADH is converted to NAD.

Staining for PK Activity.

Principle:



Under long wavelength U.V. light, NADH fluoresces whereas NAD does not.

The reaction mixture consisted of 16mls of 0.5M Tri-HCl buffer (pH 7.4) with the following additions: 30mg phosphoenolpyruvate (trihexylammonium salt), 60mg ADP (Na_2 salt), 20mg NADH (Na_2 salt), 80mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 80mg KCl and 125 μl LDH (200U/ml). The reaction mixture was applied over the entire gel as described above for LDH. Under long wave U.V. light, dark bands of PK activity are seen due to the loss of fluorescence as NADH is converted to NAD.

Separate control gels for PK and LDH staining using the above procedures were also carried out in the absence of their respective substrates (PEP in the case of PK and sodium pyruvate in the case of LDH) and no activity could be observed in either case. Thus the bands seen under long-wave U.V. were specific for either PK or LDH.

RESULTS

ANALYSIS OF CPK ISOENZYMES DURING THE DEVELOPMENT OF CHICKEN DYSTROPHY

Three distinct isoenzymatic forms of CPK have been identified by immunological, chromatographic and electrophoretic techniques, each made up from two polypeptide subunits and designated as MM, MB and BB. The MM isoenzyme is the only form seen in mature skeletal muscle whereas the BB isoenzyme is the predominant form seen in the brain. The MB hybrid form is usually present only in foetal or embryonic muscle and disappears after muscle differentiation. Therefore, during foetal or embryonic muscle development, a complete changeover of the CPK isoenzymes takes place in the direction : BB $\rightarrow$ MB $\rightarrow$ MM.

To analyse quantitatively the isoenzyme transition of CPK during muscle development in the dystrophic chicken, the isoenzyme changes in the pectoralis major muscles of both normal and dystrophic chickens were investigated from 11 days in ovo to about 9 months ex ovo by the method of isoelectric focussing on a flat-bed of Ultrodex.

At day 11 in ovo, both normal and dystrophic embryonic muscle gave one major peak of CPK activity (BB isoenzyme) which had a pI of 5.35 and also a very minor peak (MB isoenzyme) having a pI of 5.80 (Figs. 16a and 16b). At this stage of development, normal muscle also contain trace amounts of MM activity which is absent in the dystrophic muscle; the MM peak being more basic than either of the BB or MB peaks, having a pI of about 6.4. By 12 days in ovo, a dramatic increase in the synthesis of the MM and MB

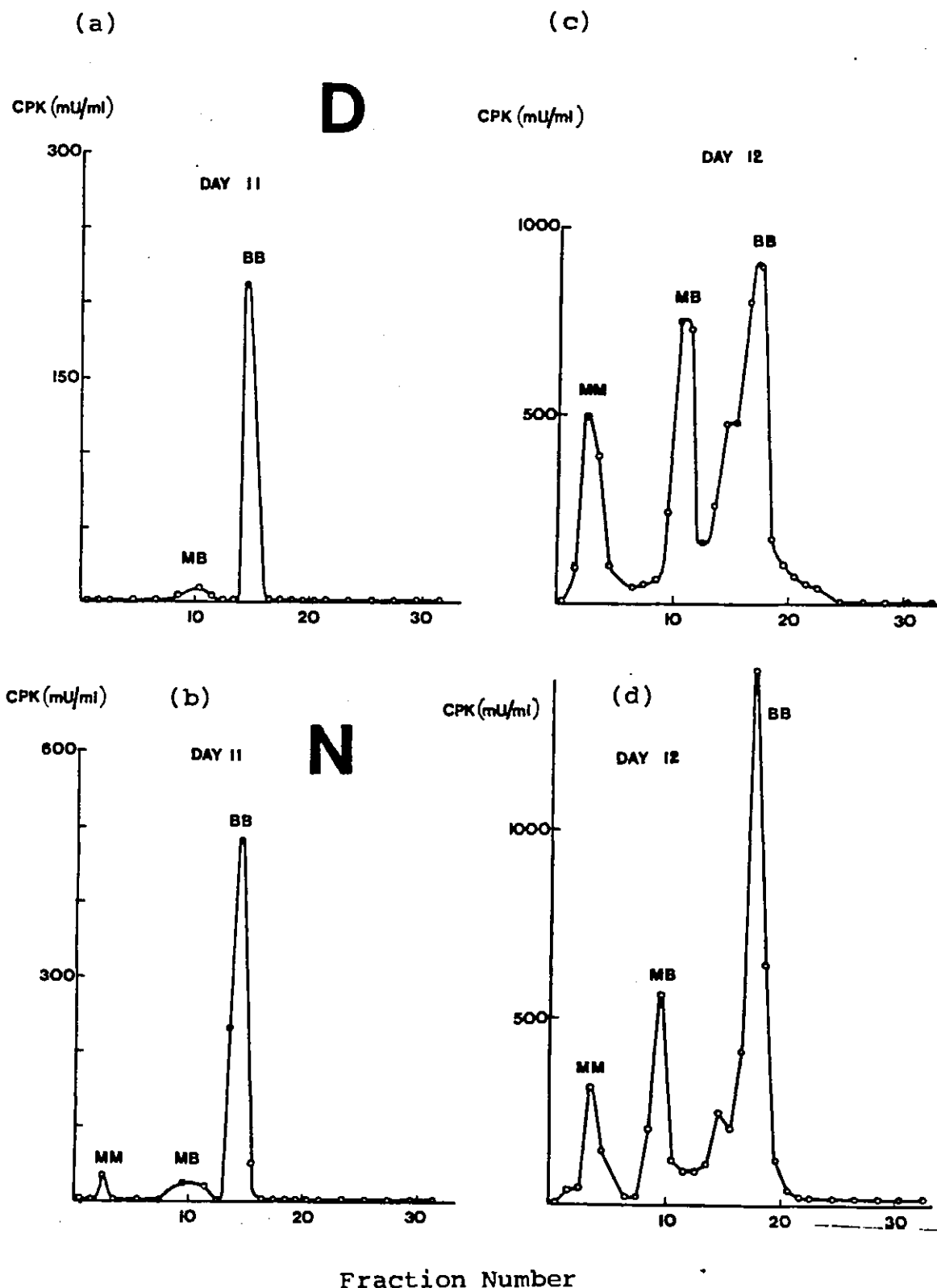


Fig. 16. Isoenzyme profiles of embryonic muscle creatine phosphokinase (CPK) activity in normal and dystrophic chick embryos. D - dystrophic, N - normal. Number of days in ovo is shown. Ampholines having a pH range of 5 - 7 were used.

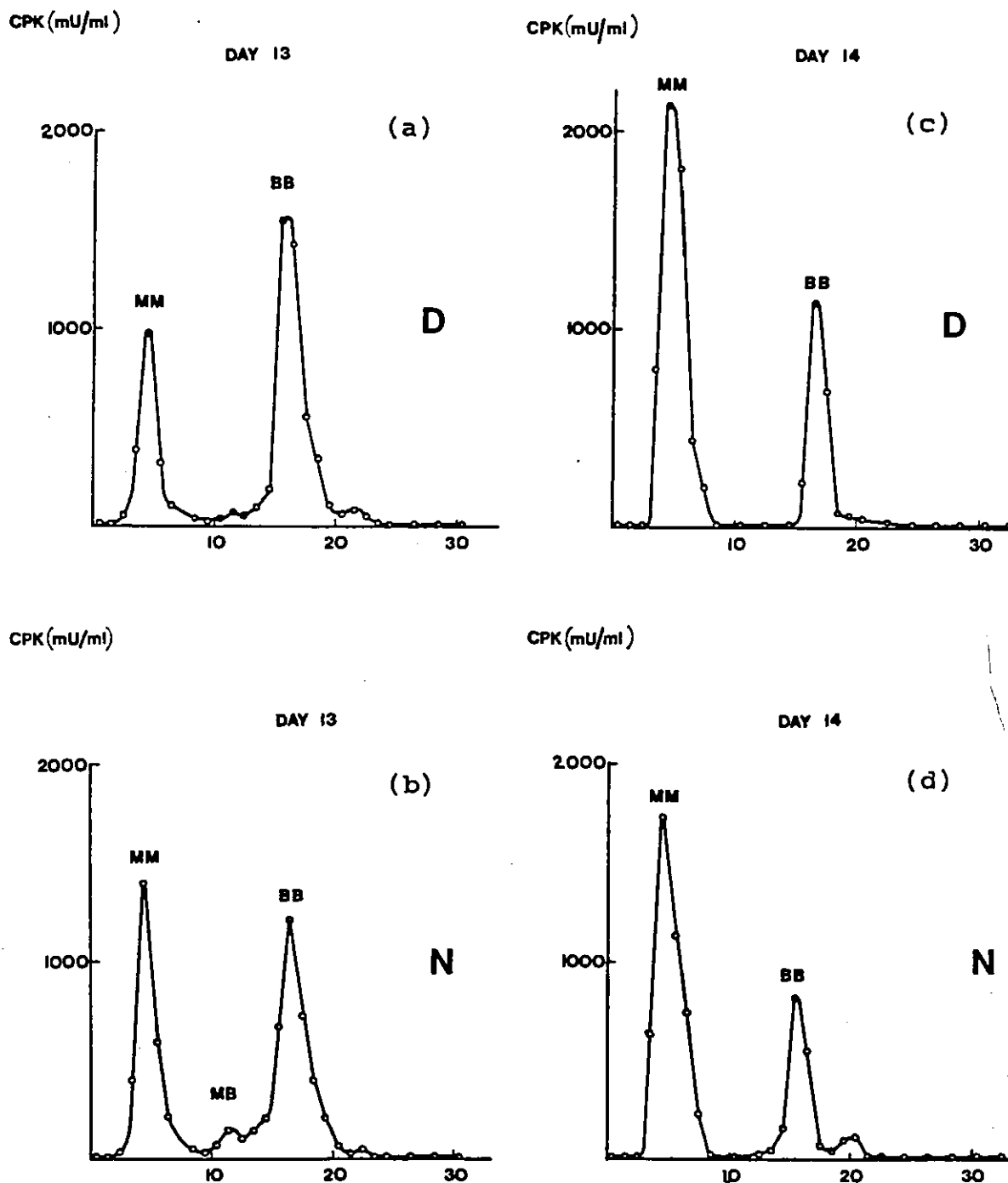


Fig. 17. Isoenzyme profiles of embryonic muscle creatine phosphokinase (CPK) activity in normal and dystrophic chick embryos. D - dystrophic. N - normal. Number of days in ovo is shown. pH 5 - 7 ampholines were used.

isoenzymes was observed in both normal and dystrophic chick embryonic muscle (Figs. 16c and 16d), with the BB form still predominating in both cases. After 13 days in ovo, the synthesis of the MB form in both normal and dystrophic embryonic chick muscle had declined markedly and the two major forms that were observed in the two different muscles were the MM adult muscle form and the BB brain form (Figs. 17a and 17b). At 14 days in ovo, the MB isoenzyme was completely absent in normal and dystrophic embryonic muscle (Figs. 17c and 17d) and the major component of CPK activity found in both cases was the MM form. However, both normal and dystrophic chick embryonic muscle still contained sizeable amounts of the BB isoenzyme at this stage. From 15 to 18 days in ovo, a rapid increase in the synthesis of the MM isoenzyme occurs (Figs. 18a+b and 19) with a corresponding decrease in the synthesis of the BB form. By two days ex ovo, the only isoenzyme seen in both normal and dystrophic chick muscle was the MM form with the BB form essentially absent (Fig. 20). Further maturation of normal and dystrophic chick muscle from 2 days ex ovo to about 9 months ex ovo gave only the MM peak (Figs. 21 and 22) with the MB and BB isoenzymes completely absent.

Reported in Table 11 are the relative percentages of the BB, MB and MM isoenzymes of CPK as a function of age in both normal and dystrophic chick embryonic muscle. Up to 11 days in ovo, the BB isoenzyme accounted for more than 90% of the total CPK activity in both normal and dystrophic chick embryonic muscle, with the MB and MM isoenzymes accounting for only about 5% each. By 12 days in ovo, the

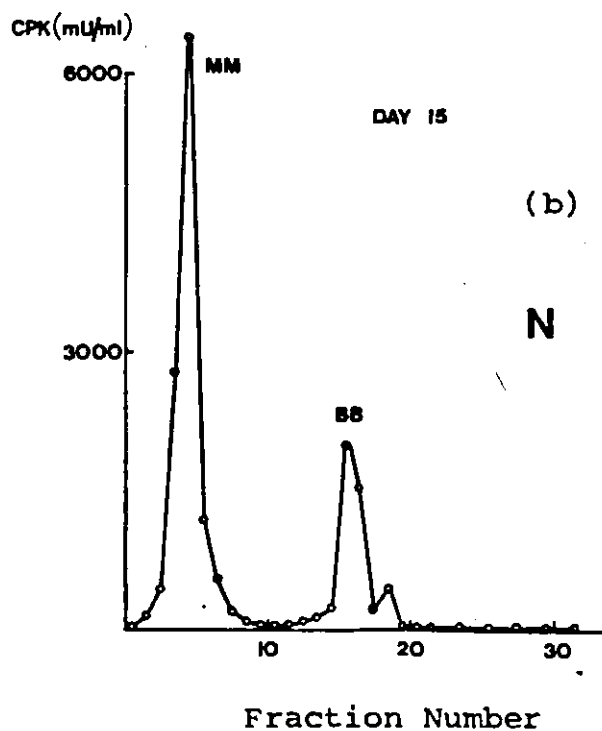
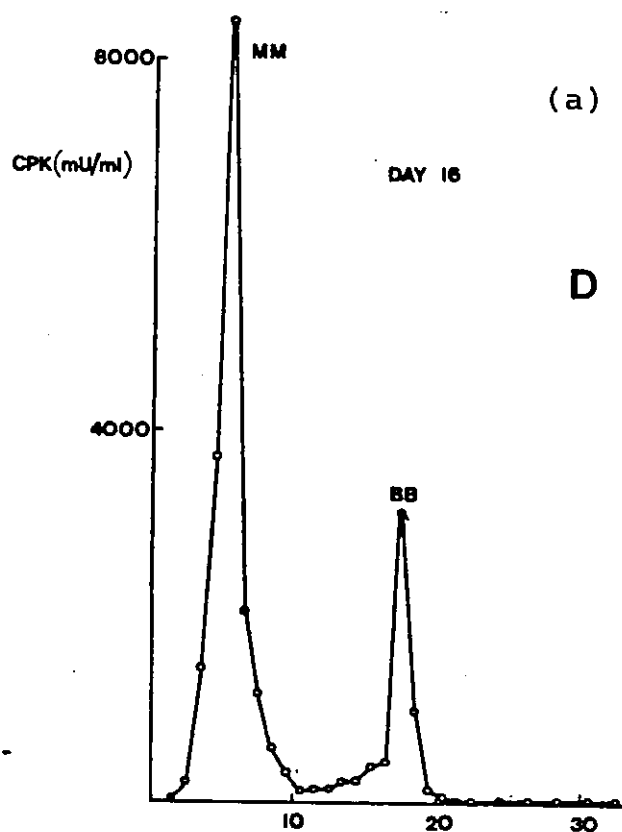


Fig.18 . Isoenzyme profiles of embryonic muscle creatine phosphokinase (CPK) activity in normal and dystrophic chick embryos. D - dystrophic. N - normal. Number of days in ovo is shown. pH 5 - 7 ampholines were used.

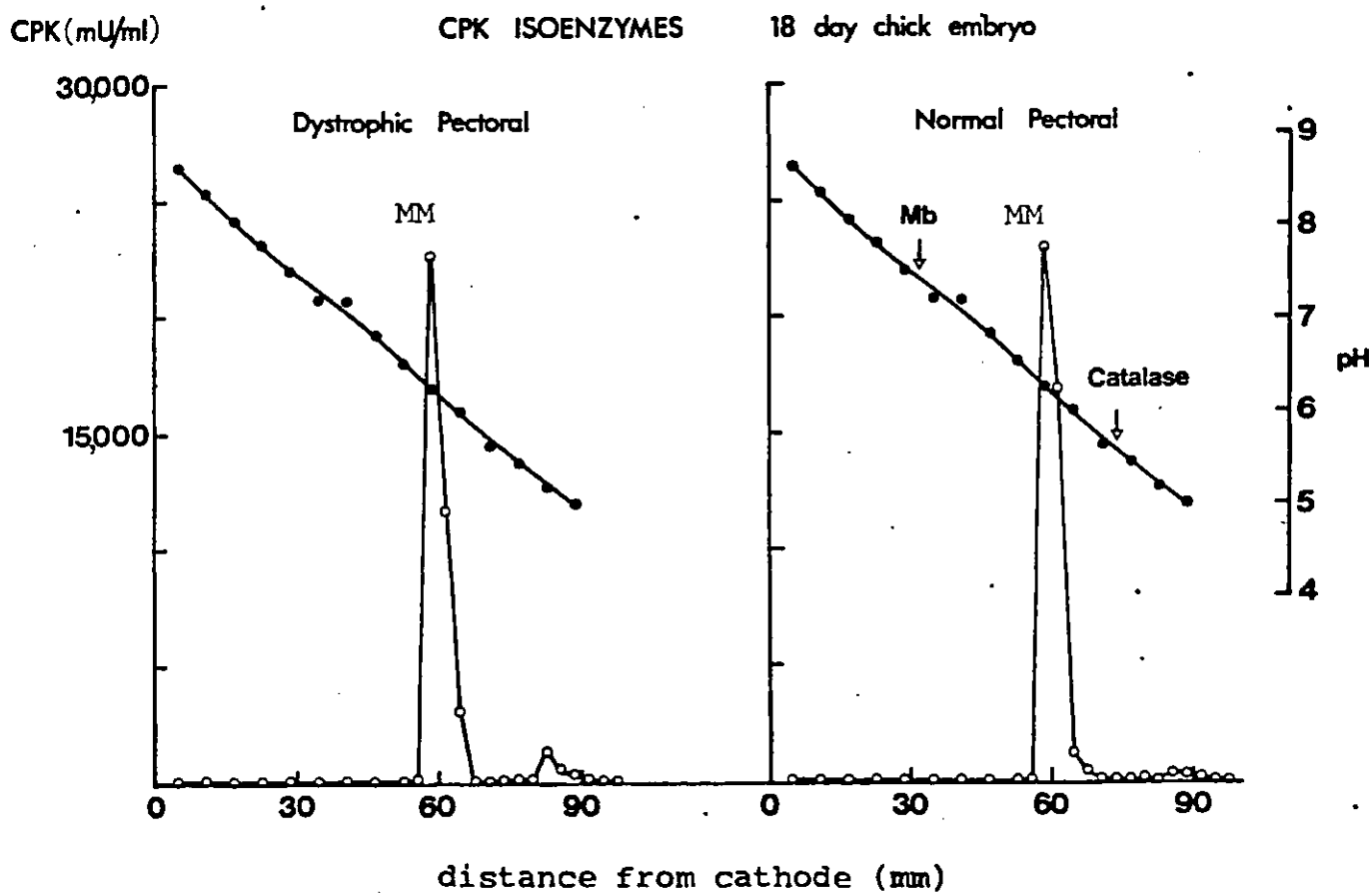


Fig. 19. Isoenzyme profiles of embryonic muscle creatine phosphokinase (CPK) activity in normal and dystrophic chick embryos. Ampholines having a pH range of 5 - 9 were used. pI of major peak (MM) is around 6.3.

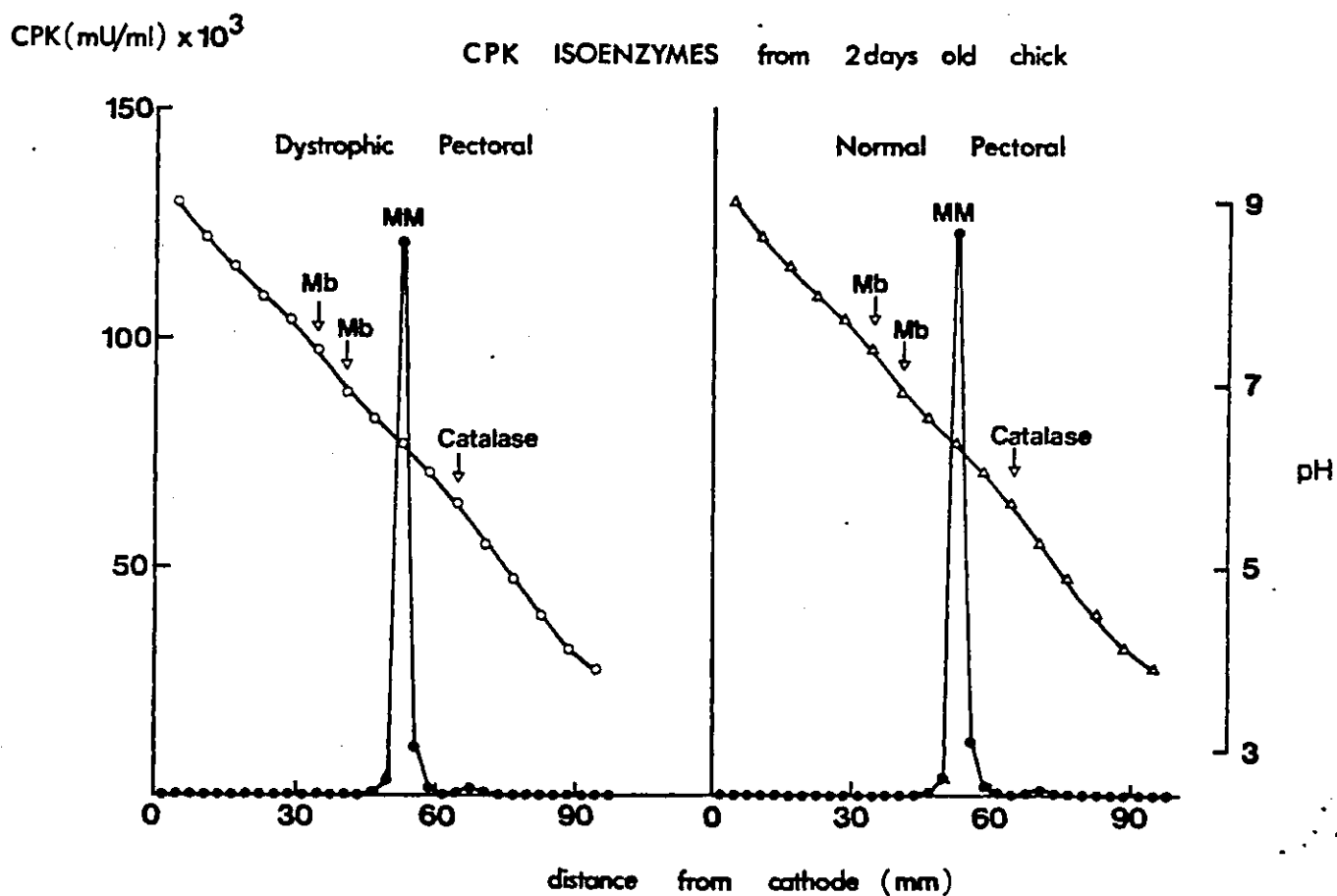


Fig. 20. Isoenzyme profiles of muscle creatine phosphokinase (CPK) activity in 2-day-old normal and dystrophic chicks. pI of MM peak is around 6.4. Ampholines having a pH range of 5 - 9 were used.

1 month old chicken

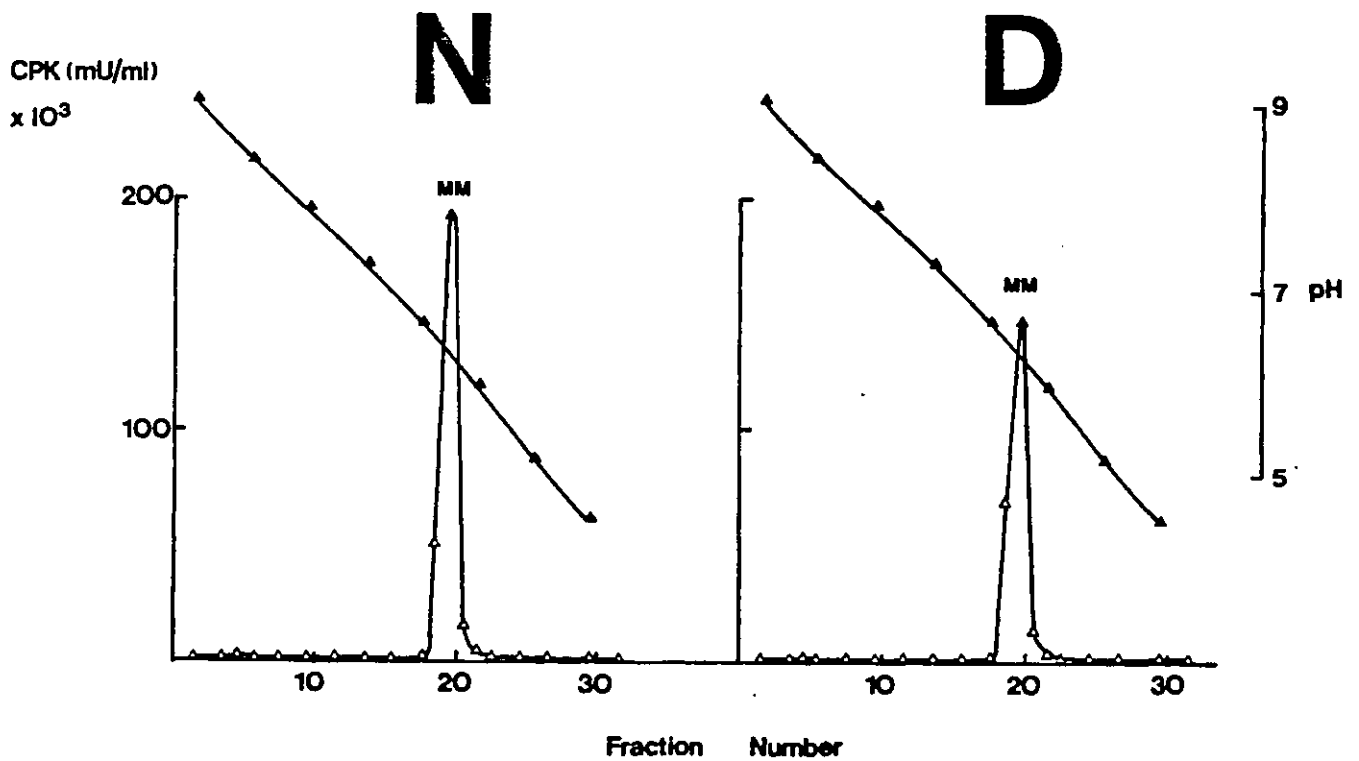


Fig. 21 . Isoenzyme profiles of muscle creatine phosphokinase (CPK) activity in 4-week-old normal and dystrophic chickens. pI of MM peak is around 6.4. Ampholines having a pH range of 5 - 9 were used.

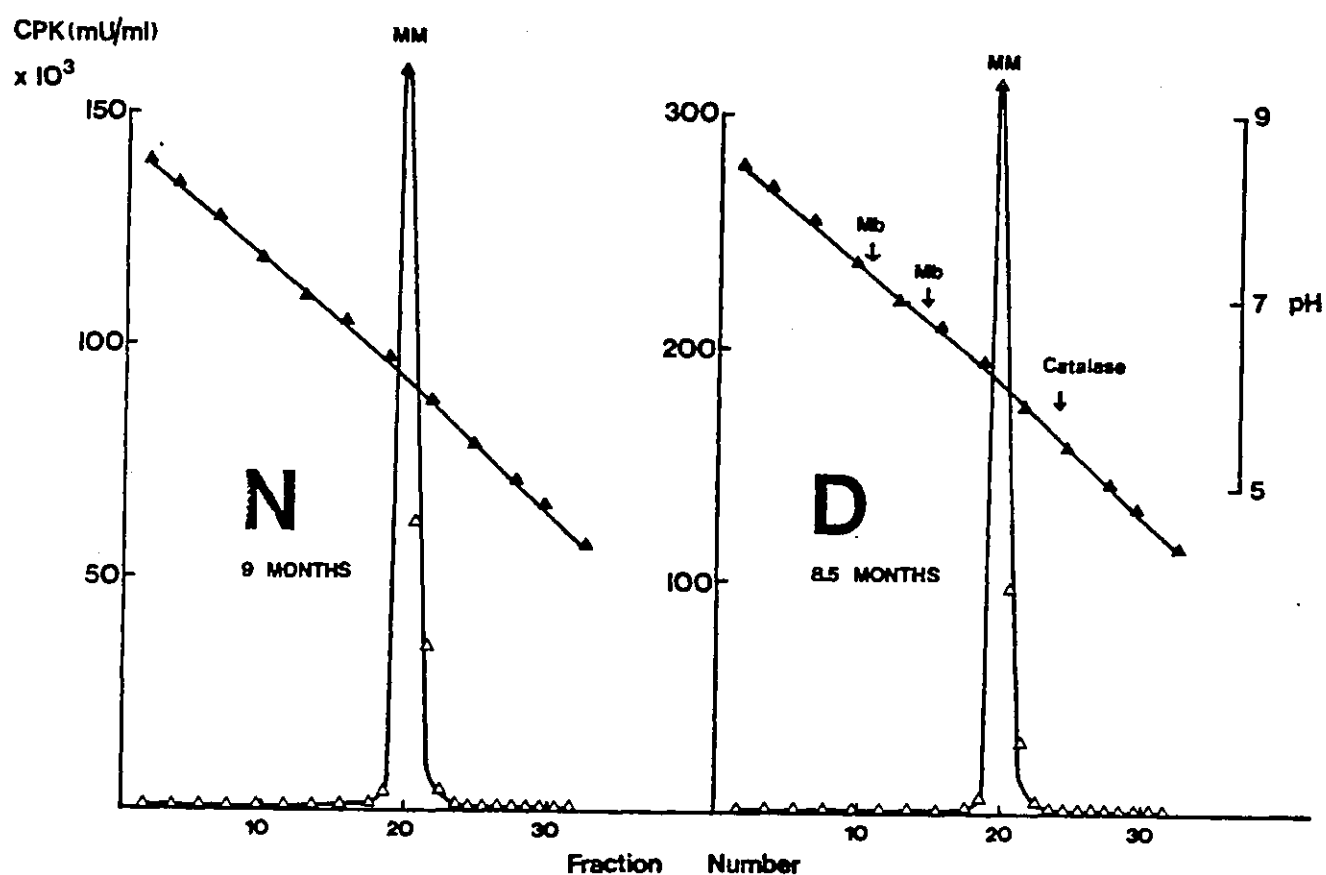


Fig. 22. Isoenzyme profile of muscle creatine phosphokinase (CPK) activity in adult chickens: N - normal. D - dystrophic. pI of MM peak is around 6.25. Ampholines having a pH range of 5 - 9 were used.

TABLE 11

Relative Percentages of CPK Isoenzymes in Normal and Dystrophic Chick Embryonic Muscle as a Function of Age

Age*	% BB ISOZYME		% MB ISOZYME		% MM ISOZYME	
	D	N	D	N	D	N
11	94	90	6	6	0	4
12	52	65	31	22	17	12.5
13	54	54	4	5	37	40
14	29	26	0	0	71	73.5
15		24		0		71
16	22		0		74	
18	7	3	0	0	93	96.5
2d <u>ex ovo</u>	~1	~1	0	0	99	99

* Days in ovo. N - Normal. D - Dystrophic.

percentage of the MB isoenzyme increased sharply and accounted for somewhere between 20 - 30% of the total CPK activity present in normal and dystrophic embryonic muscle. A corresponding decrease in the synthesis of the BB isoenzyme was also observed and now accounted for about 50 - 60% of the total CPK activity present in the two muscles. From 12 days in ovo to about 16 days in ovo, a rapid increase in the synthesis of the MM isoenzyme occurred in both muscles and by 18 days in ovo, accounted for more than 90% of the total CPK activity in normal and dystrophic embryonic muscle. Two days after hatching, the MM isoenzyme was essentially the only isoenzyme present and accounting for almost 100% of the total CPK activity present in either muscle. During the transition of CPK isoenzymes, no consistent differences in the relative percentages of the individual CPK isoenzymes could be observed between normal and dystrophic chick embryonic muscle.

LDH ISOENZYME CHANGES DURING THE DEVELOPMENT OF CHICKEN DYSTROPHY

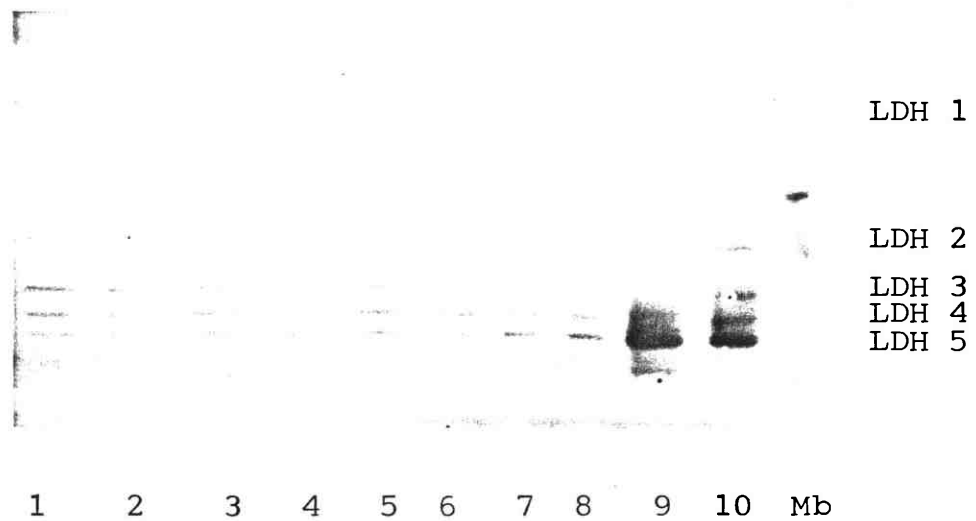
In humans, six isoenzymes of LDH have been identified, each probably being determined by three gene loci, which have been designated LDH_A , LDH_B and LDH_C . The six isoenzymes are all tetramers and may contain polypeptides derived from the same or different loci. Their subunit structures may be written as follows: $LDH_1 = B_4$, $LDH_2 = A_1B_3$, $LDH_3 = A_2B_2$, $LDH_4 = A_3B_1$, $LDH_5 = A_4$ and $LDH_x = C_4$. At pH 7.0, LDH_{1-4} migrates anodally whereas LDH_5 migrates cathodally in gel electrophoresis. LDH_x migrates between LDH_3 and LDH_4 . The isoenzymes are widely distributed in all tissues but their relative amounts vary depending on the

particular tissue. In normal human skeletal muscle, LDH₁₋₅ are all present with LDH₅ predominating. In the pectoral muscle of the chicken, however, only one isoenzyme (LDH₅) was observed by Schapira and associates (173). These same workers found, in contrast, that myopathic pectoral muscle contain three LDH isoenzymes, one identical to the isoenzyme present in normal pectoral muscle and the other two are presumably embryonic type isoenzymes.

To find out whether these additional isoenzymes of LDH that are observed in the dystrophic chicken muscle could be due to a defect in the mechanism controlling differentiation in chick embryonic muscle, the pectoral muscles of both normal and dystrophic chickens were investigated by the method of isoelectric focussing. Fig. 23 shows the LDH isoenzyme patterns of normal and dystrophic chick embryonic muscle from 14 days in ovo to 18 days in ovo. It is clearly apparent that both muscles contain five isoenzymatic forms of LDH, and these have been designated from 1 to 5 as indicated in Fig. 23. During the period investigated, no difference in the isoenzymatic patterns of LDH was observed between normal and dystrophic chick embryonic muscle. From 14 days in ovo, LDH₁ appeared to be present in reduced amounts in both muscles and this continued to day 18 in ovo. By 20 days in ovo, the LDH₅ isoenzyme became more prominent in both normal and dystrophic chick embryonic muscle (Fig. 24), though no difference in the isoenzymatic patterns could be observed. Two days post-hatching, the LDH₅ isoenzyme became the predominant band in both normal and dystrophic chick muscle (Fig. 25) but

Fig. 23

LDH Isoenzyme Transition During the Development of
Normal and Dystrophic Chicken Muscle



<u>Track</u>	<u>Sample</u>	<u>Days in ovo</u>
1	Normal pectoral	14
2	Dystrophic pectoral	14
3	Normal pectoral	15
4	Dystrophic pectoral	15
5	Normal pectoral	16
6	Dystrophic pectoral	16
7	Normal pectoral	18
8	Dystrophic pectoral	18
9	Normal pectoral	8 weeks <u>ex ovo</u>
10	Denervated pectoral	8 weeks <u>ex ovo</u>
Mb	Myoglobin	

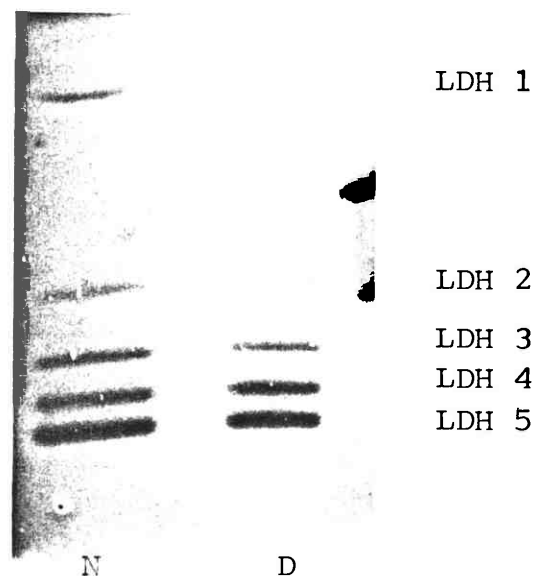


Figure 24

Isoenzymes of muscle LDH in 20-day-old normal and dystrophic chick embryos.

N - normal pectoral muscle.

D - dystrophic pectoral muscle.



Figure 25

Isoenzymes of muscle LDH in 2-day-old normal and dystrophic chicks.

N - normal pectoral muscle

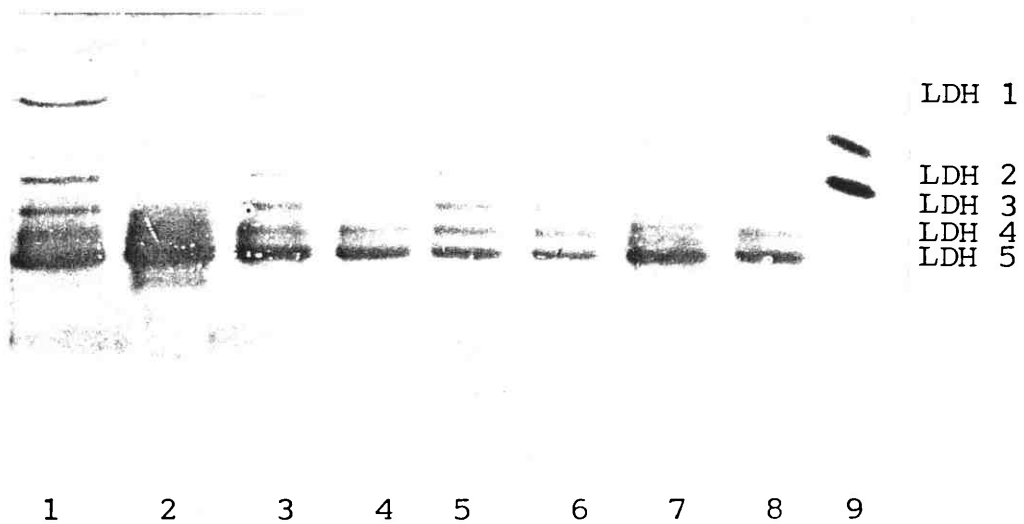
D - dystrophic pectoral muscle

again, no difference in the isoenzymatic pattern was observed between the two muscles. However, LDH₂₋₄ did appear to be present in reduced amounts and also LDH₁ seems to be absent in both muscles. Thus unlike CPK, the isoenzyme transition of chick muscle LDH takes place on or just after hatching and is not complete even after 2 days post hatching.

Reported in Fig. 26 are the isoenzymatic patterns of muscle LDH in normal and dystrophic chickens at different ages. Dystrophic pectoral muscle on focussing (10-week-old) gave three main bands (corresponding to LDH 3, 4 and 5) with the major band being LDH₅. Traces of LDH 1 and 2 could also be detected. Normal pectoral muscle (26-week-old), however, gave only one main band (LDH₅) with possible traces of LDH 3 and 4. LDH isoenzymes 1 and 2 seem to be completely absent in the normal muscle. Normal pectoral muscle also showed traces of LDH activity in a position that is slightly more basic than LDH₅. To discount that the difference between 10-week-old dystrophic and 26-week-old normal chickens could be due to age only, both normal and dystrophic pectoral muscles were electrofocussed again and the LDH isoenzyme pattern is shown in Fig. 31a. It is clearly apparent (c.f. tracks 2 and 3) that the dystrophic pectoral muscle contains LDH 1 and 2 activity which are absent in the normal muscle. Further, innervated pectoral muscle (normal, 8-week-old) reported in Fig. 31b shows no LDH 1 or 2 activity. Thus, there is a consistent absence of LDH 1 and 2 isoenzymes in normal pectoral muscle from 5 weeks ex ovo to 26 weeks ex ovo.

Figure 26

Muscle LDH Isoenzymes in Normal and Dystrophic Chickens



<u>Track</u>	<u>Sample</u>	<u>Age(weeks)</u>
1	Normal thigh muscle	26
2	Normal pectoral muscle	26
3	Dystrophic thigh muscle	10
4	Dystrophic pectoral muscle	10
5	Dystrophic thigh muscle	2
6	Normal thigh muscle	2
7	Normal pectoral muscle	26
8	Dystrophic pectoral muscle	10
9	Myoglobin	

Also reported in Fig. 26 are the LDH isoenzyme patterns in the thigh muscles of normal and dystrophic chickens. Unlike normal pectoral muscle, thigh muscles from 2 and 26-week-old normal chickens gave, on focussing, all five LDH isoenzymes (LDH_{1-5}) that were seen in the embryonic pectoral muscle of the chicken. Likewise, dystrophic thigh muscles from 2 and 10-week-old dystrophic chickens also contain all the five LDH isoenzymes seen in chick embryonic muscle. Thus, unlike the pectoral muscles of normal and dystrophic chickens where differences in the LDH isoenzyme content occurs, it appears that in the thigh muscles of normal and dystrophic chickens, the isoenzyme patterns of LDH are identical.

PK ISOENZYME TRANSITIONS DURING THE DEVELOPMENT OF DYSTROPHIC CHICK EMBRYONIC MUSCLE

In mammals, at least three distinct forms of PK have been recognised and these have been referred to as the L type (mainly liver origin), M_1 type (exclusively found in skeletal muscle, heart muscle and brain) and M_2 or K type (found in kidney and other tissues). PK in erythrocytes may represent a fourth isoenzyme (designated R type) but it has been suggested recently that it may be a proteolytically modified form of the L type. The L, M_1 and M_2 isoenzymes are probably determined by separate gene loci and all three isoenzymes occur predominantly as tetramers made up of subunits of MW between 50,000 and 60,000. At pH 8.6, the L type migrate relatively rapidly in gel electrophoresis towards the anode, M_2 remains close to the origin and M_1 migrates slightly more anodally than M_2 .

Similarly, three isoenzymes of PK have been identified in various tissues of the chicken and these also have been referred to as M_1 (exclusive to skeletal muscle, heart muscle and brain), M_2 (mainly kidney and some in liver) and L (mainly liver and other tissues). During the development of normal chick embryonic muscle, Strandholm et al. (1975) reported a transition from the embryonic M_2 -type isoenzyme at 15 days in ovo to the M_1 form at 18 days in ovo. After hatching, only the M_1 form was found in the muscle.

It was, therefore, of interest to compare the transitional changes of PK isoenzymes during the early development of normal and dystrophic chick embryonic muscle and for this purpose, the high resolution method of isoelectric focussing was used. Using this system, normal muscle from 14-day-old chick embryos gave one band of PK activity which presumably corresponds to the M_2 -type isoenzyme (Fig. 27). Focussing of dystrophic muscle of the same age also gave one band of PK activity (Fig. 27) which is identical to the band seen in normal chick embryonic muscle. At 15 days in ovo, two main forms of PK activity were detected in both normal and dystrophic chick embryonic muscle (Fig. 28); one form comigrating with the isoenzyme seen in the muscles of 14-day-old chick embryos and the other form electrofocussed in a more basic position than the first isoenzyme. It is presumed that this more basic form corresponds to the M_1 -type isoenzyme. By 17 days in ovo, both normal and dystrophic chick embryonic muscle gave one major band of PK activity (Fig. 29) corresponding to the M_1 form and also another minor band of PK activity which presumably is the M_2 -type isoenzyme. At 18 days



Fig.27.Isoenzymes of muscle PK in 14-day-old normal and dystrophic chick embryos. N - normal pectoral muscle. D - dystrophic pectoral muscle

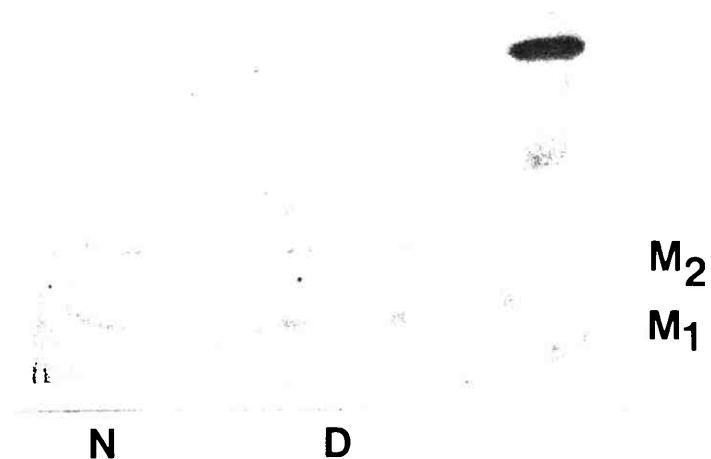


Fig.28.Isoenzymes of muscle PK in 15-day-old normal and dystrophic chick embryos. N - normal pectoral muscle. D - dystrophic pectoral muscle.



Fig.29 .Isoenzymes of muscle PK in 17-day-old normal and dystrophic chick embryos. N - normal pectoral muscle. D - dystrophic pectoral muscle



Fig.30 .Isoenzymes of muscle PK in 18-day-old normal and dystrophic chick embryos. N - normal pectoral muscle. D - dystrophic pectoral muscle.

in ovo, the changeover from the embryonic M_2 form to the adult muscle M_1 form in the muscles of both normal and dystrophic chick embryos is essentially complete (Fig. 30). Thus, during the early development of dystrophic chick embryonic muscle, the isoenzyme changes of PK that accompany muscle differentiation is completely unaffected by the dystrophic process that may be occurring within the muscle by this stage.

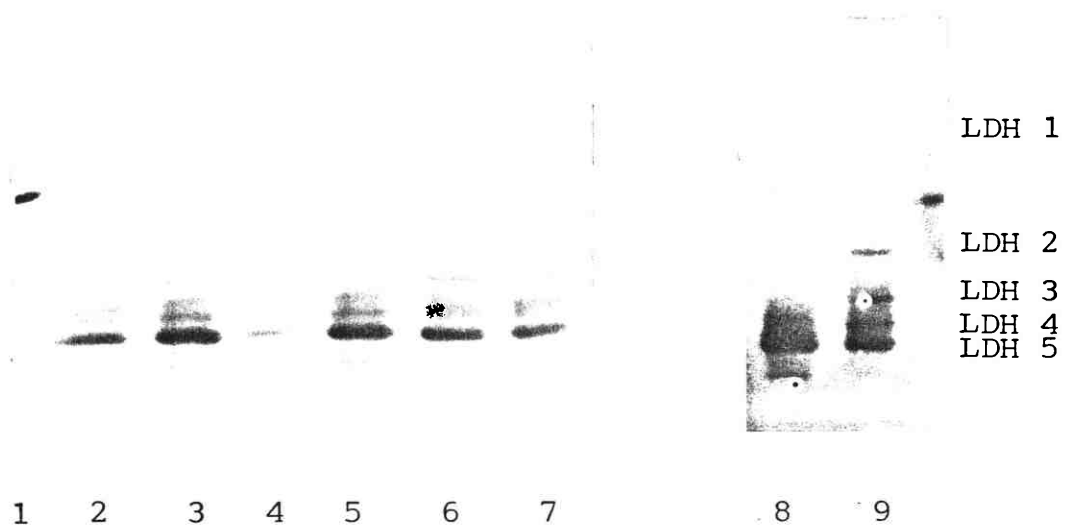
EFFECTS OF DENERVATION ON ISOENZYMES OF LDH AND PK IN NORMAL CHICKEN MUSCLE

The above studies on the isoenzyme changes of CPK, PK and LDH during the development of dystrophic chick embryonic muscle showed no evidence of a defect in the mechanism controlling differentiation in these muscles. Thus, the presence of foetal-like isoenzymes of CPK in the muscle and serum of Duchenne humans may be attributed to the presence of regenerating fibres in the affected muscle. Evidence in support of this hypothesis is provided by Wiesmann et al. (1969) when they found isoenzyme transitions of CPK and LDH in regenerating rabbit muscle that are reminiscent of the changes seen in developing rabbit foetal muscle. To see if this reversion to the embryonic-like state could be achieved in the muscle of the chicken, the pectoralis major muscle of a normal adult chicken was denervated by careful sectioning of the nerve supplying that muscle. After allowing adequate time (8 - 12 days) for regeneration to take place, the denervated pectoral muscle which can be easily recognised as being very pale and tough was subjected to isoelectric focussing on a thin-layer of acrylamide and the isoenzymatic

LDH Isoenzymes in Normal, Dystrophic and
Denervated Chicken Muscle

Figure 31a

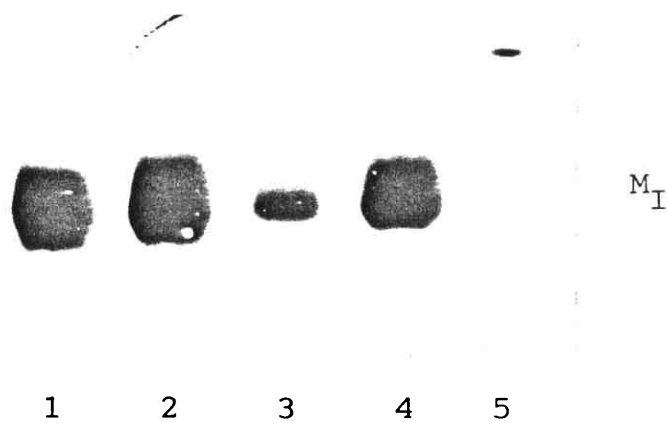
Figure 31b



<u>Track</u>	<u>Sample</u>	<u>Age(weeks)</u>
1	Myoglobin	5
2	Dystrophic pectoral muscle	5
3	Normal pectoral muscle	5
4	Dystrophic plasma	5
5	Innervated pectoral muscle	8
6	Denervated pectoral muscle (11 days)	8
7	Denervated pectoral muscle (11 days)	8
8	Innervated pectoral muscle	8
9	Denervated pectoral muscle (12 days)	8

Figure 32

PK Isoenzymes in Normal and Denervated Chicken Muscle



<u>Track</u>	<u>Sample</u>	<u>Age(weeks)</u>
1	Dystrophic pectoral muscle	8
2	Normal pectoral muscle	8
3	11d Denervated pectoral muscle	8
4	Innervated pectoral muscle	8
5	Myoglobin	

changes of LDH and PK were compared to the normal innervated control muscle.

Illustrated in Figs. 31a and 31b are the LDH isoenzyme patterns in denervated and innervated chicken muscle. It is clearly apparent from the two figures that denervated muscle contain all five LDH isoenzymes that were previously seen in chick embryonic muscle. In contrast, normally innervated adult chicken pectoral muscle as shown contains only one major isoenzyme (LDH_5) and small amounts of LDH_4 . LDH_{1-3} appears to be completely absent. Normal pectoral muscle also contains, as previously reported, two very weak bands of LDH activity that are slightly more basic than the LDH_5 isoenzyme. Also included in Fig. 31a for comparison is the isoenzymatic pattern for dystrophic muscle and as can be seen, the LDH isoenzyme pattern is very similar to the pattern observed in the denervated pectoral muscle of a normal chicken. The LDH isoenzyme pattern in dystrophic plasma is shown in Fig. 31a and found to contain LDH 3, 4 and 5. Failure to detect LDH 1 and 2 could be due to the rather low activities of these individual isoenzymes present in the blood.

Pyruvate kinase isoenzyme patterns were also investigated in the muscles of normal and denervated chickens and the findings are shown in Fig. 32. Normal adult pectoral muscle gave only one band of PK activity corresponding to the M_1 -type. Likewise, normal chicken pectoral muscle after 11 days of denervation also gave one band of PK activity on focussing which is identical to the form seen in the innervated muscle. When a sample of adult dystrophic pectoral muscle was electrofocussed for comparison, the same M_1 form

appeared. Thus, unlike LDH, it appears that the isoenzymes of PK are not affected either by dystrophy or the effects of denervation on the chicken muscle.

DISCUSSION

The transition of CPK isoenzymes during muscle development has been used as an index of muscle differentiation (Eppenberger et al., 1964). The results of the present study shows that in chick embryonic muscle, this transitional change is the result of a rapid increase in the synthesis of the M subunit, combining first with the B subunit to form the MB hybrid isoenzyme and then immediately after, combining with other M subunits to form the MM adult muscle isoenzyme. During this transitional period, the MB isoenzyme only appeared in significant amounts at day 12 in ovo. A previous report (Stewart et al., 1981) on Connecticut chickens showed that large amounts of the MB form remained present for several days in the embryonic muscle during this changeover period. It appears, therefore, that in the Californian birds, the transitional period from BB to MM is shorter than that observed in the Connecticut chickens.

During the entire CPK transitional period (from 11 days in ovo to 2 days ex ovo), no consistent differences in the relative percentages of the individual CPK isoenzymes were observed between normal and dystrophic embryonic pectoral muscle. The findings here contrast with the observations of an earlier study (Stewart et al., 1981) which found that the proportion of MM isoenzyme in dystrophic embryonic pectoral muscle was significantly lower than normal. These differences may arise due to a difference in the developmental rate and extent of the pathogenesis between the Californian and Connecticut lines of dystrophic chickens. In support of this view, it has been observed in this laboratory (Liu et al., 1980) that the degree of

manifestation of certain dystrophic characteristics (e.g. plasma PK or CPK) is quite different between the two dystrophic chicken strains.

The observation that the transition of CPK isoenzymes from the embryonic BB form to the adult MM form in the developing dystrophic chick muscle is essentially complete 2 days after hatching implies that differentiation of the affected muscle is also complete by this stage. The absence of embryonic-like isoenzymes of CPK in adult muscles of dystrophic chickens also supports this view. These results are consistent with the findings of Schapira et al. (1968) who found only the MM isoenzyme in dystrophic chick muscle. These results do, however, differ from the findings of Weinstock et al. (1978) who found a significant amount of CPK activity (between 10 - 13%) due to MB and BB isoenzymes in the pectoral muscles of dystrophic chickens at seven to ten months of age. Further, although Stewart et al. (1981) concluded that the CPK isoenzyme transition in dystrophic chick muscle is essentially complete after one week ex ovo, they do still find between 3 - 6% of CPK isoenzymes due to the MB and BB isoenzymes in one week to five-month-old dystrophic pectoral muscles. Again, the dystrophic chicken line used by both Weinstock et al. (1978) and Stewart et al. (1981) is different from the dystrophic line used in the present study and this may account for the differences that are observed.

From the results of the LDH isoenzyme studies, it appears that in early embryonic chick muscle (both normal and dystrophic), both the A and B subunits are synthesised in equal amounts so that all five isoenzymes of LDH (LDH₁₋₅)

are present in the embryonic muscle. From about 15 to 16 days in ovo, however, there is an apparent increase in synthesis of the A subunit with a corresponding decrease in the synthesis of the B subunit, the net result being a shift towards the increase production of the LDH₅ isoenzyme. Thus, by 2 days ex ovo, LDH₅ isoenzyme activity has increased enormously and becomes the most prominent band seen in the chick muscle. During this entire period of muscle development (12 days in ovo to 2 days ex ovo), no abnormal isoenzyme pattern was observed in the dystrophic chick muscle.

Although the transition of LDH isoenzymes was not entirely complete in the muscles of 2-day-old normal and dystrophic chicks, the observed disappearance of LDH₁ together with the diminishing activities of LDH₂, 3 and 4 in the two muscles at this stage indicate that the isoenzyme transition of LDH is proceeding normally to completion in the affected muscle. These findings, together with those reported above for CPK isoenzyme transitions, do not support the suggestion that differentiation of the dystrophic embryonic pectoral muscle may be impaired in some way. Further, the complete transition of PK isoenzymes in dystrophic chick embryonic pectoral muscle from the M₂ form to the adult M₁ form by 18 days in ovo also adds support to the view that the mechanism controlling differentiation of muscle is not affected in chicken dystrophy.

Dystrophic pectoral muscle was found in this study to contain all five LDH isoenzymes, a situation that is reminiscent of embryonic muscle. As reported earlier, this difference between normal and dystrophic pectoral muscle

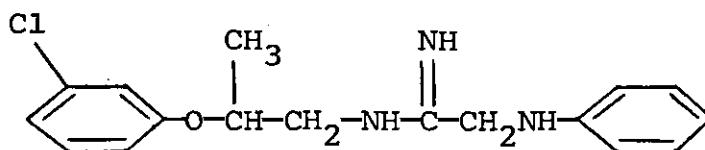
could not have been due to age only, since a difference in LDH isoenzyme pattern also occurred in the two different muscles when age-matched controls were used. An earlier study by Schapira et al. (1977) also found embryonic-like isoenzymes in the pectoral muscle of the dystrophic chicken. This abnormal LDH pattern may be due to the presence of regenerating fibres in the dystrophic chicken muscle. The appearance of all five LDH isoenzymes in the denervated muscle of a normal chicken is consistent with this view. Further, when normal skeletal muscle is provoked to regenerate either by ligation of the arteries (Wiesmann et al., 1969) or by injection of high concentrations of saline (Kitahare et al., 1977), the normal isoenzyme pattern for some enzymes (e.g. CPK, LDH and glycogen phosphorylase) is disturbed and becomes accompanied by the appearance of small amounts of embryonic or foetal-like isoenzymes. All these observations suggests that the presence of foetal or embryonic-like isoenzymes found in muscle and serum of both the human and animal dystrophies are due to regenerative processes occurring within the affected muscle and not due to an impairment of muscle differentiation.

CHAPTER 5

PHARMACOKINETIC STUDIES ON BW 501C67 AND PIZOTIFEN IN THE CHICKEN

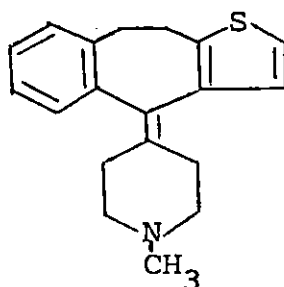
INTRODUCTION

Compound BW 501C67 (α -anilino-N(2-m-chlorophenoxypropyl)acetamidine), developed at the Wellcome Research Laboratories, Beckenham, has been reported to possess marked anti-serotonin properties.



Plasma and tissue concentrations of BW 501C67 have been studied in the rat and the patas monkey (Jones and Lees, 1978). Plasma half-lives for the compound in these species were not reported. The absorption and excretion of BW 501C67 in both species and the possible effects of this drug on rat hepatic microsomal enzyme system have also been reported (Jones and Lees 1978, Nimmo-Smith and Savage 1978). No work on this compound has been reported in the chicken.

Pizotifen (4-(9,10-dihydrobenzo(4,5)cyclohepta-(1,2-b) thien-4-ylidene)-1-methylpiperidine) is also a powerful serotonin antagonist which has been shown to be effective against migraine in chemotherapy trials (Arthur and Hornabrook 1971, Krakowski and Engisch 1973). It has a structural formula that is very similar to the very potent serotonin antagonist, cyproheptadine



Pharmacokinetic studies on this compound have been reported in the dog, the rat and also in man. The elimination half-life in man for the pharmaceutical form was reported to be around 20h. Like BW 501C67, no work on this compound has been reported in the chicken.

The object of this study serves two purposes. The first is to establish the elimination half-life for BW 501C67 and pizotifen in chicken blood for chemotherapy purposes and the second, is to observe whether there is any significant difference in the rate of removal of either of these compounds in the blood plasma between normal and dystrophic chickens.

MATERIALS AND METHODS

Animals. Five week-old normal chickens (White Leghorn strain) that weighed around 300g and of either sex were used in experiments with ^{14}C -501C67. For studies on pizotifen, strain 413 (homozygous, dystrophic) and its closely related normal control strain 412 chickens (approx. 8 weeks old) originally purchased from the University of California, Davis, and now bred and maintained at Imperial College, London, were used.

Drugs. ^{14}C -501C67 was synthesised at the Wellcome Research Laboratories, Beckenham, and supplied by Dr. C.R. Jones. Pizotifen hydrogen malate was obtained from Sandoz Pharmaceuticals, Leeds, U.K.

Chemicals. All chemicals for ^{14}C -501C67 studies were supplied by the Wellcome Research Laboratories, Beckenham, Kent.

^{14}C -501C67 Preparation. ^{14}C -501C67 hydrochloride at a specific activity of 4.7 uCi/mg was used. For a 15 mg/Kg dosage, each bird weighing approximately 300g was given 4.5 mg of the drug. Since the amount of radiolabelled drug available was small, 2.5 mg of 501C67 was added to 2.0 mg of ^{14}C -501C67 to make up a total of 4.5 mg per bird. The drug was dissolved in physiological saline (0.85%) and the injection volume used for both intravenous and intraperitoneal administration was 0.5ml. The radioactive dose per bird was therefore 9.4 uCi.

Pizotifen hydrogen malate was made up in deionised water and administered intraperitoneally to both normal and dystrophic chickens at 30 mg/Kg bodyweight.

Blood Samples. Blood (approx. 0.5ml) was taken from the jugular vein and collected into heparinized tubes. Plasma was separated by centrifugation and frozen immediately in cardice. Samples containing ^{14}C -501C67 were analysed as soon as possible or were stored overnight at -20°C . For total radioactivity determinations, plasma samples (0.2ml in duplicate) were diluted with water (0.8ml) and 10mls of toluene/triton (2:1) scintillant added. Radioactivity was measured by liquid scintillation counting. Samples containing pizotifen were sent immediately to Dr. R. Silman, Department of Metabolism, St. Bartholomew's Hospital Medical School, London, for mass spectrometry analysis.

Tissues. Whole tissues or organs were removed immediately after killing, weighed and then frozen rapidly in cardice. Tissues were either processed immediately or stored over-

night at -20°C . On thawing, tissues were homogenised in 9 volumes of water and the total homogenates weights recorded. For liver and kidney, weighed portions of the homogenates in duplicate (approx. 0.2ml), were solubilised in S.H.T. (sodium hydroxide/triton mixture, 0.2ml) by sonication at 50°C for 2 - 3h (Johnson and Dent, 1973). After cooling and neutralisation with 4.4M HNO_3 (0.1ml), the volume was adjusted to 1.0ml with water and the samples counted in toluene/triton (2:1) scintillant (10mls). For analysis of muscle, weighed aliquots (approx. 0.4ml), in duplicate, were solubilised in S.H.T. (0.4ml) by sonication at 50°C , cooled and neutralised with 4.4M HNO_3 (0.2ml). These samples were similarly counted in toluene/triton (2:1) scintillant (10mls).

Before counting, all solubilised samples/scintillant mixtures were stored in the dark for at least 48h after preparation to allow for the decay of chemiluminescence. Radioactivity was measured by liquid scintillation counting using Intertechnique ABAC SL40 and Nuclear Chicago Mk II liquid scintillation spectrophotometers. For quench correction, selected samples are spiked with ^{14}C -toluene or hexadecane as internal standard and then re-counted. Quench correction factors are then worked out by computer.

Radiochemical Purity of ^{14}C -501C67. The radiochemical purity of ^{14}C -501C67 was measured by a combination of thin layer chromatography/autoradiography. (^{14}C)-501C67 was applied to the gel plate (0.25mm thick; Merck GF₂₅₄) and developed in the following solvent systems:

Solvent System	% Purity
$\text{CHCl}_3 - \text{MeOH} - \text{NH}_3$ (60:12:13 v/v)	95.5%
$\text{CHCl}_3 - \text{MeOH} - \text{H}_2\text{O}$ (65:25:4 v/v)	97.0%
$\text{BuOH} - \text{AcCOOH} - \text{H}_2\text{O}$ (40:10:10 v/v)	99.0%

The radiochemical purity was greater than 95% in all three systems.

RESULTS

PLASMA AND TISSUE LEVELS OF ^{14}C -501C67 IN NORMAL CHICKENS AFTER A SINGLE INTRAVENOUS DOSE OF 15mg/Kg

The rate of disappearance of radioactivity from plasma of normal chickens after a single intravenous administration of 15 mg/Kg is shown in Fig. 33. The initial rate of disappearance of radioactivity is very rapid and follows an exponential pattern of decline. Twenty-four hours after drug administration, virtually all of the radioactivity has been removed from the blood plasma. A semi-logarithmic plot of the mean concentration of the base equivalent in plasma versus time (Fig. 34) shows that the decline in radioactivity in plasma is bi-exponential. Therefore, the experimental data appears to be consistent with an open two-compartment model system.

The rate constant (α) for the first exponential component of decline was computed to be -0.62h^{-1} and is a measure of the speed of distribution of radioactivity (or drug) into the second compartment shortly after drug administration. The rate constant (β) for the second exponential component of decline that is dominant at later stages was calculated to be -0.078h^{-1} , giving a biological

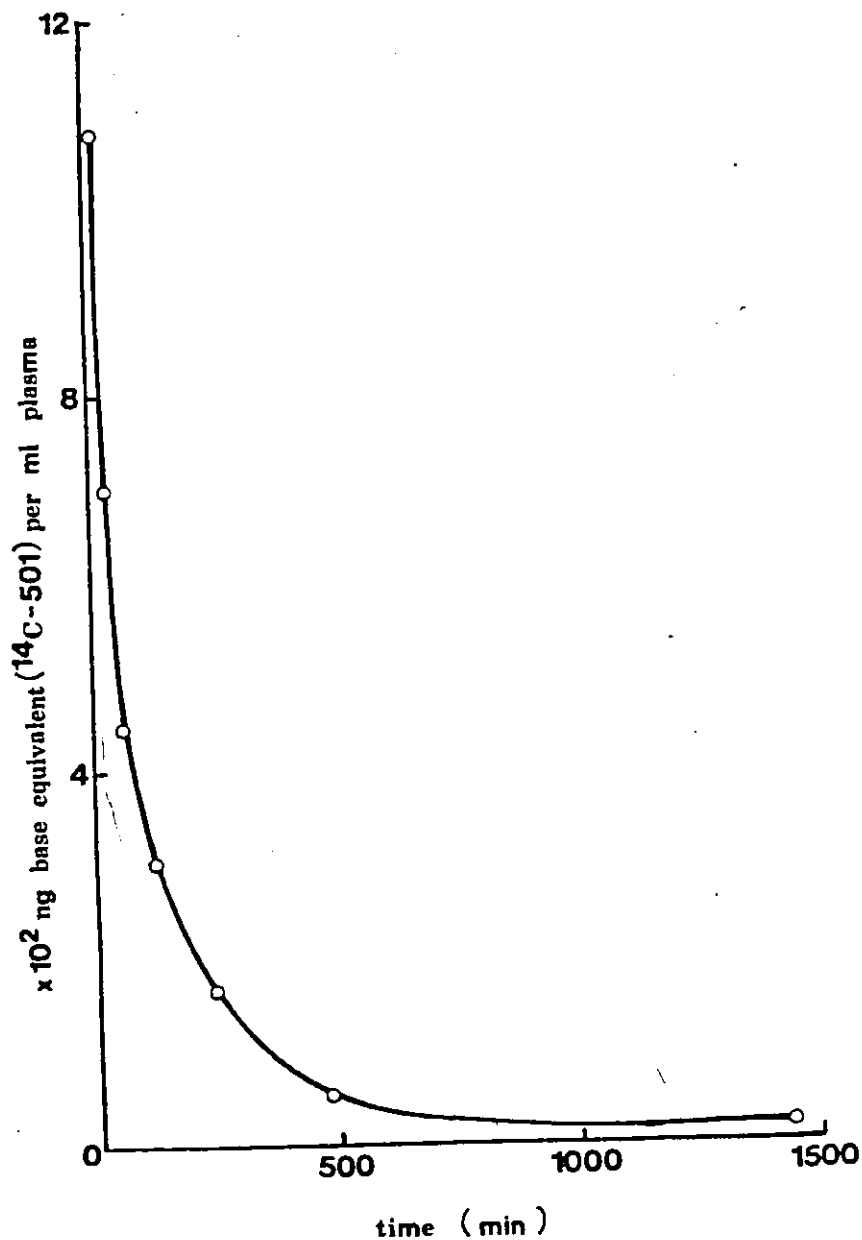


Fig.33 .Rate of disappearance of ^{14}C -501C67 in normal chicken plasma after a single i.v. dose of 15 mg/Kg. Each point along the curve is the mean of three chickens.

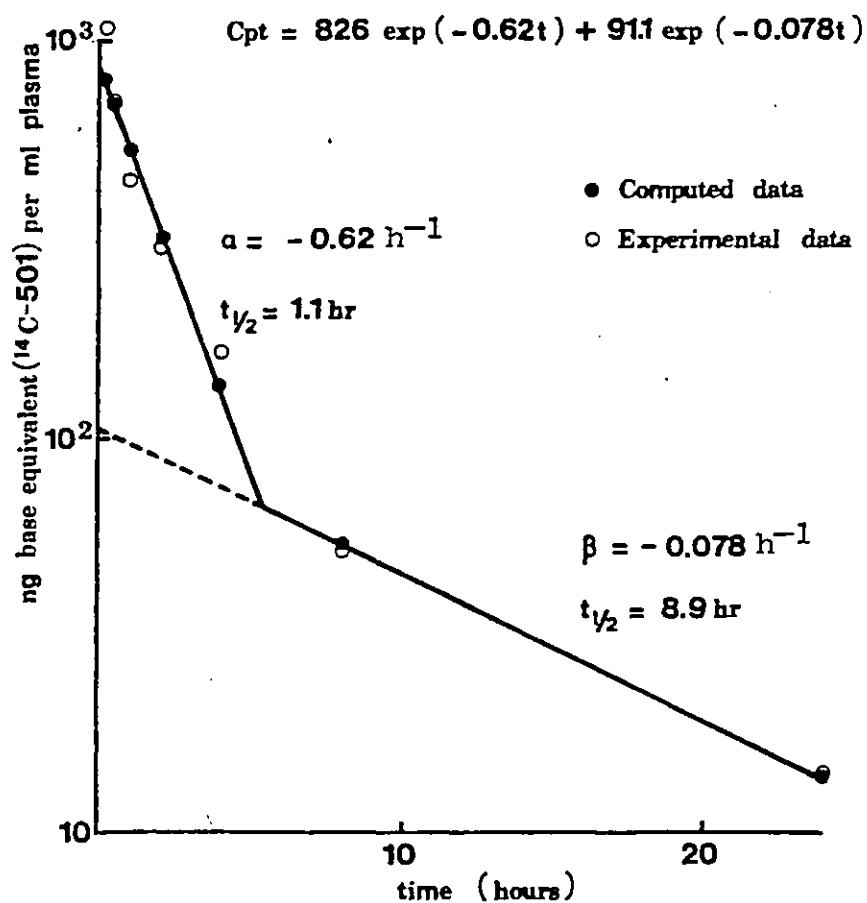


Fig.34. Semi-logarithmic plot of the rate of elimination of ^{14}C -501C67 in normal chicken plasma after a single i.v. dose of 15 mg/Kg. Each point is the mean of three birds.

half-life of 8.9h. β , therefore, measures the rate of elimination of the drug due to metabolic and excretory factors.

The distribution of radioactivity in plasma and the various tissues of the chicken over a 24-hour period following the intravenous administration of ^{14}C -501C67 at 15 mg/Kg is shown in Table 12. The equilibrium concentration ratios for kidney, liver, plasma, anterior latissimus dorsi (ALD) muscle and the posterior latissimus dorsi (PLD) muscle were all achieved 15 mins after drug administration. At equilibrium, although diffusion of drug between a particular tissue and plasma continues, their relative concentrations with time do not change. The pectoralis major muscle, however, did show an initial rise in the mean concentration of radioactivity with equilibrium being reached 30 mins after drug administration. The relatively slower achievement of equilibrium in the pectoral muscle probably reflects the poorer vascularity of this tissue, as opposed to the kidney, liver and the ALD muscle, slow muscles being more vascular than fast ones. Like the pectoral muscle, the PLD is also a fast muscle and one would expect it to behave similarly. Due to its much smaller size, however, diffusion of radiolabelled drug into the PLD may be less impeded.

When the tissue to plasma ratios of radioactivity were considered for the various tissues (Table 13), very little retention was observed in the skeletal muscles until about 24-hours after drug administration. At this point of time, the ALD and to a lesser extent, the PLD

Table 12

<u>Time after</u> <u>Dose</u>	<u>Plasma</u>	<u>ALD Muscle</u>	<u>PLD Muscle</u>	<u>Pectoral</u>	<u>Liver</u>	<u>Kidney</u>
		ug Base Equivalent g ⁻¹ Tissue				
15 min	* 1.08±0.01	3.80±2.15	1.18±0.29	0.29±0.02	16.56±3.09	27.67±5.81
30 min	0.70±0.03	1.76±0.19	0.87±0.29	0.88±0.16	8.48±1.83	20.13±2.88
60 min	0.44±0.09	1.77±0.46	0.41±0.18	0.62±0.05	4.66±0.14	7.90±0.94
2 hr	0.30±0.02	1.37±0.20	0.54±0.08	0.48±0.07	5.20±0.68	4.31±0.67
4 hr	0.16±0.008	0.86±0.23	0.36±0.02	0.26±0.04	1.45±0.29	1.64±0.19
8 hr	0.05±0.010	0.20±0.06	0.16±0.01	0.11±0.02	0.51±0.04	0.59±0.02
24 hr	0.01± 0.004	0.21±0.07	0.08±0.02	0.04±0.01	0.46±0.12	0.32±0.11

Distribution of total radioactivity in plasma and tissues after a single intravenous administration of ¹⁴C-501C67 at 15 mg/Kg. *Mean of three birds ± S.E.M.

Table 13

<u>Time after Dose</u>	<u>ALD</u>	<u>PLD</u>	<u>Pectoral</u>	<u>Liver</u>	<u>Kidney</u>
Tissue to plasma ratios					
15 min	3.53	1.10	0.27	15.36	25.67
30 min	2.51	1.23	1.25	12.10	28.72
60 min	3.99	0.93	1.39	10.49	17.78
2 hr	4.53	1.78	1.59	17.2	14.27
4 hr	5.24	2.16	1.61	8.84	10.00
8 hr	3.87	3.02	2.02	9.89	11.40
24 hr	15.10	5.43	2.71	33.00	22.79

Tissue to plasma ratios of ^{14}C -501C67 in normal chickens after the single intravenous administration of ^{14}C -501C67 at 15 mg/Kg.

aswell, both showed an increase tendency to retain the radiolabelled compound relative to plasma. Whether this effect is attributable to the small number of animals used in the study or due solely to the physico-chemical properties of this compound is yet uncertain. The two highly perfused tissues, liver and kidney, both showed a much greater retention of radioactivity than the skeletal muscles and these findings are consistent with their functions as the major organs involved in metabolism and excretion.

PLASMA AND TISSUE LEVELS OF ^{14}C -501C67 IN NORMAL CHICKENS AFTER THREE DAILY INTRAVENOUS DOSES OF 15mg/Kg

In this experiment, chickens were injected intraperitoneally with ^{14}C -501C67 at 15 mg/Kg and repeated every 24 hours for a further two days. Immediately after the third and final dose, birds in groups of three were bled at 0.25, 0.5, 1, 2, 4, 8 and 24-hour intervals. Fig. 35 depicts the time course of radioactivity derived from ^{14}C -501C67 in normal chicken plasma after three daily i.v. doses at 15 mg/Kg. Like Fig. 33 reported earlier for a single intravenous dose at 15 mg/Kg, the decline in plasma radioactivity with time is exponential in character. Replotting the time points of Fig. 35 on a semi-logarithmic scale showed that the rate of disappearance of radioactivity (or base equivalent) in normal chicken plasma also consist of two exponential decay processes (Fig. 36). The initial decline process was very rapid, having a rate constant (α) of -2.56h^{-1} . The second exponential decline process which became dominant at later stages has a rate

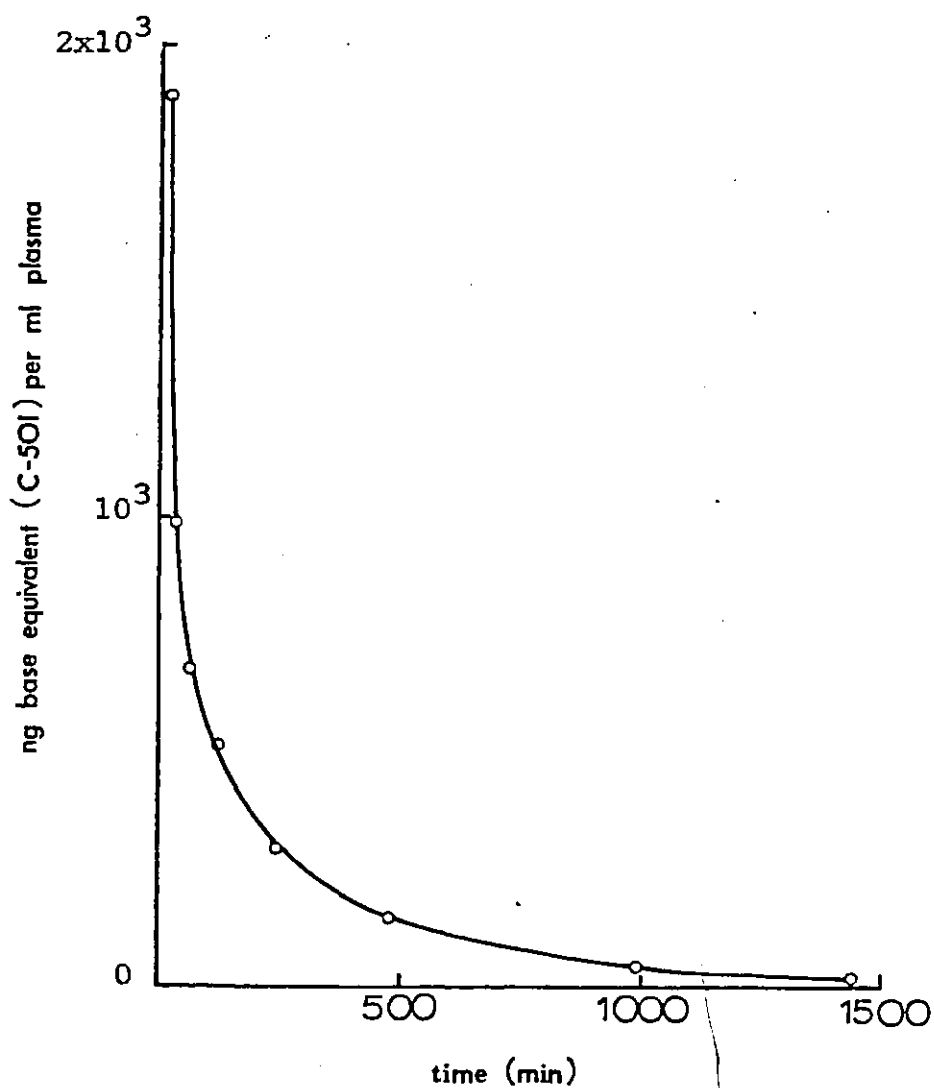


Fig.35 .Rate of disappearance of ^{14}C -501C67 in normal chicken plasma after three daily i.v. doses at 15 mg/Kg. Each point along the curve is the mean of three birds.

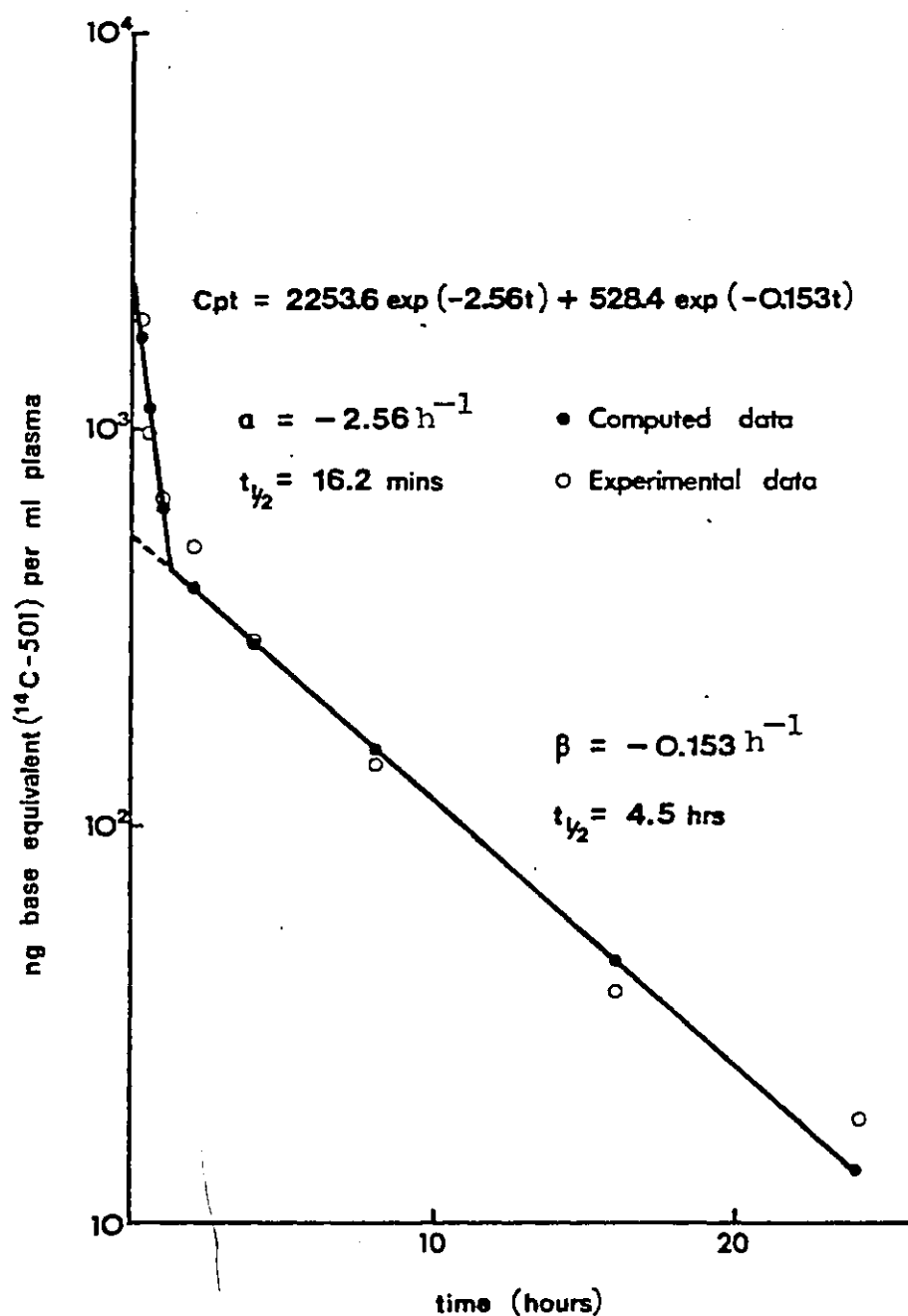


Fig.36. Semi-logarithmic plot of the rate of elimination of ^{14}C -501C67 in normal chicken plasma after three daily i.v. doses at 15 mg/Kg. Each point is the mean of three chickens.

constant (β) of -0.153h^{-1} and a biological half-life of 4.5h. It is clearly evident that after multiple dosing (c.f. Figs. 34 and 36), the rate of elimination (β) of radioactivity in the blood of normal chickens increases markedly and this effect may be attributable to the induction of hepatic microsomal enzyme systems.

The tissue distribution of radioactivity in the chicken after three daily i.v. injections of ^{14}C -501C67 at 15 mg/Kg is reported in Table 14. Like the single intravenous administration of 15 mg/Kg reported earlier, the equilibrium concentration ratios for kidney, plasma, liver, ALD and PLD muscles were all achieved 15 mins after drug administration. On comparing the data contained in Table 14 with that of Table 12, no evidence for the accumulation of radioactivity in either the blood plasma or the various tissues of the chicken was obtained after three daily i.v. administrations.

The tissue to plasma ratios for the skeletal muscles, liver and kidney were similarly examined for any signs of radioactivity accumulation and are reported in Table 15. Like that observed earlier for a single intravenous dose, very little accumulation of radioactivity was found in the ALD, PLD or the pectoral muscles. Much higher tissues to plasma ratios were observed for the two highly perfused tissues, namely, the liver and kidney and these findings are consistent with their functions as the major organs involved in metabolism and excretion.

Table 14

<u>Time after</u> <u>Dose</u>	<u>Plasma</u>	<u>ALD Muscle</u>	<u>PLD Muscle</u>	<u>Pectoral</u>	<u>Liver</u>	<u>Kidney</u>
	ug Base Equivalent g ⁻¹ Tissue					
15 min	* 1.89±0.27	2.7±0.18	1.24±0.04	0.97±0.007	18.09±3.37	36.47±0.49
30 min	0.99±0.03	2.27±0.32	0.86±0.09	0.60±0.18	10.22±2.58	23.89±4.73
60 min	0.67±0.06	1.61±0.10	0.95±0.12	0.75±0.09	11.40±2.66	11.84±2.90
2 hr	0.51±0.07	3.05±1.83	0.69±0.02	0.45±0.10	4.67±0.33	3.85±0.36
4 hr	0.29±0.09	1.24±0.21	0.41±0.04	0.36±0.05	6.02±3.16	3.05±0.51
8 hr	0.14±0.004	0.30±0.06	0.20±0.18	0.19±0.02	2.58±0.12	1.17±0.14
16 hr	0.04±0.002	0.10±0.01	0.05±0.004	0.07±0.005	0.90±0.04	0.58±0.02
24 hr	0.02±0.004	0.07±0.02	0.03±0.009	0.02±0.005	0.80±0.03	0.36±0.03

Distribution of total radioactivity in plasma and tissues after three daily intravenous administrations of ¹⁴C-501C67 at 15 mg/kg. *Mean of three birds ± S.E.M.

Table 15

<u>Time after Dose</u>	<u>ALD</u>	<u>PLD</u>	<u>Pectoral</u>	<u>Liver</u>	<u>Kidney</u>
Tissue to Plasma Ratios					
15 min	1.41	0.65	0.51	9.57	19.29
30 min	2.30	0.87	0.61	10.37	24.23
60 min	2.41	1.41	1.12	17.03	17.69
2 hr	5.98	1.36	0.88	9.15	7.55
4 hr	4.27	1.42	1.24	20.74	10.51
8 hr	2.13	1.43	1.30	18.16	8.25
16 hr	2.44	1.21	1.71	23.68	15.24
24 hr	3.61	1.78	0.94	44.17	20.17

Tissue to plasma ratios of ^{14}C -501C67 in normal chicken after three daily i.v. administrations of 15 mg/Kg.

KINETICS OF ^{14}C -501C67 REMOVAL IN NORMAL CHICKEN PLASMA
AFTER A SINGLE INTRAPERITONEAL DOSE AT 15mg/Kg

Since most of the drugs used in chemotherapy trials of dystrophic chickens have been administered intraperitoneally, it was important to find out whether there is any significant difference in the rate of elimination of radioactivity in normal chicken plasma following intravenous and intraperitoneal administration.

Reported in Fig. 37 is a semi-logarithmic plot of the concentration of radioactivity in normal chicken plasma as a function of time after a single intraperitoneal administration of 15mg/Kg. Like the pharmacokinetic data reported earlier for a single intravenous dose, the kinetics of the rate of disappearance of radioactivity in plasma after a single i.p. dose is also a double exponential process that appears to be compatible with a two-compartment open system. The first exponential decline process was found to be very rapid, having a rate constant (α) of -0.81h^{-1} . This was followed by a much slower decline that became dominant at later stages, having a rate constant (β) of -0.072h^{-1} and a biological half-life of 9.6h. Comparing this $t_{1/2}$ value for ^{14}C -501C67 with that obtained earlier for a single intravenous dose ($\beta_{iv} = 8.9\text{h}$ and $\beta_{ip} = 9.6\text{h}$), no appreciable difference in the rate of elimination of this drug was found using the two different routes of administration.

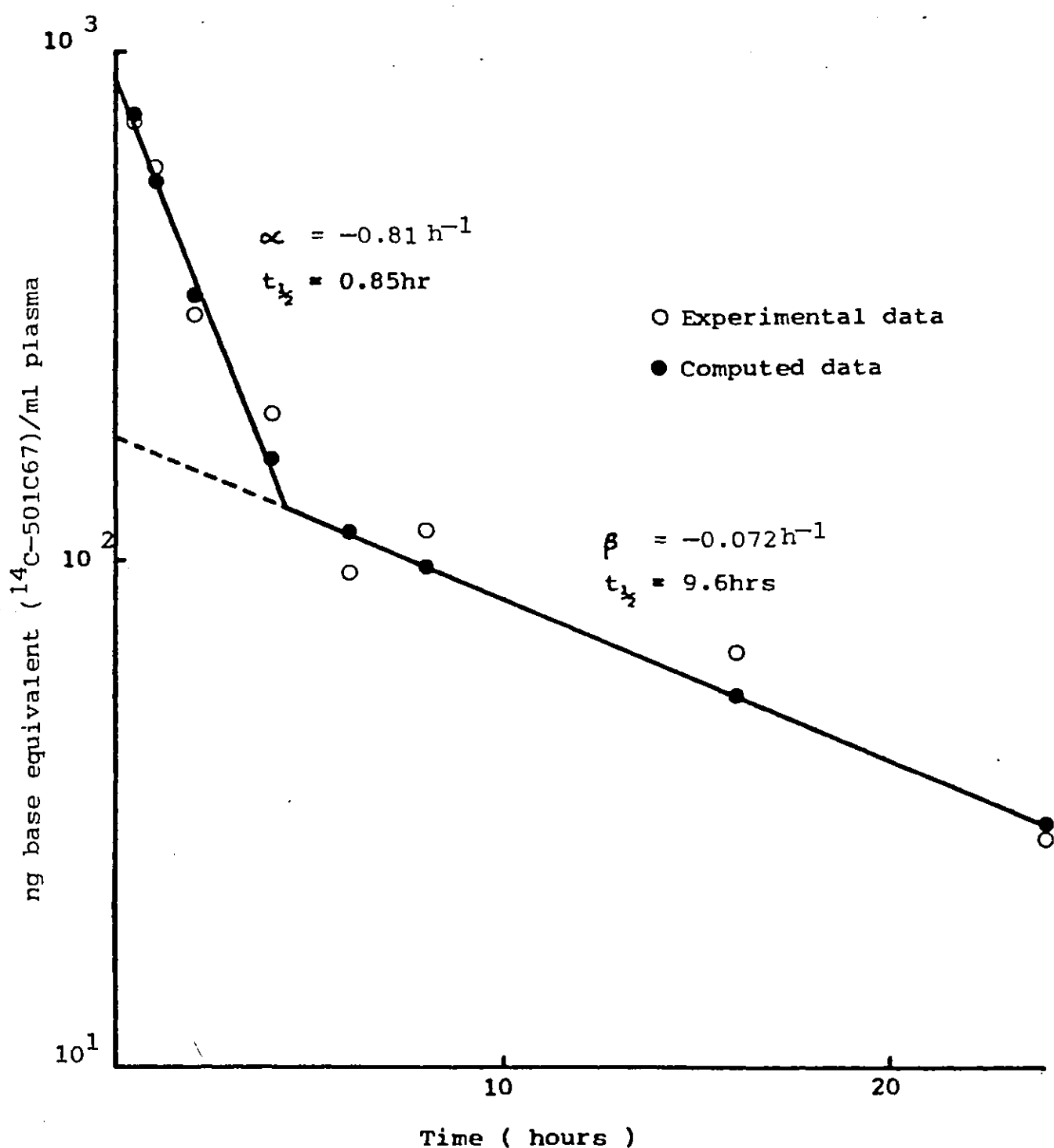


Fig. 37. Elimination of ^{14}C -501C67 in normal chicken plasma after a single intraperitoneal administration of ^{14}C -501C67 at 15 mg/Kg. Each point is the mean of three birds.

ELIMINATION OF PIZOTIFEN IN NORMAL AND DYSTROPHIC CHICKEN BLOOD

All of the pharmacokinetic data reported above for ^{14}C -501C67 were obtained using normal healthy chickens. In the dystrophic chicken, however, where a myopathy of the skeletal muscles is present along with other cell abnormalities (e.g. red blood cells), the rate of breakdown or removal of a particular drug in the blood plasma may be different. Since pizotifen has been found to have some positive effects on the course of the dystrophic process in genetically dystrophic chickens, it was of interest to see if there is any difference in the rate of removal of this drug in blood plasma between normal and dystrophic chickens.

In this study, both normal and dystrophic chickens were given daily intraperitoneal injections of pizotifen at 30 mg/Kg starting from day 4 ex ovo. After about 8 weeks of continuous treatment and immediately following the last and final dose, birds in groups of three were bled at 0.25, 0.5, 1, 7 and 24-hour intervals. Like the removal of ^{14}C -501C67 reported earlier, the elimination of pizotifen in normal chicken plasma with time is also bi-exponential (Fig. 38). The initial decline phase was very rapid, having a rate constant (α) of -0.72h^{-1} . This was followed by a much slower decline phase having a rate constant (β) of -0.073h^{-1} and an overall elimination half-life of 9.6h.

When the kinetics of pizotifen removal in dystrophic chicken plasma was examined (Fig. 39), a similar double exponential decay process was observed with time. The

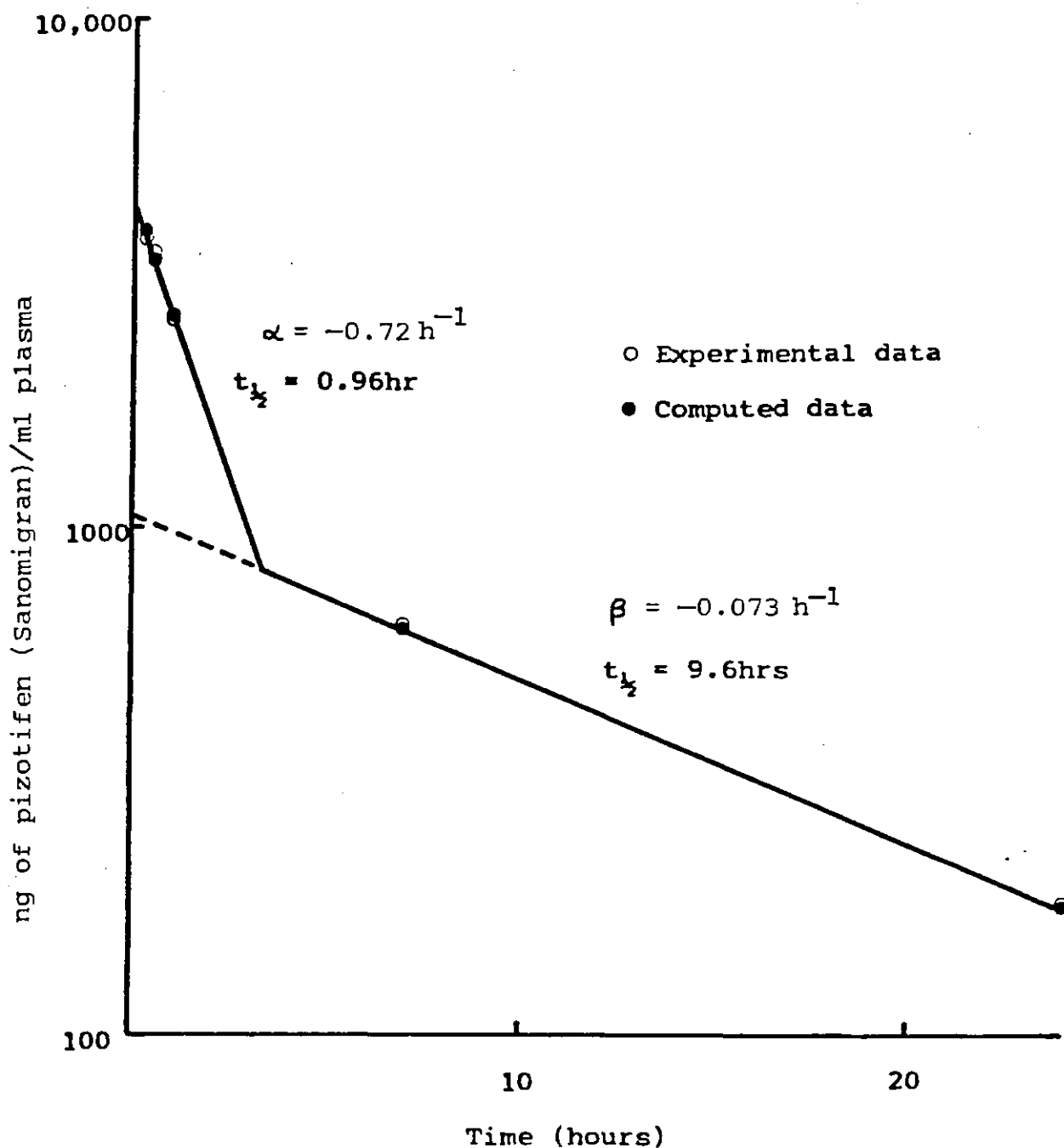


Fig.38 . Elimination of pizotifen (Sanomigran) in normal chicken plasma after daily intraperitoneal administrations of 30 mg/Kg. Each point along the double exponential line is the mean of three birds.

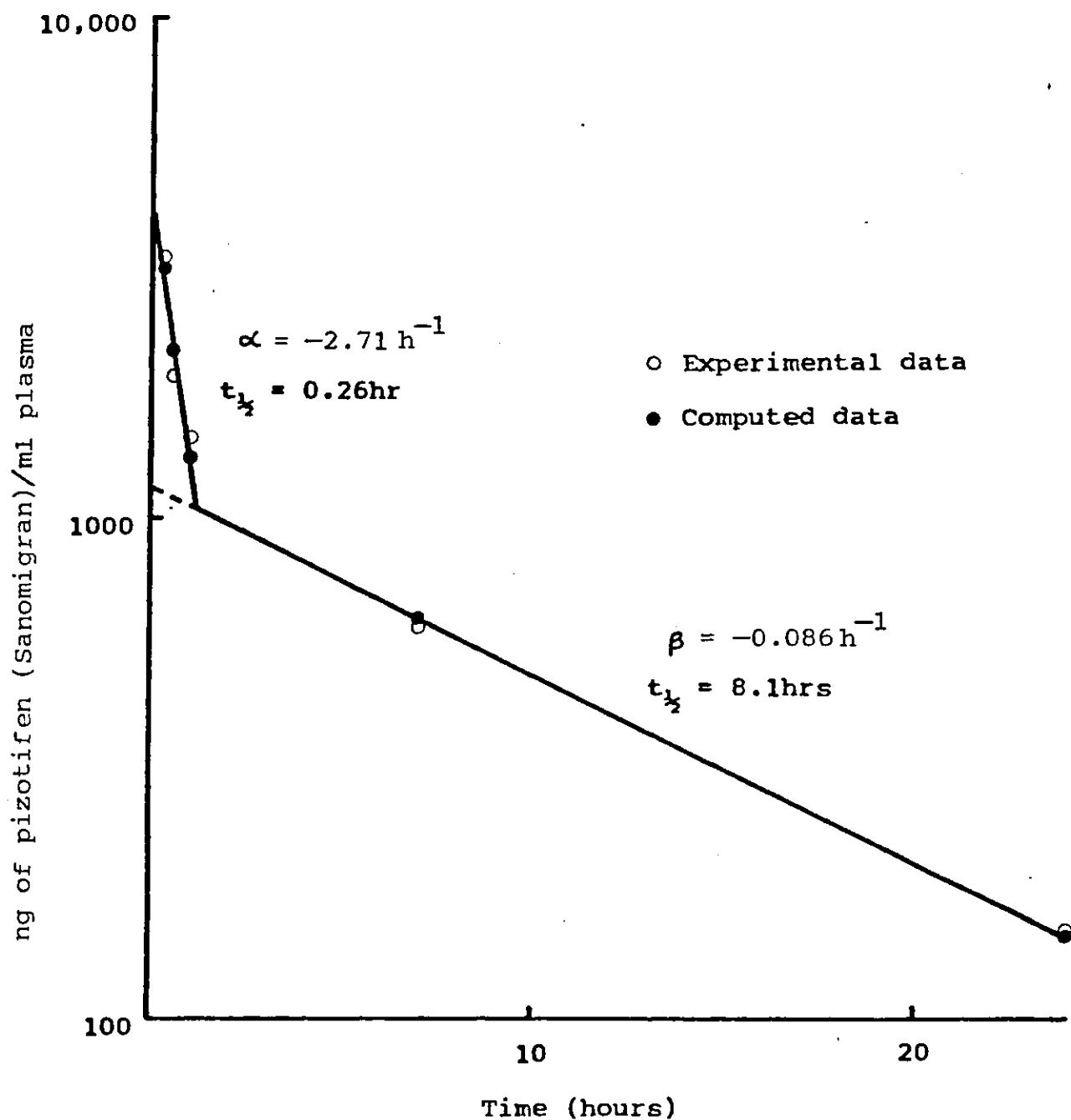


Fig.39 . Elimination of pizotifen (Sanomigran) in dystrophic chicken plasma after daily intraperitoneal administrations of 30 mg/Kg. Each point along the double exponential line is the mean of three birds.

initial decline phase was markedly faster than that observed in normal chicken plasma, having a rate constant (α) of -2.71h^{-1} . The second and much slower exponential decline process has a rate constant (β) of -0.086h^{-1} , giving a half-life value (8.1h) that is almost identical to that observed in normal chicken plasma.

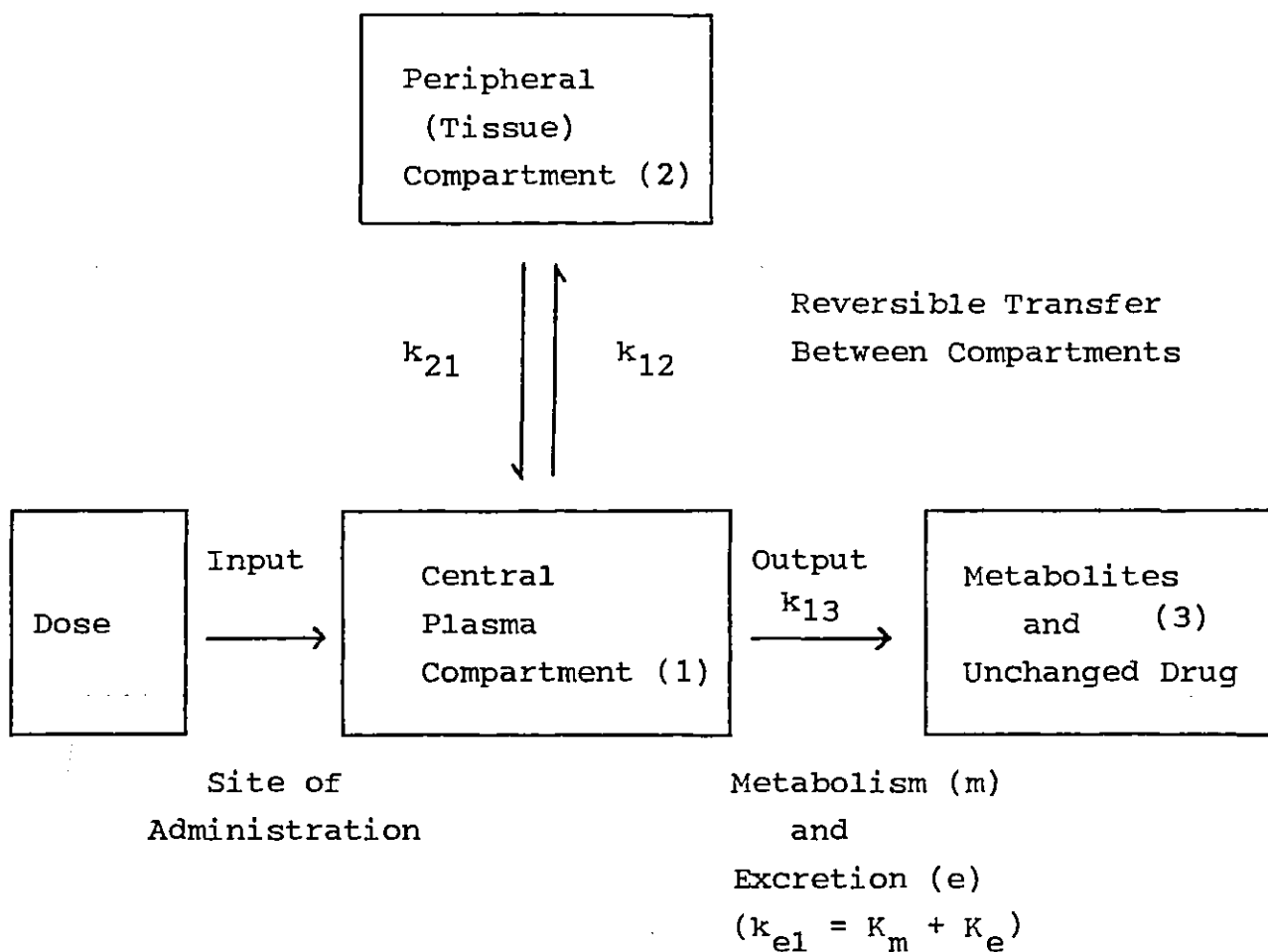
The results of this study clearly indicate that the rates of elimination of pizotifen in the plasma of both normal and dystrophic chickens are very similar if not identical. The distribution of pizotifen into the second compartment was, however, markedly faster in dystrophic chickens when compared to normal control chickens.

Discussion

From the results of these pharmacokinetic studies, a number of interesting observations have emerged concerning the elimination of ^{14}C -501C67 and pizotifen in normal and dystrophic chicken plasma. First, the experimental data obtained for the removal of ^{14}C -501C67 and pizotifen in chicken blood plasma follows a bi-exponential pattern of decline that is compatible with an open two-compartment model system. In this model system (Fig. 41), the body is considered to consist of first, a central plasma compartment that also comprises a number of freely perfused tissues (e.g. lung, brain, liver, kidney and spleen), and a second compartment comprising the remaining organs and tissues into which the drug may penetrate. The drug first enters the central plasma compartment after administration and is then ultimately eliminated by the combined effects of excretion and metabolism. The second compartment acts as a storage site into which the drugs pass reversibly, the direction of net transfer being governed by the concentrations and rate constants.

Thus the rapid initial decline of drug in chicken plasma is due mainly to the rapid distribution of drug into the second compartment, although some metabolism of drug also takes place. Therefore, the rate constant (α) is a measure of the initial speed of transfer or distribution of these drugs into the second compartment. The rate constant (β), on the other hand, measures the rate of elimination of the drug due to metabolic and excretory factors.

In these studies, the radioactive compound ^{14}C -501C67 was found to have a relatively long half-life of about 9h in normal chicken plasma after a single administration of the



k_{21} = rate constant of transfer from the peripheral compartment into the central compartment

k_{12} = rate constant of transfer from the central compartment into the peripheral compartment

k_{13} = elimination rate constant

Fig. 40. Two-compartment open model system

drug (8.9h for i.v. and 9.6h for i.p.). Although Jones and Lees (1978) have reported that BW 501C67 is metabolised rapidly in both the rat and the patas monkey, no reports on the half-life value for this drug were given in either animal. Whether the half-life value obtained in this study for radioactivity represents that for the parent compound (^{14}C -501C67) or its metabolites is impossible to say and further studies involving specific assay methods for BW 501C67 (e.g. HPLC) need to be employed.

Multiple dosing of ^{14}C -501C67 markedly reduced the elimination half-life of radioactivity in chicken blood from about 9h to about 4.5h. Although no activation of rat hepatic microsomal enzymes by BW 501C67 was observed by Nimmo-Smith and Savage (1978), the presence of an inducing effect of ^{14}C -501C67 or its metabolites on the chicken hepatic microsomal enzymes is inferred from this study. The rate of elimination of drug may even increase further with continual drug administration and this should be taken into account in any chemotherapy trials involving this drug. No accumulation in the levels of radioactivity was observed in either the blood plasma or the tissues of the chicken after multiple dosing.

As reported above, both the kinetics of removal of the radioactivity and its biological half-life after an intraperitoneal dose were very similar to those found following intravenous administration. This suggests that absorption of the drug into the central plasma compartment after an intraperitoneal injection is very rapid.

That the rate of elimination of pizotifen was found to be very similar in the blood plasma of normal and dystrophic chickens suggests that a defect in the metabolic or excretory

pathway in the dystrophic animals is very unlikely. Further, it was observed that the initial decline phase (α) of pizotifen in dystrophic chicken plasma was appreciably faster than that observed in normal chicken plasma, indicating that some tissues of the dystrophic chicken are more permeable to the drug. In both the animal and human dystrophies, a generalised membrane defect may be present (De Kretser and Livett 1977, Butterfield 1977, Lucy 1980) and the rapid fall in pizotifen levels seen initially in dystrophic chicken blood may be a consequence of this abnormality.

To conclude, these pharmacokinetic studies show that of the two serotonin antagonists investigated, BW 501C67 is eliminated about twice as fast as that observed for pizotifen in chicken blood. Further, the rate of elimination of BW 501C67 in chicken blood may even be increased on long-term drug administration as pizotifen had been and this has to be taken into account when comparing the elimination rates of these two drugs. This and other factors such as dosage levels used need to be considered when comparing the chemotherapeutic effectiveness of these two compounds as antidystrophic agents in the chicken.

CHAPTER 6

CHEMOTHERAPY OF DYSTROPHIC CHICKENS

INTRODUCTION

The dystrophic chicken provides us with an animal model of dystrophy that shows closely the functional, biochemical and histopathological features that are characteristic of human Duchenne muscular dystrophy. Unlike other animal models, the genetically dystrophic chicken has a number of attributes in its favour for studying the effects of potential antidystrophic drugs on the disease process and these are:

- 1) The functional ability or disability of these birds could be easily monitored in a quantitative manner by use of either the flip (FN) or wing apposition (WA) tests.

- 2) Biochemical markers of dystrophy have been identified in the dystrophic plasma and used to monitor chemotherapy trials. These markers of the dystrophic muscle include creatine phosphokinase (CPK), pyruvate kinase (PK), acetylcholinesterase (AChE) and possibly also phosphoglyceromutase (PGM).

- 3) Histopathological changes may be observed and quantitated both before and after drug therapy.

- 4) The present dystrophic line (413 chickens) is selected for early onset of symptoms and therefore, more drugs can be tested than otherwise.

Using the dystrophic chicken as an experimental test system for chemotherapy purposes, three classes of possible antidystrophic drugs were investigated in vivo to see if they could arrest or alter the course of the dys-

trophic process in these animals. The first class of these drugs used in the treatment of dystrophic chickens were serotonin antagonists.

Over the last fifteen years, several lines of evidence have suggested that serotonin (5-hydroxytryptamine) may be involved in the development of dystrophy (for a review, see Hudecki and Barnard, 1976). The idea of serotonin antagonism in muscular dystrophy, however, was first developed by Barnard and associates (1976) when they managed to show positive drug-effects with two potent serotonin antagonists, cyproheptadine and methysergide. Subsequent studies by these investigators and others have shown that this effect is more general and that other potent antiserotonin drugs having widely divergent chemical structures were also beneficial to these animals (9, 10, 11, 82). The work presented here is an extension of these studies so that a more effective antidystrophic drug could be found.

Another class of compounds that were tested in these dystrophic birds were antimyotonic drugs. It had been suggested earlier that dystrophic chicken muscle fibres may be "myotonic" (Holiday et al. 1965) due to its abnormal electromyographic properties. Consistent with this suggestion, an earlier study (Entrikin et al. 1977) showed that when dystrophic chickens were treated with the antimyotonic drug, diphenylhydantoin (DPH), marked functional and biochemical improvements were observed. It was of interest, therefore, to investigate the chemotherapeutic value of this class of drugs in the dystrophic chicken.

A third class of drug used in the chemotherapy of dystrophic chickens has neurotrophic properties. Isaxonine (N-isopropylamino-2-pyrimidine-phosphate, IAPP) is a drug that has been shown to promote nerve growth in laboratory animals. It has also been shown recently that IAPP accelerates muscle reinnervation in patients suffering from leprous neuritis, a particular form of motor neuropathy (Hugelin et al. 1977). Since a neurotrophic defect cannot be entirely ruled out in both the human and animal dystrophies, it was of interest to evaluate the effects of these drugs on the disease process in the dystrophic chicken.

Having established the value of plasma PK as a sensitive and reliable marker of the condition of the dystrophic muscle in these myopathic animals (chapter 2), it was natural to use this enzyme marker to monitor the chemotherapeutic value of the three classes of drugs mentioned above. Using PK in conjunction with other measurable parameters of the dystrophic muscle discussed above, several drugs were found to have a beneficial effect on these dystrophic animals and may also be of value in the treatment of dystrophic humans.

MATERIALS AND METHODS

Animals. As reported above (Materials and Methods, chapter 2).

Drugs. Cyproheptadine HCl, hydroxindasol and benanserine were supplied by Merck Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A.

Prajineline was a gift from Miles Laboratories Ltd., Stoke Court, Slough, U.K.

Methysergide and pizotifen hydrogen malate (Sandomigran) were obtained from Sandoz Pharmaceuticals, Leeds, U.K.

Sodium phenytoin (DPH) was obtained from Parke, Davies and Co., Usk Road, Pontypool, Gwent.

Methiothepin maleate was obtained from Hoffmann-La-Roche Inc., Nutley, New Jersey, U.S.A.

Cinanserin was obtained from Squibb, N.J., U.S.A.

Isaxonine (IAPP) was supplied by Beaufour Dreux Laboratories, France.

Hepzidine was obtained from Broades, G.B. Ltd.

Wellcome 501C67 was a gift from Wellcome Research Laboratories, Langley Court, Beckenham, Kent.

Chemicals. PK Test Combination (U.V. method) kits were purchased from Boehringer-Mannheim Corp., Crawley, Sussex.

Drug Treatments. All drug injections were made by the Animal Unit Staff. From 4 days ex ovo, dystrophic chicks undergoing drug treatment were injected intraperitoneally once daily, unless otherwise stated, with aqueous solutions of the drugs. Dystrophic control chicks (untreated) were given similar injections of water only. The dosages used for each drug is indicated at the bottom of each table in the results section and the actual amount of drug injected into each animal was based on the average bodyweight of the group of dystrophic chickens receiving therapy. All birds (treated and untreated) were weighed once weekly to monitor any toxic effects that these drugs may have on the treated chickens.

Blood Specimens. Blood samples were collected and processed as previously described (Materials and Methods, chapter 2).

Assays. Both PK and CPK activities were measured as described previously (Materials and Methods, chapter 2).

Enzyme Dilutions. All enzyme dilutions were made with 0.85% physiological saline solution.

Physical Testing of Chickens. As described above.

RESULTS

EFFECTS OF SEROTONIN ANTAGONISTS ON PLASMA PK

ACTIVITY AND RELATED PARAMETERS

CYPROHEPTADINE

Cyproheptadine, a very potent serotonin receptor antagonist, had been shown previously to be effective in delaying the onset of muscular dystrophy in the genetically dystrophic chicken (Barnard et al. 1976). In the present study, cyproheptadine was administered at two different doses and its effect on the disease process was monitored by plasma PK and a number of other well-established parameters of dystrophy. Dystrophic chickens treated with cyproheptadine alone showed a dose related response to the drug in terms of their plasma PK activities and their functioning abilities (FN). When dystrophic chickens are administered intraperitoneally with cyproheptadine at a daily dosage of 10 mg/Kg starting from day 1 ex ovo to about 60 days ex ovo, large reductions in plasma PK were observed after about 40 days ex ovo but only slight functional improvements accompanied this change (results not shown).

Table 16

Effect of Cyproheptadine on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
18 - 19	* 251 $\pm$ 61 (7)	* 212 $\pm$ 21 (40)	+ 18%	N.S.
22 - 26	511 $\pm$ 107 (6)	525 $\pm$ 41 (99)	- 3%	N.S.
28 - 33	405 $\pm$ 46 (7)	1196 $\pm$ 61 (203)	- 66%	<0.02
35 - 39	783 $\pm$ 151 (7)	1988 $\pm$ 100 (151)	- 61%	0.01
41 - 46	1216 $\pm$ 196 (7)	2857 $\pm$ 141 (152)	- 57%	<0.02
50 - 54	828 $\pm$ 177 (7)	2844 $\pm$ 219 (85)	- 71%	<0.02
56 - 61	1154 $\pm$ 309 (6)	3522 $\pm$ 168 (122)	- 67%	<0.01

Dystrophic chickens were treated daily with 20 mg/Kg of cyproheptadine from 4 days ex ovo. Untreated dystrophic control chickens were given similar injections of water only.

* Mean PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

However, when the dosage was increased to 20 mg/Kg/day using the same route of administration and for the same length of time, similar reductions in plasma PK occurred, but this time the treated birds performed significantly better than the corresponding untreated dystrophic control birds in terms of the flip test (FN data donated by Dr. P. J. Barnard).

Presented in Table 16 are the plasma PK activities of dystrophic chickens treated with 20 mg/Kg of cyproheptadine for a period of about 60 days. It is clearly evident that after about 4 weeks ex ovo, a marked reduction in the mean plasma PK activity of cyproheptadine-treated chickens was observed when compared to untreated dystrophic control chickens and this difference was found by statistical analysis (Students 't' test) to be highly significant (i.e. $P < 0.02$). The plasma PK levels in the treated birds remained significantly lower than the corresponding control chickens for the remaining period that was investigated.

Plasma CPK activities were also measured in the cyproheptadine-treated chickens and were also found to be substantially reduced (between -3% to -81%) when compared to untreated dystrophic control chickens (Data donated by Dr. J. Lyles).

That the two plasma enzyme activities follow very closely to one another in the cyproheptadine-treated chickens is indicated by the very high degree of association that is observed in these chickens throughout the period of treatment. Fig. 41 shows the degree of association between plasma CPK and PK at 25 days ex ovo. The correl-

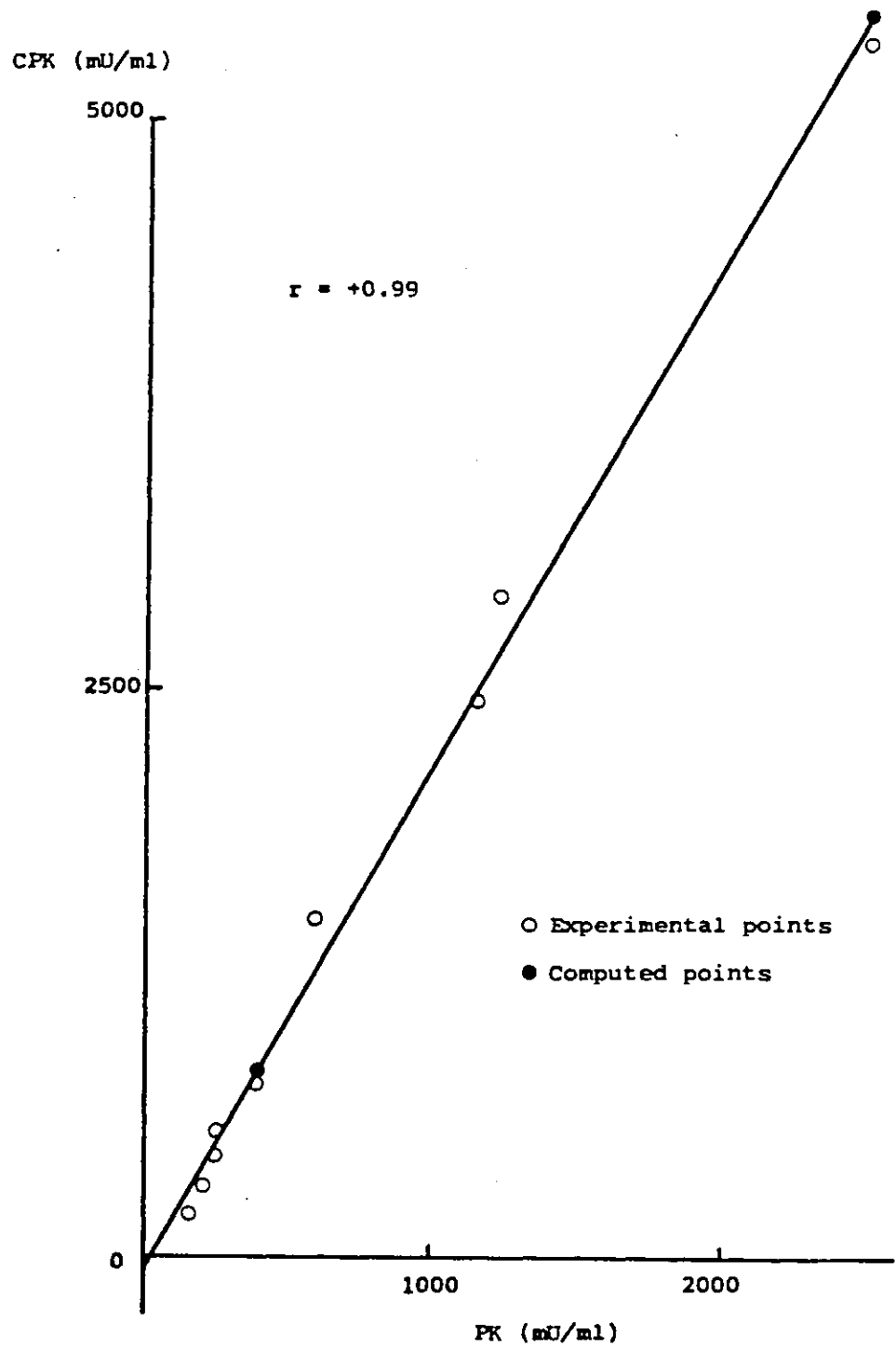


Figure 41. Association of CPK and PK activities in plasma from cyproheptadine-treated dystrophic chickens. Points represent single birds of various weights and either sex at age 25 days ex ovo. The correlation coefficient (r) was +0.99.

ation coefficient (r) for this association was computed to be +0.99. Although this association was not shown for a later age, the two enzyme activities in almost all cases moved in concert during the entire period of treatment.

Cyproheptadine-treated dystrophic chickens showed no appreciable weight-loss when compared to untreated dystrophic control birds.

CINANSERIN

Daily intraperitoneal administration of cinanserin (another potent serotonin antagonist) at 40 mg/Kg, starting from day 4 ex ovo and continued until about 60 days ex ovo, markedly reduced the mean plasma PK activity in the treated animals from about 28 days ex ovo to about 50 days ex ovo (Table 17). Statistical analysis (Students 't' test) showed that the difference in PK activity between the treated and untreated groups during this period was highly significant ($P < 0.02$). After this period, however, the mean plasma PK activity in the treated birds increased to about the same level as the untreated control chickens.

Like cyproheptadine-treated dystrophic chickens, these birds responded well on treatment with cinanserin and performed significantly better in the flip test when compared to the untreated dystrophic control chickens. The functional improvement in these animals was maintained up to about 60 days ex ovo when treatment was discontinued.

In addition to plasma PK, plasma CPK activities were also examined in the cinanserin-treated chickens and found to decline substantially (between -25% to -60%) when compared to untreated dystrophic control chickens (Data

Table 17
Effect of Cinanserin on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY</u>
28 - 33	* 605 $\pm$ 104 (12)	* 1196 $\pm$ 61 (203)	-49%	0.02
35 - 39	592 $\pm$ 104 (12)	1988 $\pm$ 100 (151)	-70%	<0.001
41 - 46	1472 $\pm$ 338 (11)	2857 $\pm$ 141 (152)	-49%	0.01
50 - 54	3022 $\pm$ 602 (11)	2844 $\pm$ 219 (85)	+6%	N.S.
56 - 61	3407 $\pm$ 574 (11)	3522 $\pm$ 168 (122)	-3%	N.S.

Dystrophic chickens were treated daily with 40 mg/Kg of cinanserin from 4 days ex ovo. Dystrophic control chickens were given similar injections of water only. Plasma PK activities are expressed in mU/ml plasma.

*Mean PK activity $\pm$ SEM, No. of birds used in parenthesis.

N.S. = Not Statistically Significant (P > 0.05)

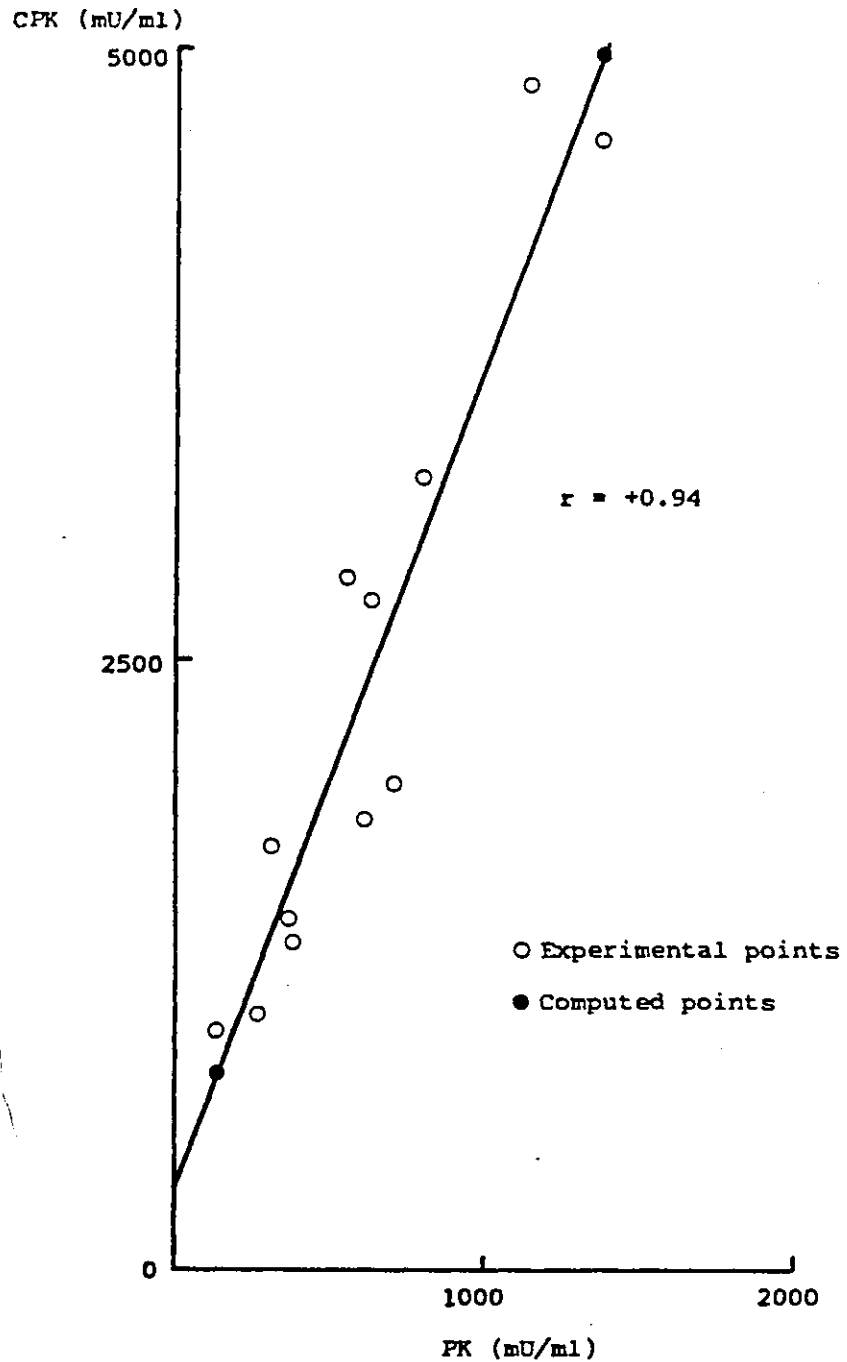


Fig.42 . Association of CPK and PK activities in plasma from cinanserlin - treated dystrophic chickens. Points represent single birds of various weights and either sex at age 28 days ex ovo. The correlation coefficient was +0.94.

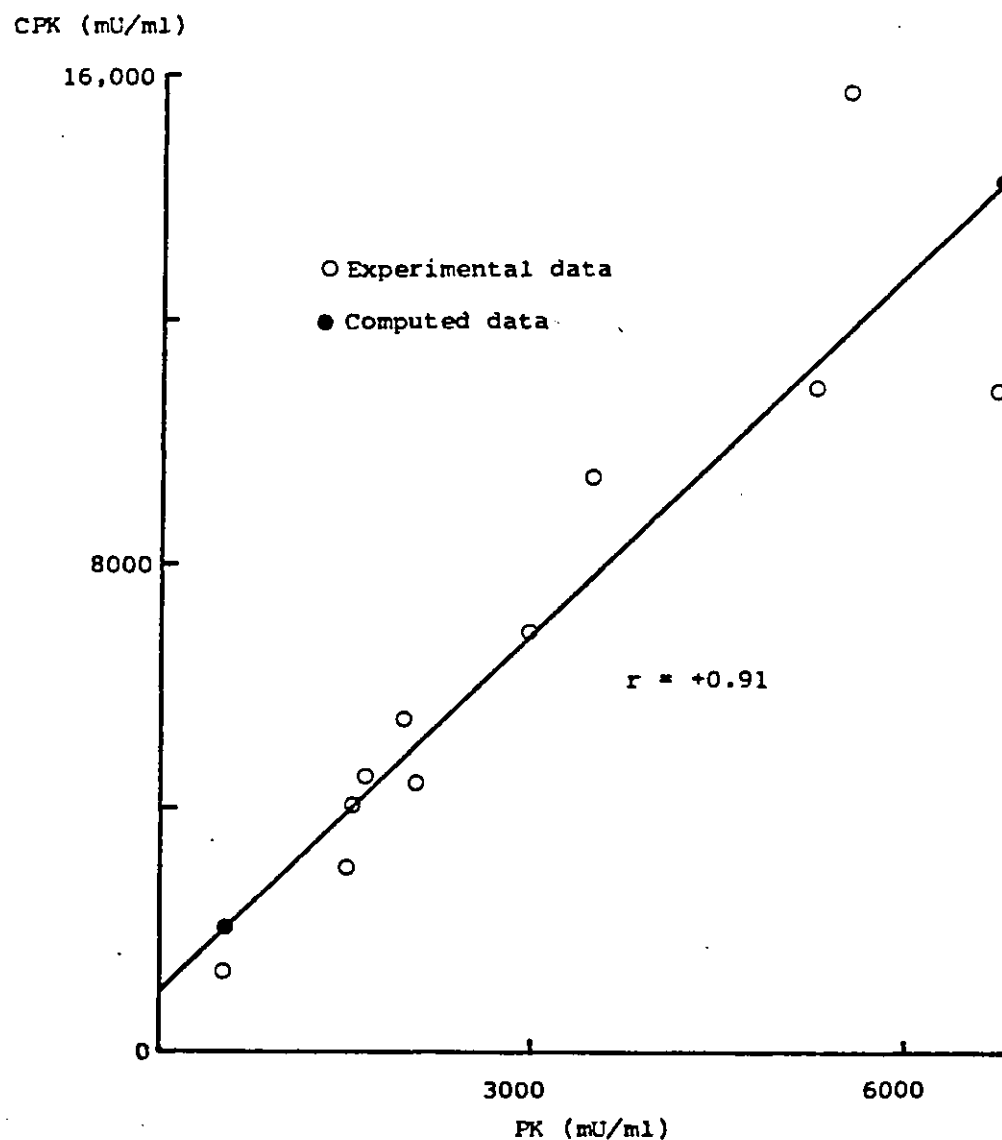


Fig. 43. Association of CPK and PK activities in plasma from cinanserin - treated dystrophic chickens. Points represent single birds of various weights and either sex at age 49 days ex ovo. The correlation coefficient was +0.91.

kindly supplied by Dr. J. Lyles).

Like cyproheptadine-treated birds, a similar correlation was observed between the enzyme activities of plasma CPK and PK in the cinanserin-treated chickens. Whenever examined, the two enzyme activities moved in concert during the entire period of drug treatment. Figs. 42 and 43 illustrate at two different ages the very high degree of association between plasma CPK and PK in the cinanserin-treated animals. The correlation coefficient (r) at day 28 ex ovo and day 49 ex ovo were calculated to be +0.94 and +0.91 respectively. The ratio of CPK to PK efflux varied between 2 and 3 during this period of investigation.

Cinanserin-treated dystrophic chickens showed little signs of toxicity in terms of weight loss at the dosage used when compared to untreated birds of the same age.

PIZOTIFEN

Pizotifen (Sanomigran), a drug which is known to have powerful antiserotonin properties was administered daily to dystrophic chickens at two different doses starting from day 4 ex ovo. When given intraperitoneally at a very low dosage of 1mg/Kg/day for about 60 days, substantial reductions in plasma PK activities were observed in the treated birds after about 35 days ex ovo. When the dosage was increased to 20 mg/Kg/day using the same route of administration and for the same length of time, even greater reductions in plasma PK levels were observed in the treated chickens when compared to untreated dystrophic

Table 18

Effect of Pizotifen on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
18 - 19	* 235 $\pm$ 24 (12)	* 212 $\pm$ 21 (40)	+ 11%	N.S.
22 - 26	423 $\pm$ 53 (11)	525 $\pm$ 41 (99)	- 19%	N.S.
28 - 33	611 $\pm$ 99 (10)	1196 $\pm$ 61 (203)	- 49%	<0.05
35 - 39	653 $\pm$ 88 (10)	1988 $\pm$ 100 (151)	- 67%	<0.001
41 - 46	1426 $\pm$ 255 (10)	2857 $\pm$ 141 (152)	- 50%	0.01
50 - 54	1197 $\pm$ 180 (10)	2844 $\pm$ 219 (85)	- 58%	<0.02
56 - 61	1056 $\pm$ 175 (9)	3522 $\pm$ 168 (122)	- 70%	<0.002

Dystrophic chickens were treated daily with 20 mg/Kg of pizotifen (sanomigran) from 4 days ex ovo. Untreated dystrophic control chickens were given similar injections of water only. * Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

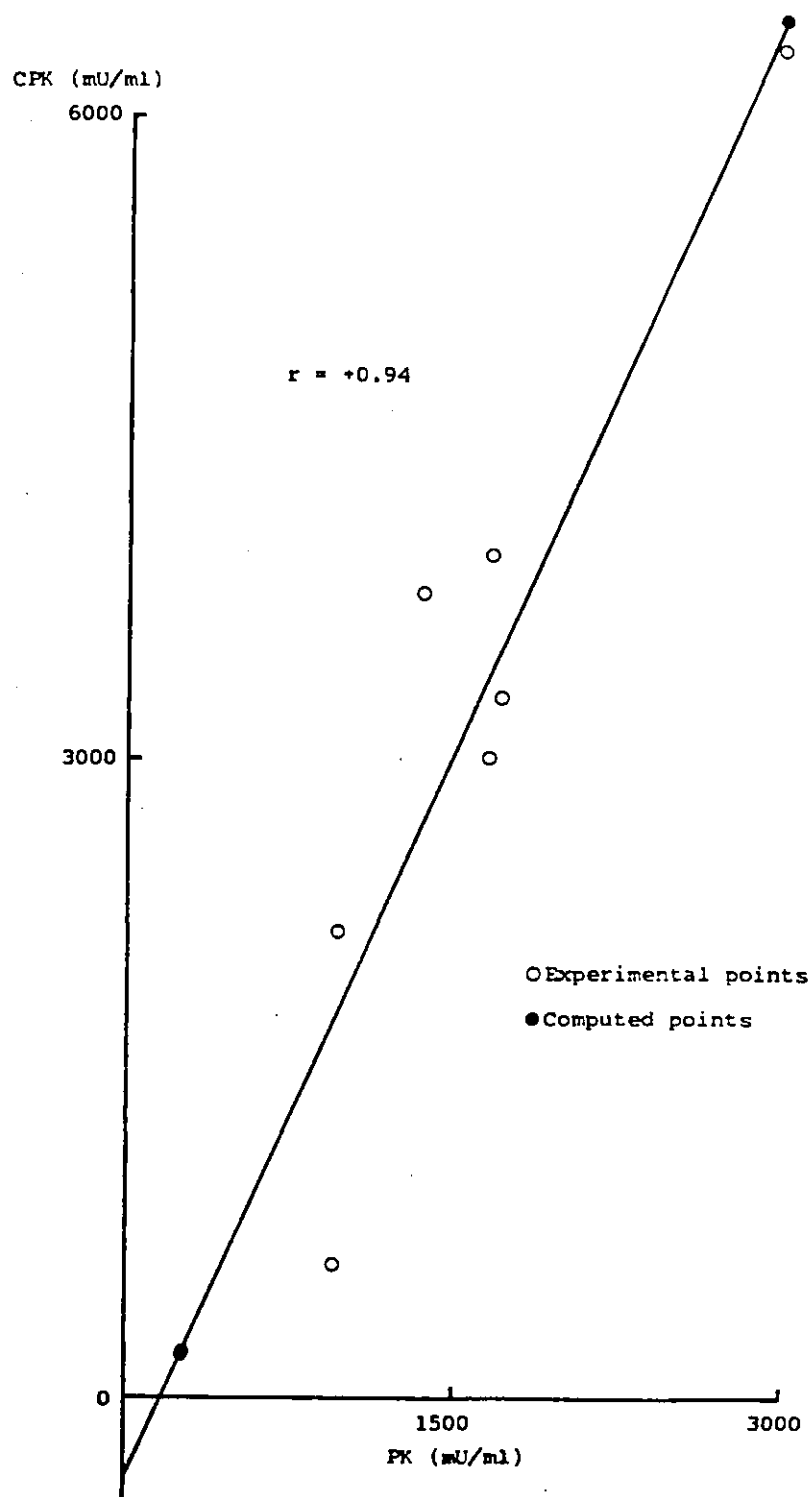


Fig. 44. Association of CPK and PK activities in plasma from pizotifen-treated dystrophic chickens. Points represent single birds of various weights and either sex at age 41 days ex ovo. The correlation coefficient (r) was +0.94.

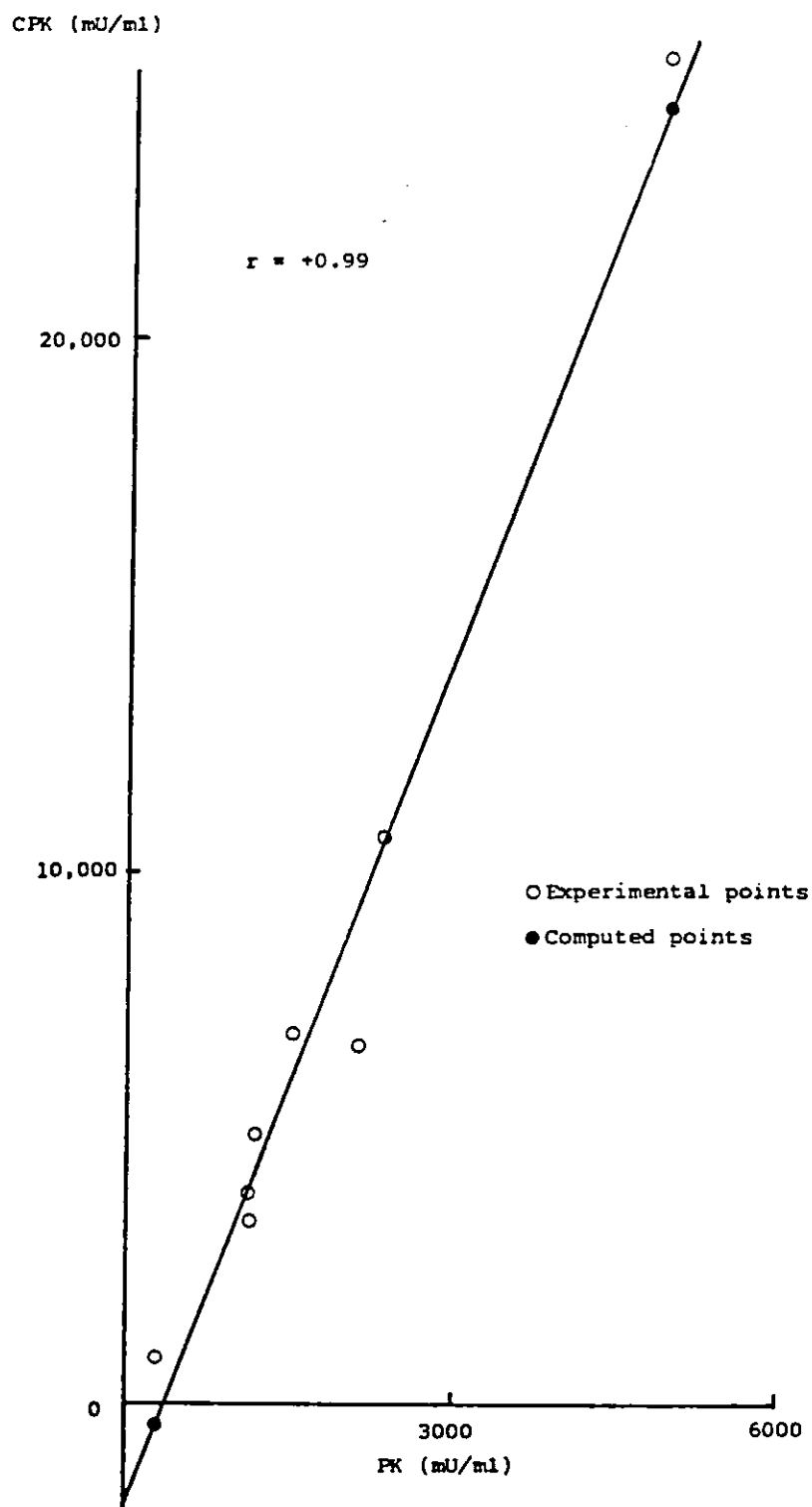


Fig. 45. Association of CPK and PK activities in plasma from pizotifen-treated dystrophic chickens. Points represent single birds of various weights and either sex at age 57 days *ex ovo*. The correlation coefficient (r) was +0.99.

control chickens (Table 18). The difference in plasma PK levels between the two groups was found to be statistically significant ($P < 0.05$).

In line with the biochemical improvements that were observed above, pizotifen-treated chickens also showed marked functional improvements in terms of their righting ability when compared to untreated control animals. This improvement in muscular performance was maintained up to the period when drug treatment was discontinued (Data donated by Dr. P.J. Barnard).

In addition to the reduction in plasma PK levels mentioned above in pizotifen-treated chickens, plasma CPK levels when examined were also suppressed considerably (between -14% to -67%) when compared to dystrophic control chickens (Data from Dr. J. Lyles).

Like the cyproheptadine and cinanserin treated chickens, a very high degree of correlation was observed between plasma CPK and PK in pizotifen-treated chickens. This is illustrated at days 41 ex ovo (Fig. 44) and 57 days ex ovo (Fig. 45) in the same batch of treated chickens. The correlation coefficient (r) for the two ages were computed to be +0.94 and +0.99 respectively. The ratio of CPK to PK output in this case varied between 2 and 5.

Pizotifen-treated birds showed no appreciable loss in weight (less than -15%) during treatment when compared to the corresponding dystrophic controls of the same age.

BW 501C67

Compound BW 501C67 (α -anilino-N(2-m-chlorophenoxy propyl) acetamidine), developed at the Wellcome Research

Laboratories, Beckenham, has been reported to possess marked anti-serotonin properties. Dystrophic chickens treated with this drug from 4 days ex ovo to about 60 days ex ovo at a daily intraperitoneal dose of 10 mg/Kg bodyweight showed no appreciable reduction in their mean plasma PK activities or an improvement in their mean flip values when compared to the corresponding untreated dystrophic control animals (results not shown). When the dosage was increased to 15 mg/Kg/day using the same route of administration and for about the same length of time in a different batch of dystrophic chickens, large reductions in plasma PK activities were observed (Table 19). Statistical analysis (Students 't' test) showed that the difference in PK levels between the treated and untreated groups was highly significant ($P < 0.01$).

Inconsistent with the biochemical measurements observed above, BW 501C67-treated dystrophic chickens showed very little or no functional improvements in terms of their righting abilities over the entire period investigated (Data from Dr. P.J. Barnard).

Plasma CPK activities were also investigated in BW 501C67-treated chickens and although its activity in dystrophic plasma was depressed considerably (between -15% to -40%), these reductions were smaller than those observed for plasma PK (Data from Dr. J. Lyles).

A similar correlation between the enzyme activities of CPK and PK in the plasma of BW 501C67-treated dystrophic chickens was observed and illustrated at day 25 ex ovo (Fig. 46) and day 32 ex ovo (Fig. 47). The correlation

Table 19

Effect of BW 501C67 on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
12 - 15	* 103 $\pm$ 10 (12)	* 146 $\pm$ 10 (27)	- 30%	<0.01
22 - 26	409 $\pm$ 65 (10)	525 $\pm$ 41 (99)	- 22%	N.S.
28 - 33	917 $\pm$ 119 (10)	1196 $\pm$ 61 (203)	- 23%	N.S.
35 - 39	831 $\pm$ 135 (10)	1988 $\pm$ 100 (151)	- 58%	<0.01
41 - 46	1147 $\pm$ 201 (10)	2857 $\pm$ 141 (152)	- 60%	<0.01
50 - 54	504 $\pm$ 102 (9)	2844 $\pm$ 219 (85)	- 82%	<0.001
56 - 61	1776 $\pm$ 329 (8)	3522 $\pm$ 168 (122)	- 50%	<0.01
63 - 67	2083 $\pm$ 262 (8)	3467 $\pm$ 197 (51)	- 40%	<0.01

Dystrophic chickens were treated daily with 15 mg/Kg of BW 501C67 from day 4 ex ovo. Untreated dystrophic control chickens were given similar injections of water only.

* Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

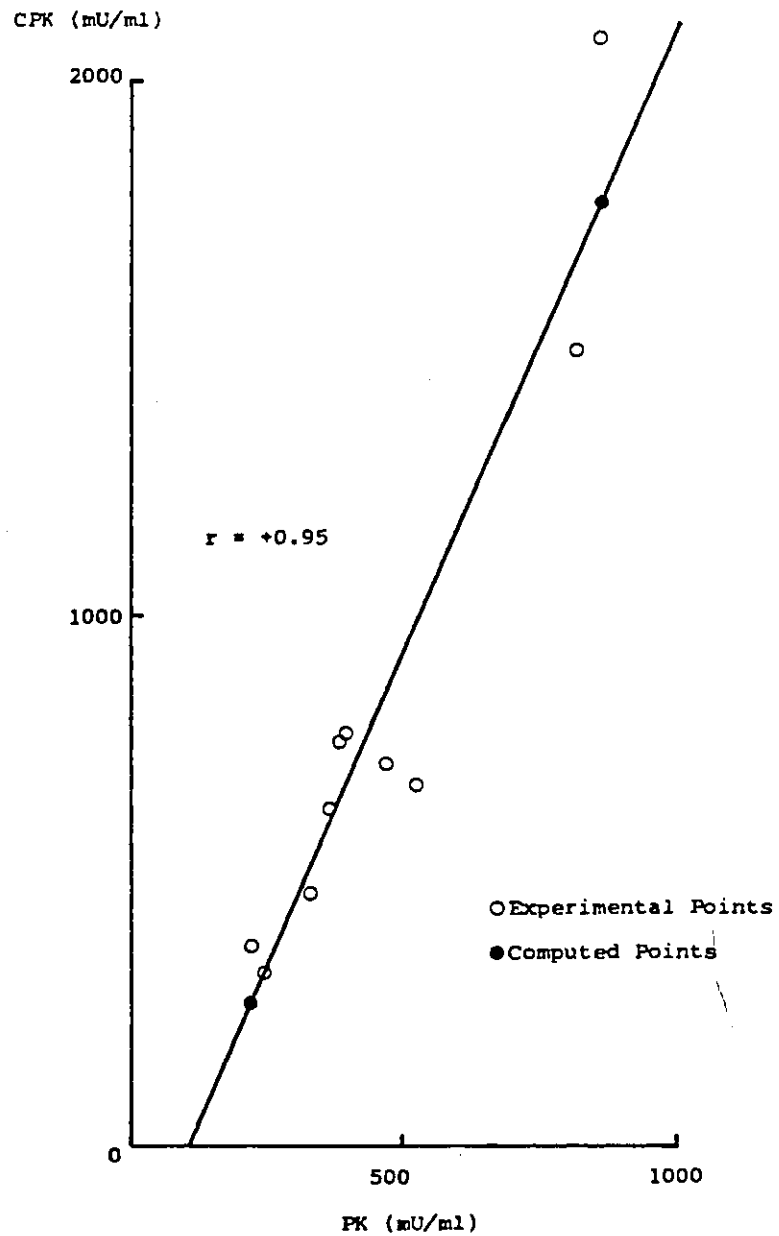


Fig. 46. Association of CPK and PK activities in plasma from BW 501C67-treated dystrophic chickens. Points represent single birds of various weights and either sex at age 25 days ex ovo. The correlation coefficient (r) was +0.95.

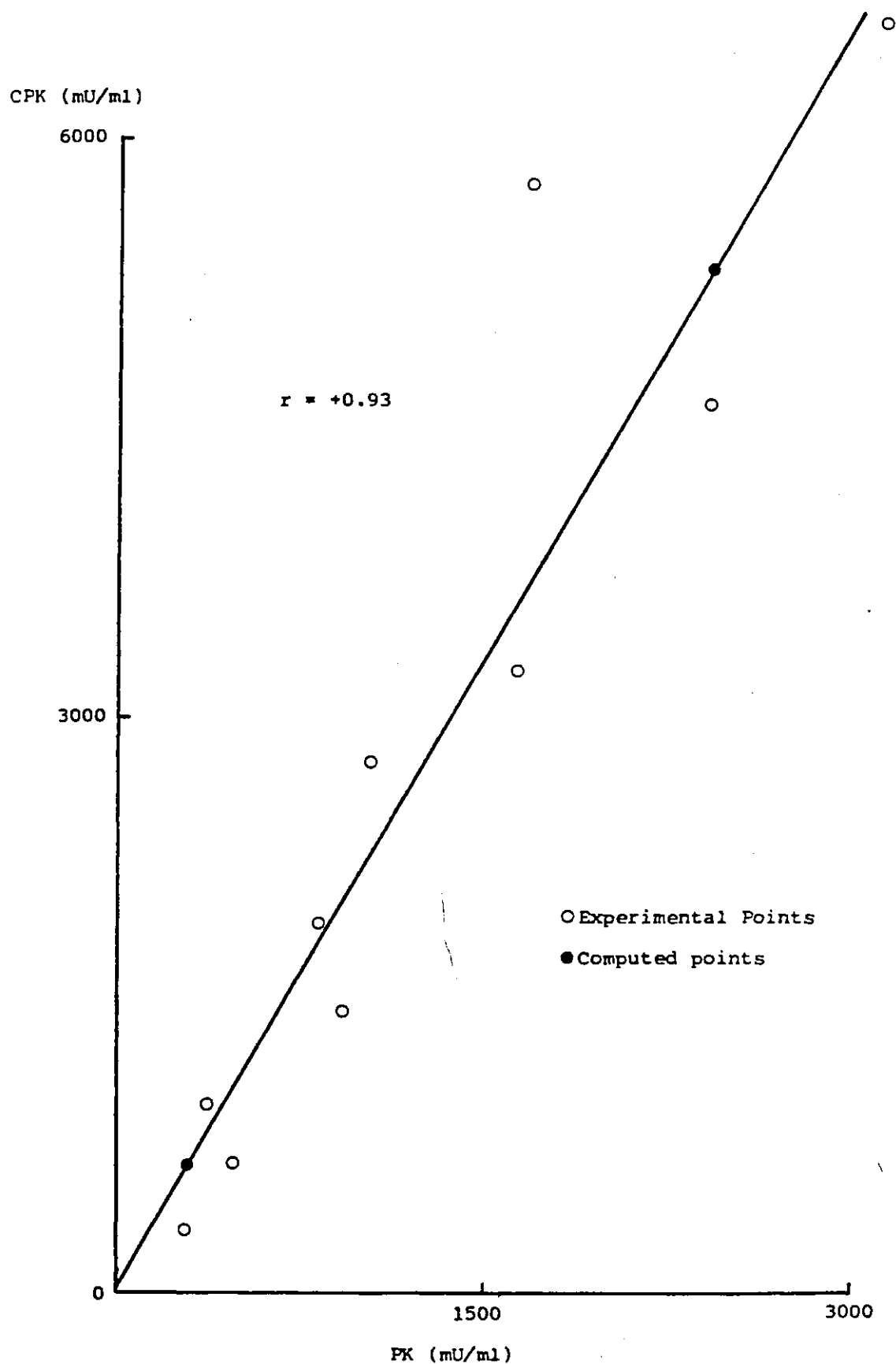


Fig. 47. Association of CPK and PK activities in plasma from BW 501C67-treated dystrophic chickens. Points represent single birds of various weights and either sex at age 32 days ex ovo. The correlation coefficient (r) was $+0.93$.

coefficient (r) for these two ages were computed to be +0.95 and +0.93 respectively. The ratios of CPK to PK activities in plasma varied between 2 and 3 during this period.

The compound BW 501C67 was found to be rather toxic to these animals at the dosage used and birds receiving treatment showed a loss in bodyweight of up to 30% when compared to untreated control birds.

METHIOTHEPIN

Daily intraperitoneal administration of methiothepin maleate, another potent serotonin receptor antagonist, to dystrophic chickens at a dose of 20 mg/Kg starting from day 4 ex ovo, resulted in large reductions in plasma PK activities when compared to untreated dystrophic chickens of the same age (Table 20). These reductions in plasma PK levels in the methiothepin-treated group became statistically significant (Students 't' test) when compared to the untreated animals after about 22 days ex ovo.

Methiothepin maleate also improved the righting ability of these treated chickens and this functional improvement was maintained up to the period when drug treatment was discontinued (Data from Dr. P.J. Barnard).

Although not included, a similar decrease in the mean CPK activity was observed in methiothepin-treated chickens and this reduction correlated well with the mean plasma PK activity observed above (CPK data from Dr. J. Lyles).

Methiothepin was found to be rather toxic to these dystrophic animals when it was observed that a loss in

Table 20

Effect of Methiothepin Maleate on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY</u>
18 - 19	* 199 $\pm$ 25 (8)	* 212 $\pm$ 21 (40)	- 6%	N.S.
22 - 26	271 $\pm$ 45 (10)	525 $\pm$ 41 (99)	- 48%	0.05
28 - 33	418 $\pm$ 76 (9)	1196 $\pm$ 61 (203)	- 65%	<0.01
41 - 46	696 $\pm$ 153 (9)	2857 $\pm$ 141 (152)	- 76%	<0.001
50 - 54	1516 $\pm$ 224 (15)	2844 $\pm$ 219 (85)	- 47%	<0.02

Dystrophic chickens were treated daily with 20 mg/Kg of methiothepin maleate from 4 days ex ovo. Untreated dystrophic control chickens were given similar injections of water only.

*Mean PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$)

bodyweight of up to 30% occurred with these birds on comparison with untreated birds of the same age.

CYPROHEPTADINE + BENANSERIN

Benanserin, a powerful serotonin receptor antagonist, had been shown recently to delay the onset of muscular disability in dystrophic chickens when administered daily at a dose between 6 - 30 mg/Kg. Combination of this drug with cyproheptadine in the present study at a dose of 5 and 15 mg/Kg respectively, considerably reduced the mean plasma PK activity in these birds when compared to the mean value of the untreated control animals (Table 21). Statistical analysis ('t' test) showed that the difference in PK activity between the treated and untreated groups became highly significant after about 28 days ex ovo and remained so up to about 70 days ex ovo. After this period, the mean plasma PK activity in these treated animals remained substantially depressed but the difference was not found to be significant (i.e. $P > 0.05$).

Consistent with the plasma PK changes observed above, these treated animals performed substantially better in the flip test than the corresponding dystrophic control animals (Data supplied by Dr. P.J. Barnard). Statistical analysis confirmed that the difference in FN values between these two groups was highly significant and remained so for up to about 100 days ex ovo.

Plasma CPK activities in these treated chickens when examined were also depressed markedly when compared to the dystrophic control chickens and this fall in CPK activity correlated well with the concomitant decline in PK activity (Data from Dr. J. Lyles).

Table 21

Effects of Cyproheptadine + Benanserin on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
12 - 15	* 134 $\pm$ 8 (11)	* 146 $\pm$ 10 (27)	- 8%	N.S.
22 - 26	330 $\pm$ 40 (11)	525 $\pm$ 41 (99)	- 37%	N.S.
28 - 33	648 $\pm$ 77 (11)	1196 $\pm$ 61 (203)	- 46%	<0.05
35 - 39	698 $\pm$ 75 (11)	1988 $\pm$ 100 (151)	- 65%	<0.001
41 - 46	935 $\pm$ 89 (11)	2857 $\pm$ 141 (152)	- 67%	<0.001
50 - 54	539 $\pm$ 77 (9)	2844 $\pm$ 219 (85)	- 81%	0.001
56 - 62	1127 $\pm$ 209 (9)	3522 $\pm$ 168 (122)	- 68%	<0.001
63 - 67	1976 $\pm$ 258 (8)	3467 $\pm$ 197 (51)	- 43%	<0.01
70 - 76	1990 $\pm$ 305 (8)	3125 $\pm$ 267 (39)	- 36%	0.074
92 - 96	1823 $\pm$ 519 (3)	3113 $\pm$ 201 (38)	- 41%	<0.10

Dystrophic chickens were treated daily with a combination of cyproheptadine and benanserin at a dosage of 15:5 mg/Kg respectively, starting from day 4 ex ovo. Untreated dystrophic control chickens were given similar injections of water only. * Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

This drug combination was found to be slightly more toxic and dystrophic chickens receiving drug therapy showed a loss in weight of about 30% or under when compared to untreated chickens of the same age.

CYPROHEPTADINE + BW 501C67

Combination of cyproheptadine with BW 501C67 was also investigated in these dystrophic animals at a daily I.P. dosage of 10:10 mg/Kg, respectively, starting from day 4 ex ovo. Chickens treated with this drug combination also showed substantial reductions in their plasma PK levels when compared to dystrophic control chickens and these reductions became statistically significant (by use of 't' test) from about 35 days ex ovo to about 80 days ex ovo when treatment was discontinued (Table 22).

Although plasma PK activities in cyproheptadine + BW 501C67 -treated chickens were considerably depressed, only moderate improvements were observed in these treated animals in terms of their functional ability in the flip test (Data supplied by Dr. P.J. Barnard). Compared to dystrophic control chickens, these birds only showed moderately higher flip values after about 35 days ex ovo and remained so up to about 60 days ex ovo.

When plasma CPK activities were examined in these treated animals, similar reductions occurred in the dystrophic plasma (up to 60% down when compared to controls) and these correlated well with the reductions seen for PK above (CPK data from Dr. J. Lyles).

Table 22

Effects of Cyproheptadine + BW 501C67 on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY</u>
18 - 19	*189 $\pm$ 23 (9)	*212 $\pm$ 21 (40)	- 11%	N.S.
22 - 26	501 $\pm$ 90 (10)	525 $\pm$ 41 (99)	- 5%	N.S.
28 - 33	916 $\pm$ 142 (10)	1196 $\pm$ 61 (203)	- 23%	N.S.
35 - 39	1106 $\pm$ 134 (9)	1988 $\pm$ 100 (151)	- 44%	<0.05
50 - 54	491 $\pm$ 53 (6)	2844 $\pm$ 219 (85)	- 83%	<0.01
56 - 61	1071 $\pm$ 176 (6)	3522 $\pm$ 168 (122)	- 70%	<0.01
69 - 76	1407 $\pm$ 208 (6)	3125 $\pm$ 267 (39)	- 55%	<0.02

Dystrophic chickens were treated daily with 10mg/Kg cyproheptadine and 10mg/kg of BW 501C67 from 4 days ex ovo. Untreated dystrophic control chickens were given similar injections of water only. *Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

The cyproheptadine + BW 501C67 drug combination proved to be much more toxic than the other drugs tested so far and dystrophic chickens treated with this drug combination showed a loss in bodyweight of up to 50% when compared to the corresponding untreated control animals.

HEPZIDIN HYDROCHLORIDE

Another serotonin receptor antagonist that was tested in these dystrophic chickens was hepzidin hydrochloride. Although no results are included, when this drug was administered daily at an I.P. dosage of 10 mg/Kg starting from day 4 ex ovo, no biochemical (i.e. PK or CPK levels) or functional improvements (i.e. righting ability) could be observed in these treated animals after about 60 days ex ovo. It could be that the effective therapeutic level in these dystrophic animals had not been reached and a higher dose may have some effects on the dystrophic process in these animals. The small difference in weight between the treated and untreated chickens (less than 10% down on controls) may be indicative of this.

EFFECT OF ISAXONINE ON PLASMA PK ACTIVITY AND RELATED PARAMETERS

Daily administration of isaxonine (IAPP) by the I.P. route at a dosage of 40 mg/Kg starting from day 4 ex ovo resulted in very large reductions in the mean plasma PK activity of IAPP-treated chickens when compared to untreated chickens of the same age. Table 23 shows the mean plasma PK levels in both the treated and untreated chickens according to their age groups and it is clearly evident from the table that during the treatment period of between

Table 23

Effect of Isaxonine (IAPP) on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
18 - 19	* 153 $\pm$ 11 (7)	* 212 $\pm$ 21 (40)	- 28%	N.S.
22 - 26	191 $\pm$ 11 (7)	525 $\pm$ 41 (99)	- 64%	<0.05
28 - 33	249 $\pm$ 37 (7)	1196 $\pm$ 61 (203)	- 79%	<0.01
35 - 39	442 $\pm$ 150 (6)	1988 $\pm$ 100 (151)	- 78%	<0.01
41 - 46	819 $\pm$ 336 (6)	2857 $\pm$ 141 (152)	- 71%	<0.01
50 - 54	1704 $\pm$ 548 (6)	2844 $\pm$ 219 (85)	- 40%	N.S.
56 - 61	2307 $\pm$ 716 (6)	3522 $\pm$ 168 (122)	- 35%	N.S.

Dystrophic chickens were treated daily with 30 mg/Kg of Isaxonine from 4 days ex ovo.

Untreated dystrophic control chickens were given similar injections of water only.

* Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$)

20 days ex ovo to about 50 days ex ovo, very significant reductions in plasma PK activities are seen in the treated birds. Although not statistically significant, plasma PK activities in IAPP-treated birds continued to be depressed well after 50 days ex ovo.

In parallel with the decline in PK, plasma CPK activities were also found to be suppressed considerably in IAPP-treated dystrophic chickens (CPK reduced by up to 70%) when compared to the corresponding control chickens (CPK data from Dr. J. Lyles).

Consistent with the PK data reported above, IAPP-treated dystrophic chickens also showed a substantial improvement in functional ability when compared to untreated chickens of the same age (Data from Dr. P.J. Barnard). The difference in flip values between the two groups became highly significant (Students 't' test) from about 40 days ex ovo to about 70 days ex ovo.

Dystrophic chickens treated with IAPP showed very little signs of toxicity in terms of weight loss when compared to dystrophic control chickens of the same age.

Although not included in the present study, results of dystrophic chickens treated with higher doses of IAPP (70, 160, 200 and 300 mg/Kg bodyweight) by the I.P. route did not show any more improvements in terms of PK or FN than the dose used at 40 mg/Kg.

IAPP was also administered orally to dystrophic chickens at a daily dosage of 1g/Kg and treatment was maintained for up to about 60 days ex ovo. Plasma PK levels

in these orally-treated animals were also depressed markedly (between -26% to -50%) and the difference in PK values between the treated and untreated groups became statistically significant from about 28 days ex ovo and remained so up to about 60 days ex ovo when treatment was discontinued (results not included). The plasma PK reductions seen here are slightly less than those observed when IAPP was administered intraperitoneally at 40 mg/Kg.

In line with these observations, dystrophic chickens treated orally with IAPP also showed improved functional ability in terms of the flip test when compared to the dystrophic control animals (Data from Dr. P.J. Barnard). The magnitude of this functional improvement was, however, less than that observed earlier for the I.P. route (i.e. 40 mg/Kg).

Dystrophic chickens treated with IAPP by the oral route showed very little signs of any toxicity in terms of their weight loss when compared to the corresponding dystrophic control animals (less than 7% down in weight).

EFFECTS OF ANTIMYOTONIC DRUGS ON PLASMA PK ACTIVITY AND RELATED PARAMETERS

DIPHENYLHYDANTOIN (DPH)

DPH is an antimyotonic drug and is believed to work by stabilising the cell membrane against depolarising influences. A recent report by Entrikin et al. (1977) had shown that when dystrophic chickens were given DPH twice daily at a dosage of 20 mg/Kg from day 1 ex ovo onwards, dramatic improvements in the righting abilities of these dystrophic birds were observed along with other biochemical

Table 24

Effect of Diphenylhydantoin (DPH) on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
22 - 26	* 391 $\pm$ 68 (9)	* 525 $\pm$ 41 (99)	- 26 %	N.S.
28 - 33	596 $\pm$ 80 (9)	1196 $\pm$ 61 (203)	- 50 %	<0.05
35 - 39	941 $\pm$ 147 (8)	1988 $\pm$ 100 (151)	- 53 %	<0.02
41 - 46	1458 $\pm$ 143 (9)	2857 $\pm$ 141 (152)	- 49 %	<0.02
50 - 54	1779 $\pm$ 193 (9)	2844 $\pm$ 219 (85)	- 37 %	0.10
57 - 62	2119 $\pm$ 120 (9)	3522 $\pm$ 168 (122)	- 40 %	<0.05

Dystrophic chickens were treated daily with 20 mg/Kg of Diphenylhydantoin from 4 days ex ovo. Untreated dystrophic control chickens were given similar injections of water only. * Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

parameters that they have measured.

In the present study, DPH was administered intraperitoneally to dystrophic chickens at a daily dosage of either 20 mg/Kg or 40 mg/Kg respectively, starting at 4 days ex ovo. At 20 mg/Kg, marked reductions in the mean plasma PK activities of the treated birds were observed and these became statistically significant ('t' test) after 28 days ex ovo and remained so up to about 60 days ex ovo (Table 24).

Similarly, plasma CPK levels were also depressed by about 40% in these treated animals, though the magnitude of these reductions were slightly smaller than that observed for plasma PK (CPK data from Dr. J. Lyles).

The suppression of plasma PK activity in DPH-treated chickens was also associated with an improvement in the muscular performance of these birds as shown by the flip test (FN data from Dr. P.J. Barnard). The improved functional ability of these birds was maintained up to about 60 days ex ovo when drug treatment was discontinued.

Dystrophic chickens treated with DPH at a dosage of 20 mg/Kg/day showed no appreciable loss in weight (less than 16% down) when compared to chickens that have not received treatment.

When the dosage was increased to 40 mg/Kg, similar reductions in plasma PK activities occurred in the DPH-treated animals. These treated birds also displayed improved functional ability when compared to control animals though no increased effect was observed due to the higher dosage used.

PRAJMALINE

Another antimyotonic drug that was investigated in these dystrophic chickens was prajmaline. This drug due to its rather toxic effect on bodyweight was administered daily to dystrophic birds via the I.P. route at a relatively low dosage of 1 mg/Kg bodyweight, starting from day 4 ex ovo. Reported in Table 25 are the plasma PK activities in prajmaline-treated and untreated dystrophic chickens. It is clear that after about 22 days ex ovo, significant reductions in the mean plasma PK activities of the treated chickens occurred and these levels remained significant up to about 46 days ex ovo. Although plasma PK activity in the treated group remained depressed after this period, the difference between the two groups became statistically insignificant ($P > 0.05$).

Parallel with plasma PK reductions, plasma CPK levels were also observed to be lowered considerably in prajmaline-treated dystrophic chickens (CPK data from Dr. J. Lyles).

Dystrophic chickens treated with prajmaline showed, in general, very little improvement in functional ability (i.e. righting ability) when compared to untreated dystrophic control chickens. Although an initial improvement in the righting abilities of these treated animals was observed at around 30 days ex ovo, this functional improvement was not maintained (FN data from Dr. P.J. Barnard).

The dosage used for prajmaline treatment (1 mg/Kg) did not have any appreciable effect on the weight of these animals (less than 15%) when compared to untreated controls.

Table 25

Effect of Prajmaline on Plasma PK Activities

<u>AGE(days)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROLS</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY</u>
18 - 19	* 176 $\pm$ 8.5 (10)	* 212 $\pm$ 21 (40)	- 17%	N.S.
22 - 26	279 $\pm$ 21 (10)	525 $\pm$ 41 (10)	- 47%	0.06
28 - 33	496 $\pm$ 86 (10)	1196 $\pm$ 61 (203)	- 59%	<0.02
35 - 39	783 $\pm$ 128 (10)	1988 $\pm$ 100 (151)	- 61%	<0.01
41 - 46	1200 $\pm$ 253 (10)	2857 $\pm$ 141 (152)	- 58%	<0.01
50 - 54	1964 $\pm$ 319 (10)	2844 $\pm$ 219 (85)	- 31%	N.S.

Dystrophic chickens were treated daily with 1 mg/Kg of prajmaline from 4 days ex ovo.

Untreated dystrophic control chickens were given similar injections of water only.

* Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P>0.05$).

EFFECTS OF OTHER DRUG COMBINATIONS

DPH + CYPROHEPTADINE

Dystrophic chickens receiving the DPH + cyproheptadine drug combination at a daily I.P. dose of 15:15 mg/Kg respectively, starting from day 4 ex ovo, showed marked biochemical improvements in terms of plasma PK activities (Table 26). These reductions in PK activity in the treated birds were found to be highly significant using the Students 't' test.

When plasma CPK activities in these treated animals were examined, similar reductions were also observed (up to about 80% down) when compared to dystrophic control birds (CPK data from Dr. J. Lyles). In general, a good correlation between plasma CPK and PK activities in the treated group was observed during the entire period investigated.

When functional ability was measured, the DPH + cyproheptadine-treated chickens performed only slightly better than the corresponding controls in the flip test. (Data from Dr. P.J. Barnard). The small difference in righting ability between the treated and untreated groups was found not to be statistically significant (i.e. $P > 0.05$) for all the ages tested.

The DPH + cyproheptadine drug combination was found to be moderately toxic to these dystrophic animals in terms of weight loss (up to 30% down on control animals).

Table 26

Effects of Cyproheptadine + DPH on Plasma PK Activities

<u>AGE(DAYS)</u>	<u>TREATED DYSTROPHICS</u>	<u>DYSTROPHIC CONTROL</u>	<u>% DIFFERENCE</u>	<u>PROBABILITY(P)</u>
18 - 19	* 154 $\pm$ 5 (12)	* 212 $\pm$ 21 (40)	- 27%	N.S.
22 - 26	182 $\pm$ 6 (12)	525 $\pm$ 41 (99)	- 65%	0.01
28 - 33	219 $\pm$ 15 (11)	1196 $\pm$ 61 (203)	- 82%	0.001
35 - 39	635 $\pm$ 56 (12)	1988 $\pm$ 100 (151)	- 68%	0.001
41 - 46	1321 $\pm$ 137 (11)	2857 $\pm$ 141 (152)	- 54%	0.01
50 - 54	1791 $\pm$ 132 (10)	2844 $\pm$ 219 (85)	- 37%	0.10
56 - 61	2111 $\pm$ 239 (10)	3522 $\pm$ 168 (122)	- 40%	0.02
63 - 67	2004 $\pm$ 204 (10)	3467 $\pm$ 197 (51)	- 42%	0.01

Dystrophic chickens were treated daily with 15:15 mg/Kg of cyproheptadine + DPH respectively starting from day 4 ex ovo. Untreated dystrophic chickens were given similar injections of water only. * Mean plasma PK activity $\pm$ S.E.M., no. of birds used in parenthesis. Plasma PK activities are expressed in mU/ml plasma. N.S. = Not statistically significant ($P > 0.05$).

DISCUSSION

The results of these chemotherapy studies clearly demonstrate the value of plasma PK as a tool for monitoring the effectiveness of various drugs in the treatment of dystrophy in genetically dystrophic chickens. Like plasma CPK and some other indices of the dystrophic muscle, plasma PK can also give either false positive or false negative results as has been observed for one or two of the drugs tested (e.g. prajmaline and BW 501C67). Therefore, these biochemical parameters alone are not sufficiently reliable to interpret the results of a particular drug trial and that other additional parameters (e.g. functional or histopathological) must be included in any drug therapy assessment if misinterpretation of the results are to be avoided. In all the drug trials reported above, both functional and histopathological changes in the dystrophic birds have been quantitatively measured by colleagues in this laboratory and in the majority of cases, they are consistent with the changes seen for plasma PK and will be reported in full elsewhere.

The high degree of association that was observed between plasma CPK and PK activities in almost all of the drug-treated chickens suggest that the mechanism of their suppression by a particular drug must be similar if not the same in these birds. That this suppression of enzyme leakage from the pathological muscle is due to a direct improvement of the muscle fibre itself and in particular, the sarcolemma membrane, and not due to any other effects that the drug may have on the activities of these plasma

enzymes is inferred by a number of observations. First, in the more therapeutically responsive chickens, not only are plasma PK activities significantly reduced, but these birds also show a real improvement in functional activity such as the ability to flip. Further, histological observations of the treated muscle do in fact show an improvement in the muscle fibre towards the normal state (personal communication).

Of the serotonin antagonists that were tested, cyproheptadine (either alone or in combination with benanserin), pizotifen, cinanserin and methiothepin exerted marked functional and biochemical improvements in these dystrophic animals. These findings are consistent with earlier reports which showed that both cyproheptadine and another serotonin antagonist, methysergide, gave positive drug effects in the dystrophic chicken (Barnard et al., 1976). Why the two other serotonin antagonists, BW 501C67 and hepzidine, did not produce any real improvement in these dystrophic animals is uncertain. One possible explanation is that these two compounds are much less effective in antagonising the actions of serotonin (i.e. weak serotonin antagonists). Another possibility is that they may be eliminated more quickly in chicken blood than the others mentioned above. Evidence for this suggestion is found in chapter 5 when BW 501C67 was observed to be eliminated twice as quickly in chicken blood as observed for pizotifen after multiple dosing.

The site of action of these serotonin antagonists in the dystrophic chicken is unclear at the present moment. It has been suggested that serotonin, which is known to

cause vasoconstriction of the microcirculation in the skeletal muscle, may have a deleterious effect on muscle and that these antiserotonin drugs antagonise these effects (Barnard and Barnard, 1979). Two recent studies, however, have failed to confirm the vascular nature of the disease (Bradley et al. 1975, Koehler 1977). Alternatively, these drugs may exert their effects in these animals by a direct blockage of serotonin activity either at the level of the sarcolemma membrane or on some component within the muscle itself. In this connection, two groups of investigators (Patten et al. 1974, Garber 1977) have shown a direct effect of serotonin on normal rat skeletal muscle that can be blocked by methysergide and cyproheptadine. Also, there have been reports of serotonin inhibiting both the Mg^{2+} -activated ATPase activity of actomyosin (Mothersill et al. 1976) and the activity of sarcolemmal ATPase (Verrill et al. 1977). The possibility of serotonin having a general inhibitory effect on ATPase activity is significant. Dystrophic muscle may accumulate abnormally high levels of serotonin either by passive diffusion across the leaky membranes or by some endocytosis mechanism. This build-up of intracellular serotonin may interfere with the transport of Ca^{2+} ions into the sarcoplasmic reticulum by inhibiting the Ca-ATPase pump, thus seriously affecting the normal intracellular ionic concentrations of calcium. The resulting effect could be hypercontraction followed by muscle fibre necrosis. This process would also lead to the activation of a number of Ca-dependent proteinases which will further enhance myofibrillar breakdown.

Of the two antimyotonic drugs tested, only DPH gave positive drug effects in these dystrophic animals in terms of a marked reduction in plasma PK activities and an improvement in righting ability. These findings on DPH are consistent with earlier reports on the beneficial action of this drug in dystrophic chickens (Entrikin et al. 1981). The mechanism of action of DPH in dystrophy of the chicken is unknown at present. It has been suggested that the functional improvement of dystrophic chickens treated with DPH is probably due to the ability of this drug to suppress abnormal electrical activity in the dystrophic muscle (Entrikin and Bryant, 1977). How this suppression of electrical activity is brought about is not known but an effect of the drug on both the sodium and chloride conductances have been postulated (Lipicky et al. 1972). DPH also has a marked effect on muscle AChE activity and this in turn may effect calcium transport into the sarcoplasmic reticulum (Cisson et al. 1978).

It was found earlier that isaxonine (IAPP) is also very effective in producing large reductions in plasma PK levels in addition to improving the righting ability of dystrophic chickens. When compared to other drugs, the isaxonine effect is even more striking because it causes little or no weight losses in these birds. Further, treatment of this drug resulted in a very substantial improvement of the muscle fibre itself (Dr. J. Pizzey, personal communications). Preliminary trials giving relatively high oral doses of IAPP to these dystrophic birds also had a beneficial effect without any concomitant weight loss

and further work is required to see whether higher doses of IAPP will be of value. The mode of action of IAPP in these dystrophic animals is unclear but an effect on muscle regeneration/or reinnervation are two likely possibilities.

To conclude, a number of beneficial drugs having widely divergent chemical structures and possessing either antiserotonin, antimyotonic or neurotrophic properties were examined in the dystrophic chicken. Many of these positive drug effects were, however, only temporary and complete arrest of the disease was never observed with any of the drugs tested. Nevertheless, these drugs do offer some form of treatment for these dystrophic animals and may also be of value in the treatment of dystrophic humans. Regarding the latter point, some of these drugs have gone into clinical trial (Dr. P.J. Barnard, personal communications). If these drugs are also shown to be effective in the treatment of dystrophic humans, then this would suggest that the primary biochemical lesion in both the human and animal dystrophies are very similar if not identical.

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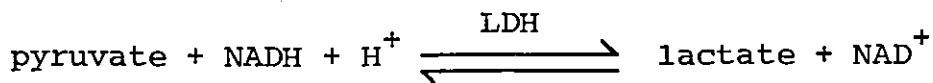
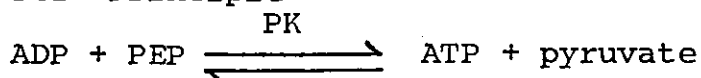
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APPENDIX I

The Boehringer Protocol for PK Assays

Test Principle



Reagents

Contents	Initial concentrations of solutions:	Concentrations in the test volume:
1) Triethanolamine Buffer	0.16mol/1, pH 7.5	0.123mol/1
KCl	0.12mol/1	0.092mol/1
MgSO ₄	21mmol/1	16.2mmol/1
EDTA	1.3mmol/1	1.0mmol/1
2) NADH	6mmol/1	0.18mmol/1
PEP	32.5mmol/1	1.0mmol/1
3) LDH	240U/ml	3.7U/ml
4) ADP	0.1mol/1	3.1mmol/1

Preparation and Stability of Solutions

Solution 1. Dissolve contents in 75 ml deionized water. Stable for one year at +15 to 25°C.

Solution 2. Dissolve contents in 3 ml deionized water. Stable for three weeks at +2 to +8°C.

Solution 3. Use solution undiluted. Stable up to the expiry date specified when stored at +2 to +8°C.

Solution 4. Dissolve contents in 3 ml deionized water. Stable for three weeks at +2 to +8°C.

Procedure

Wavelength: 340 nm

Cuvette: 1 cm light path

Temperature: 25°C

Measure against air.

Pipette into test tubes:

solution 1	0.625 ml
solution 2	0.025 ml
solution 3	0.0125 ml
serum or plasma	0.125 ml

Mix and incubate in water bath for ca. 5 min at 25°C. Pour into cuvette, measure initial absorbance A_1 , let stand at 25°C. Measure absorbance A_2 exactly 10 min after first reading. Add:

solution 4	0.025 ml
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Mix and measure absorbance A_3 , let stand at 25°C. Exactly 10 min after third reading, measure absorbance A_4 .

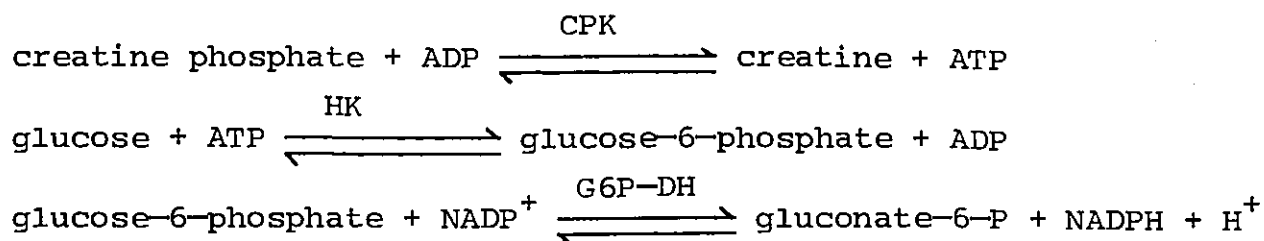
$$(A_3 - A_4) - (A_1 - A_2) = \Delta A \text{ produced by PK in 10 min.}$$

$$\text{PK Activity (mU/ml) at 25°C} = 103.2 \times \Delta A / 10 \text{ min}$$

APPENDIX II

The Boehringer Protocol for CPK Assays (Automated Method)

Test Principle



Reagents

Contents	Initial concentrations of solutions:	Concentrations in the test volume:
1) Triethanolamine Buffer	0.11 mol/l, pH 7	0.1 mol/l, pH 7
glucose	22 mmol/l	20 mmol/l
Mg-acetate	11 mmol/l	10 mmol/l
2) Buffer/Substrate		
ADP	1.1 mmol/l	1.0 mmol/l
AMP	11 mmol/l	10 mmol/l
NADP	0.66 mmol/l	0.6 mmol/l
glutathione	9.9 mmol/l	9 mmol/l
G6P-DH	1.3 U/ml	1.2 U/ml
HK	1.3 U/ml	1.2 U/ml
3) Creatine Phosphate	770 mmol/l	35 mmol/l

Preparation and Stability of Solutions

Solution 1. Use contents of one bottle 1 undiluted. Stable up to the expiry date specified when stored at +15 to 25°C.

Solution 2. Fill one bottle 2 up to the mark with solution 1. Stable for three days at +2 to +8°C or one day at +15 to +25°C.

Solution 3. Dissolve contents of one bottle 3 in 5 ml of solution 1. Stable for one week at +2 to +8°C or three days at +15 to +25°C.

Procedure

Wavelength: 340 nm; cuvette: 1 cm light path; temperature:
25°C; measure against air.

Pipette into cuvette:

solution 2 0.5 ml

serum or plasma 0.025 ml

Incubate for at least 5 min, then start reaction with:

solution 3 0.025 ml

Determine the mean absorbance change per minute ($\Delta A/\text{min}$) and use this for the calculation.

Calulation

$$\text{CPK Activity (U/l) at } 25^{\circ}\text{C} = 3491 \times \Delta A_{340 \text{ nm}}/\text{min}$$