

ENZYMATIC STUDIES IN THE DETECTION AND
CHEMOTHERAPY OF MUSCULAR DYSTROPHY

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By

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Abstract

Avian dystrophy has been utilised as an animal model for critical assessment of the therapeutic potential of particular drugs to retard dystrophic symptoms. These drugs, targeted at documented features of dystrophic pathogenesis, were categorised into six functional groups (serotonin antagonists, calcium antagonists, neurotrophic agents, antiproteases and chelating agents). The beneficial effects of drug treatment were characterised by quantifiable improvements in physical capacity and altered activities of plasma enzymes. Amelioration of symptoms following treatment with the calcium antagonist Nifedipine or the antiprotease Map-1 indicate that extended therapeutic trials were worthwhile. Activities of isocitrate dehydrogenase, known to be elevated in certain dystrophic avian lines at hatch, have been evaluated in chicken lines 412 and 413. Analyses revealed no such patterns of activity, but did reveal significant differences in older birds. This variability when extrapolated to the human condition, may have potential as diagnostic marker in the monitoring of drug therapy or quantitative assessment of the metabolism of healthy and diseased muscle.

Phosphoglycerate mutase (PGAM) activities, elevated in avian dystrophy (line 413) have been studied in human specimens from normal and 'at risk' carriers of Duchenne muscular dystrophy (DMD). Comparison with established diagnostic enzymes in 'at risk' carriers has been made with respect to the upper limit of normal female range of enzyme activities. Enzyme purification, electrophoresis and immuno-chemical methods have been applied to the development of a sensitive radioimmuno assay (RIA) specific to PGAM. Improvements to protein purification, antibody isolation and radiolabelling will establish a diagnostic assay for improved carrier and feotal screening.

A study of a novel mouse mutant (mdx), previously proposed as a unique mammalian model of DMD, was based upon analyses of selected plasma and muscle enzymes. Enzyme profiles similar to those observed in dystrophic pathogenesis were revealed in young mice. Unique 'recovery' with age was also observed.

The research has revealed aspects of dystrophic enzymology which may provide a future basis for accurate diagnosis and controlled treatment of Duchenne muscular dystrophy.

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<u>CONTENTS</u>	<u>PAGE</u>
TITLE PAGE	1
ABSTRACT	2
ACKNOWLEDGEMENTS	4
TABLE OF CONTENTS	5
LIST OF TABLES	7
LIST OF FIGURES	9
GENERAL INTRODUCTION	12
SECTION ONE CHEMOTHERAPY	16
INTRODUCTION	17
MATERIALS AND METHODS	37
RESULTS	43
DISCUSSION	53
TABLES	61
FIGURES	113
SECTION TWO ISOCITRATE DEHYDROGENASE	155
INTRODUCTION	156
MATERIALS AND METHODS	158
RESULTS	160
DISCUSSION	161
TABLES	165
FIGURES	166

SECTION THREE	PHOSPHOGLYCERATE MUTASE	174
INTRODUCTION		175
MATERIALS AND METHODS		178
RESULTS		190
DISCUSSION		198
TABLES		203
FIGURES		212
SECTION FOUR	MURINE (<u>mdx</u>) DYSTROPHY	256
INTRODUCTION		257
MATERIALS AND METHODS		260
RESULTS		262
DISCUSSION		273
TABLES		276
GENERAL CONCLUSIONS		284
REFERENCES		288

LIST OF TABLES

<u>TABLE</u>		<u>PAGE</u>
1a	Drugs and diluents	40
1b	Plasma CK activities in ketanserin-treated and untreated dystrophic chickens	62
1c	Plasma PK activities in ketanserin-treated and untreated dystrophic chickens	64
1d	Plasma enzyme activities and flip scores for R49 945-treated and untreated dystrophic chickens.	66
1e	Plasma enzyme activities for R55 667-treated and untreated dystrophic chickens.	68
1f	Plasma enzyme activities from cyproheptadine-treated and untreated dystrophic chickens.	70
1g	Plasma enzyme activities from minoxidil-treated and untreated dystrophic chickens.	72
1h	Plasma enzyme activities from minoxidil-treated (20) and untreated dystrophic chickens.	74
1i	Plasma enzyme activities for Bol 148hta-treated and untreated dystrophic chickens.	76
1j	Plasma enzyme activities for nifedipine-treated (0.1) and untreated dystrophic chickens.	78
1k	Plasma enzyme activities for nifedipine-treated and untreated dystrophic chickens.	80
1l	Plasma enzyme activities for diltiazem-treated and untreated dystrophic chickens.	82
1m	Plasma enzyme activities for flunarizine - and cinnarizine-treated and untreated dystrophic chickens.	84
1n	Plasma enzyme activities for flunarizine- and cinnarizine-treated (15) and untreated dystrophic chickens.	86
1o	Plasma enzyme activities for dantrolene-treated and untreated dystrophic chickens.	88
1p	Plasma enzyme activities and weight determinations for verapamil-treated and untreated dystrophic chickens.	90
1q	Plasma enzyme activities and flip scores for Pyl08 068n-treated and untreated dystrophic chickens.	92
1r	Plasma enzyme activities for IAPP-treated (40,80,120,200) and untreated dystrophic chickens.	94
1s	Plasma enzyme activities for IAPP-treated (150,300) and untreated dystrophic chickens.	96
1rs	Compilation of results from tables 1r and 1s.	98
1t	Plasma CK activities from BN1041-A treated and untreated dystrophic chickens.	100
1u	Plasma PK activities from BN1041-A treated and untreated dystrophic chickens.	102
1v	Plasma enzyme activities from BN1041-B treated and untreated chickens.	104

1w	Plasma enzyme activities from 4-aminopyridine and diaminopyridine-treated and untreated dystrophic chickens.	106
1x	Plasma enzyme activities from Map-1 treated and untreated dystrophic chickens.	108
1y	Plasma enzyme activities from Map-1 treated (100) and untreated dystrophic chickens.	110
1z	Plasma enzyme activities from desferal-treated and untreated dystrophic chickens.	112
2a	Normal and dystrophic muscle isocitrate dehydrogenase activities.	165
3a	Plasma phosphoglycerate mutase activities from normal females.	203
3b	Serum phosphoglycerate mutase activities from normal females.	204
3c	Comparison of plasma and serum PGAM for 9 normal females.	205
3d	Plasma enzyme activities for 23 females 'at risk' as carriers of DMD.	207
3e	Plasma phosphoglycerate mutase activities for 6 individuals, sampled at time intervals.	208
3f	Cellogel electrophoresis of iodinated phosphoglycerate mutase.	209
3g	Adsorption of phosphoglycerate mutase to cibacron-blue.	210
3h	Desorption of phosphoglycerate mutase from cibacron-blue.	211
4a	Plasma enzyme activities from normal and <u>mdx</u> mice.	277
4b	Enzyme activities from normal and <u>mdx</u> pectoral muscle.	279
4c	Enzyme activities from normal and <u>mdx</u> gastrocnemius muscle.	281
4d	Whole body and individual muscle weight analysis for normal and <u>mdx</u> mice.	283

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
II	Molecular structures of drugs.	34
III	Molecular structures of drugs.	36
IIII	Exhaustion flip scores for ketanserine-treated and untreated dystrophic chickens.	114
IIV	Exhaustion scores for R55 667 - treated and untreated dystrophic chickens.	116
IV	Flip scores for cyproheptadine -treated and untreated dystrophic chickens.	118
IVI	Exhaustion scores for minoxidil-treated and untreated dystrophic chickens.	120
IVII	Exhaustion scores for Bol 148 hta-treated and untreated dystrophic chickens.	122
IVIII	Exhaustion scores for nifedipine-treated and untreated dystrophic chickens.	124
IX	Exhaustion scores for diltiazem-treated and untreated dystrophic chickens.	126
IX	Exhaustion scores for flunarizine-treated and untreated dystrophic chickens.	128
IXI	Exhaustion scores for cinnarizine-treated and untreated dystrophic chickens.	130
IXII	Exhaustion scores for dantrolene-treated and untreated dystrophic chickens.	132
IXIII	Exhaustion scores for verapamil-treated and untreated dystrophic chickens.	134
IXIV	Exhaustion scores for IAPP-treated (40,80) and untreated dystrophic chickens.	136
IXV	Exhaustion scores for IAPP-treated (120,200) and untreated dystrophic chickens.	138
IXVI	Flip scores for IAPP-treated (150,300) and untreated dystrophic chickens.	140
IXVII	Flip scores for BN1041-A - treated (72,100) and untreated dystrophic chickens.	142

1XVIII	Exhaustion scores for BN1041-A-treated and untreated dystrophic chickens.	144
1XIX	Flip scores for BN1041-B-treated and untreated dystrophic chickens.	146
1XX	Flip scores for 4AP and DAP-treated and untreated dystrophic chickens.	148
1XXI	Flip and exhaustion scores for Map 1 - treated and untreated dystrophic chickens.	150
1XXII	Exhaustion scores for Map 1 - treated and untreated dystrophic chickens.	152
1XXIII	Exhaustion scores for desferal-treated and untreated dystrophic chickens.	154
2I	Plasma isocitrate dehydrogenase activities from normal and dystrophic chickens.	167
2II	Intra-mitochondrial isocitrate dehydrogenase activities from normal and dystrophic chickens.	169
2III	Extra-mitochondrial isocitrate dehydrogenase activities from normal and dystrophic chickens.	171
2IV	Isocitrate dehydrogenase ratios for normal and dystrophic chickens.	173
3I	Creatine kinase and phosphoglycerate mutase activities for women 'at risk' carriers of DMD.	213
3II	Carbonic anhydrase III, and phosphoglycerate mutase activities for women 'at risk' carriers of DMD.	215
3III	Carbonic anhydrase III and creatine kinase activities for women 'at risk' carriers for DMD.	217
3IV	SDS-polyacrylamide gel electrophoresis of rabbit and human PGAM.	219
3V	Ochterlony double diffusion analysis of guinea pig anti PGAM serum.	221
3VI	Ochterlony double diffusion analysis of anti-serum from guinea pig 2.	223
3VII	Column elution profiles for iodinated phosphoglycerate mutase	225
3VIII	Column elution profile for iodinated phosphoglycerate mutase.	227

3IX	Standard curve for rabbit ¹²⁵ I-PGAM.	229
3X	The relationship of ammonium sulphate saturation to protein concentration and phosphoglycerate mutase activity.	231
3XI	SDS-polyacrylamide gel electrophoresis of chicken pectoral muscle phosphoglycerate mutase.	233
3XII	Protein elution profile of dialysed rabbit muscle PGAM from cibacron blue column.	235
3XIII	Protein elution profile of rabbit muscle PGAM (in ammonium sulphate) from cibacron blue column.	237
3XIV	Elution profile for chicken pectoral muscle PGAM from cibacron blue column.	239
3XV	SDS-polyacrylamide gel electrophoresis of human (foetal and adult) muscle phosphoglycerate mutase	241
3XIV	SDS-polyacrylamide gel electrophoresis of muscle phosphoglycerate mutase after application to cibacron blue columns.	243
3XVII	Comparison of pH gradient measured by surface electrode and elution from gel slices.	245
3XVIII	The isoelectric banding pattern for rabbit muscle phosphoglycerate mutase.	247
3XIX	The isoelectric banding pattern for mouse pectoral muscle and brain phosphoglycerate mutase.	249
3XX	The isoelectric banding pattern for partially purified adult muscle phosphoglycerate mutase.	251
3XXI	The isoelectric banding pattern for partially purified foetal muscle phosphoglycerate mutase.	253
3XXII	The isoelectric banding pattern for adult muscle phosphoglycerate mutase.	255

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive and fatal wasting disease of muscle tissue which, as a consequence of an X-linked mode of inheritance, affects only male children (Tyler, 1966; Dubowitz, 1973). The disease additionally has a high incidence of spontaneous mutation which results in affected children being born into families with no previous history of the myopathy (Emery, 1980). Despite the original written diagnosis being recorded in the late 19th century, and current extensive research into the symptoms of DMD, the primary causative agent remains unknown (Meryon, 1852; Duchenne, 1862; Serratrice et al. 1984). Consequently, detection of carriers and early diagnosis of sufferers is beset with problems, many arising from false positive results and overlapping normal and pathological ranges of current diagnostic tests (Firth, 1983; Griggs et al., 1985). There is clearly a need for fundamental research towards the description of diagnostic aids to increase the accuracy of detection. In addition, full characterisation of the primary myopathy and the development of specific drugs will be beneficial to the management of DMD.

The first section of this thesis describes the evaluation of drugs of potential benefit to the management of DMD. For this purpose, the dystrophic chicken model of DMD (New Hampshire line 413) was exploited for the detailed evaluation of specific drugs. Particular categories (serotonin antagonists, calcium antagonists, neurotrophic agents, anti-proteases and chelating agents) were selected according to certain factors reported to influence aspects of the progression of the disease. Standard trial procedures were employed and the assessment of measured parameters (creatine kinase, pyruvate kinase and exhaustion scores) was based on comparisons of treated and untreated dystrophic birds.

In Section two and three, two potential diagnostic enzymes were investigated. The oxidative enzyme isocitrate dehydrogenase (ICD) was analysed in normal and dystrophic chicken plasma and muscle. Previously, the enzyme had exhibited elevated levels in dystrophic muscle of the parent strain and indicated that further research in the derived lines 413/412 would profitably be pursued. The main objective was to confirm these results in plasma and muscle and establish whether further research on human DMD tissues would be viable. The glycolytic enzyme, phosphoglycerate mutase (PGAM), has previously been shown to be a suitable candidate for DMD research (Chown, et al., 1984). In Section three, PGAM levels in normal and 'at risk' carriers of DMD female plasma and serum were analysed and comparison made to determine the suitability of the enzyme as a diagnostic aid. Methods having advantages of increased sensitivity were investigated for application to foetal and infant analyses, where volumes were necessarily at a premium. Affinity chromatography and isoelectric-focussing techniques were exploited for the preparation of homogeneous samples of the human enzyme.

Currently, no combination of diagnostic tests (CK, PK, myoglobin etc.) can provide a greater than 70% certainty of diagnosis of DMD, and this figure can be as low as 20% (Emery, 1980; Griggs et al., 1985). The development of specific and highly sensitive systems is therefore essential to the improvement of diagnostic capabilities. In the UK the protracted nature and inaccuracy of diagnoses brings unnecessary suffering to the families of DMD patients. Clearly, the development of new diagnostic procedures should allow for the practicality of screening all babies within a few weeks of birth. In this context, it is worth noting that a voluntary newborn screening program developed in West Germany has reported diagnosis of DMD as early as 2 weeks post-partum (Scheuerbrandt and Mortier, 1984).

Section four describes the particular analysis of a novel mouse mutant (mdx) proposed as an X-linked mammalian model for DMD (Bulfield et al., 1984). Initial studies revealed abnormalities including elevated levels of plasma pyruvate kinase and altered muscle histology. Thus a detailed biochemical and histological study of this strain was warranted to establish the status of the mutation. Specific plasma and muscle enzymes whose levels deviate from normal in DMD and avian muscular dystrophy (AMD), were utilised to evaluate the mdx mouse.

Overall the purpose of this research project is to focus upon the diagnosis and treatment of Duchenne muscular dystrophy in a direct manner or through the study of animal models of the disease.

SECTION ONE

CHEMOTHERAPY

INTRODUCTION

The development of drugs specific for the treatment of Duchenne muscular dystrophy (DMD) is handicapped by a lack of knowledge concerning the primary myopathy. Since it's first written diagnosis the secondary characteristics have been well documented but the initial effector remains unknown (Meryon, 1852; Tyler, 1966; Dubowitz, 1973). DMD is an X-linked recessive trait, transmitted by the female line and develops in 50% of male offspring of known carriers (Tyler, 1966). There is also a very high incidence of new mutations ($4.3-10.5 \times 10^{-5}$ per gamete per generation) (Witkowski and Jones 1981; Perry 1984; Monaco et. al. 1985). Recent advances in genetic engineering technology enable investigations of the exact location of the mutation of the X-chromosome (Roses, 1984; Emery, 1984). Should the results of these analyses reveal the mutation to involve an expressed protein then amniocentesis tests and drug therapy could be directed towards the deficiency (Harper et al., 1984; O'Brien, 1984). If the area of the chromosome, however, should code for a regulatory factor then drug testing as described in this section would remain a valid area of research.

DMD is characterised by a selective and progressive degeneration of muscle tissue. It is selective in that weakness occurs in proximal limb muscles before distal and lower limbs before upper. Diagnosis at 2-6 years is usually a result of the child's inability to rise from a sitting position unaided. The disease progresses through increased muscle weakness (5-10 years) resulting in loss of ambulation (8-12 years) and subsequent death (18-30 years).

The cause of death is usually due to pneumonia complicated by cardiac problems arising from the enforced immobilisation (Dubowitz, 1973). The present aim of drug therapy in the absence of known effector, is to hold in abeyance the fatal progression of the disease.

Drugs cannot be referred for human clinical trials without prior proven effect on an animal model of dystrophy (Cosmos and Butler, 1980). Thus the experiments described below were carried out using the dystrophic chicken (Davis line 413). There are other well documented mammalian models of the disease; dystrophic mouse (dy/dy ; dy^{2j}/dy^{2j}) and dystrophic hamster which, although, expressing dystrophic symptoms are primarily a neuropathy in the case of the mouse and cardiomyopathy in that of the hamster (Bradley and Jaros 1979; Aguayo et. al 1979; Homburger et. al 1962). The chicken model expresses only dystrophic symptoms (Wilson et. al, 1979). Another consideration when comparing these models is size. For the purposes of chemotherapy the rodents cause problems in drug administration and muscle and blood sampling. The chicken, however, can be injected intra-peritoneally with ease, blood samples taken from the readily accessible jugular vein and histological samples may be taken from the pectoral muscle.

Inherited muscular dystrophy of chickens arose spontaneously in a commercial flock of New Hampshire chickens (Asmundson and Julian, 1956). Subsequently eggs from this source were used to establish lines of chickens homozygous for dystrophy. Selective breeding resulted in the dystrophic line 413 and normal line 412 birds, the London sublimes of which were used in these trials (Wilson et. al 1979). Lines 412 and 413 have a common genetic background. They resulted from an outcross of the original dystrophic line 304 to normal New Hampshire birds. The F_2 generation progeny were

segregated and bred as two lines, one homozygous for dystrophy (413) and the other for normal (412) (Randal and Wilson, 1980; Barnard, 1980). Line 413 was specifically bred for early onset of symptoms similar to DMD. Other features in common are raised plasma enzyme levels (e.g. CK, PK), selective degeneration of fast-twitch white muscles, variability of fibre size, rounding and splitting of fibres, degeneration and initially some regeneration of cells (Ebashi, et. al, 1959; Harano et. al, 1973; Pizzey et. al, 1984; Dubowitz, 1973). Unlike DMD, AMD is an autosomal trait not X-linked. However since the mode of sex determination in birds is the reverse of that in humans it is not possible to predict whether the genes on the human X chromosome would be similar to those of the avian W chromosome (Romanoff, 1960). There are also a few rare human dystrophies with similar pathology to DMD which also have an autosomal mode of inheritance (Barnard, 1980).

Drug evaluation was based on analysis of biochemical, physiological and histological parameters. The first two categories will be discussed in detail in this thesis. The biochemical parameters plasma creatine kinase and pyruvate kinase levels were assayed in treated and untreated dystrophic birds. Both enzymes are found elevated in dystrophic plasma as a result of 'leakage' from muscle, and thus a reduction in activity in treated birds as compared to untreated animals is a measure of the beneficial effect of a drug (Zatz and Otto, 1980; Liu et. al, 1980). The physical parameters of body weight and flip performance were carefully monitored. It was important to ensure that drug treatment did not result in dramatic weight losses thereby invalidating the

the trial and also to measure the actual physical ability of the birds (Hudecki and Entrikin 1982; Julian and Asmundson, 1963). The last category, histology, directly demonstrates the effects of drugs on dystrophic muscle fibres (Pizzey and Barnard, 1983; Barnard and Barnard 1979). Beneficial effects of drug treatment on biochemical and physiological parameters must also be substantiated by improved histology (hypertrophy/atrophy, nuclei, fibre splitting, vacuolisation fat and connective tissue deposition) before consideration for human clinical trials (Barnard and Barnard, 1980).

Drugs tested in these trials fall into the following categories, serotonin antagonists, calcium antagonists, neurotrophic agents, anti-proteases and chelating agents. These groups are targeted against factors reported to influence the progress of dystrophic symptoms. The rationale for testing these drug categories is discussed fully below. The dosages of drugs chosen for the trials were determined by single i.p. injections into groups of 2 or 3 birds. Those dosages that produced least physical and behavioural effect were selected for trial procedures. Birds injected with these dosages of drugs were compared to an equivalent number of untreated dystrophic birds of the same age.

This work aims to evaluate the efficacy of selected drugs with reference to biochemical and physiological parameters. The task was complicated by lack of knowledge concerning the primary effector of the disease and thus all these drugs were targeted at secondary symptoms. The objective of treatment therefore being alleviation of symptoms rather than a cure of the disease.

SEROTONIN ANTAGONISTS

Serotonin (5-HT), a potent amine, found in various cells of the body has been implicated in the pathogenesis of muscular dystrophy. It functions singly, and synergistically with other hormones. One hypothesis concerning the involvement in dystrophy has resulted from the observation that 5-HT-induced vasodilation often occurred at the expense of muscle capillaries (Vetterlein and Schmidt, 1975). The resulting oxygen deprivation caused muscle cell necrosis (O'Steen et al. 1967). Muscle lesions similar to those found in dystrophy have been demonstrated in experimental oxygen deprivation (Mendell et al. 1971). In 1976, Meltzer proposed an animal model of dystrophy based on the treatment of normal rats with a combination of a membrane active drug and 5-HT (Meltzer, 1976). These rodents subsequently showed scattered necrosis of muscle fibres and elevated CK levels similar to those found in muscular dystrophy.

Excess 5-HT could be involved in cell necrosis, connective tissue infiltration, fibroblast proliferation and abnormal protein synthesis found in dystrophy. The high incidence of mast cells, the major source of 5-HT, in dystrophy may be involved in cell necrosis (Essman, 1978). Mast cells are always found in areas of degeneration and the action of 5-HT is to increase the rate of collagen synthesis and fibroblast proliferation (Boucek, 1978). In dystrophy, collagen which is secreted from fibroblast replaces muscle tissue during the pathogenesis of the disease and a high proportion of fibroblasts are observed in dystrophic muscle cell culture (Foidart et al. 1981; Stephens et al., 1982). Serotonin also stimulates protein turnover, which is also abnormal in DMD (Pennington, 1978). Evidence for high serotonin levels in two animal models of dystrophy has been found.

Dystrophic mouse spleens were observed to contain excess 5-HT and in the dystrophic chicken high plasma 5-HT was assayed, (Gordon and Dowben, 1966; Bhargava et. al 1977).

Muscle lesions caused by high 5-HT, in carcinoid syndrome, can be successfully treated by anti-seritonergeric drugs e.g. Cyproheptadine. However sufferers exhibited elevated 5-HT levels for at least 10 years before muscle lesions were diagnosed (Berry et. al 1974).

Previously, several "classical" 5-HT antagonists have been evaluated on Avian dystrophy (Barnard et al, 1976; Barnard and Barnard, 1979). These drugs demonstrated positive retarding effects on the progress of dystrophy but were disadvantaged by having agonistic properties i.e. methysergide caused hallucinations (Janssen, 1983; Barnard and Barnard 1980). The series investigated below contains one "classical" antagonist, cyproheptadine the most promising agent of previous trials (Barnard and Barnard, 1979). Of the other drugs, all specific 5-HT antagonists, Ketanserin selectively blocks the 5-HT₂ receptor and is devoid of agonistic properties (Leyson et. al, 1981).

The drugs investigated in this group were as follows:-

- (i) Ketanserin
- (ii) R49 945
- (iii) R55 667
- (iv) Cyproheptadine
- (v) Minoxidil
- (vi) Bol 148 hta

(i) Ketanserin

Ketanserin is a selective and potent serotonin receptor (S_2) blocker whose structure is shown in Fig. III (Leysen et. al, 1981). Receptor binding studies have shown ketanserin to bind specifically to the $5-HT_2(S_2)$ receptor which has been observed to mediate in vitro artery contractions, head twitches and cholonic in vivo (Leysen et.al, 1981). In addition, specific binding in brain slices appears only in areas of $5-HT_2$ receptors (Leysen et. al, 1982). Necrosis of ligated rat gastrocnemius muscle treated with 5-HT can be prevented by Ketanserin which makes the drug an interesting candidate for trial (Verheyen, et. al, 1981). Ketanserin is commercially available as an anti-hypertensive agent and is favoured because of minimal adverse side effects resulting from treatment.

(ii), (iii) R49-945, R55-667

Both these drugs were donated by Janssen Pharmaceuticals the manufacturers of Ketanserin. They are not commercially available and no relevant information has been published in commercial or academic literature to date.

(iv) Cyproheptadine

Cyproheptadine (structure shown Fig.1I) has been shown previously to be an effective treatment of avian dystrophy, singly and in combination with benanserin (Barnard and Barnard, 1979). Various dose regimes resulted in decreased plasma enzyme levels and improved physical performance (Barnard and Barnard, 1978). In similar receptor binding studies to those applied to ketanserin, cyproheptadine demonstrated specific affinities for both $5-HT_1$ and $5-HT_2$ receptors together with histamine (H_1) and acetylcholinesterase (AChE) receptors (Leysen et al, 1981). In keeping with the binding

studies multiple effects on treatment have been observed (Janssen, 1983). This drug was included in this group for comparative purposes, because it was one of the most effective anti-dystrophic drugs analysed in the previous trials (Barnard and Barnard, 1980).

(v) Minoxidil

Minoxidil (structure shown Fig.11) used clinically as an anti-hypertensive agent, acts primarily on vascular smooth muscle. It has no affinity for β -adrenergic, cholinergic or histaminergic receptors but can cause impairment of adrenergic neurone function. No CNS effects have been observed with this drug and it is devoid of anti-inflammatory activity (Ducharme et. al 1973, Thomas and Harpootlian, 1975).

(vi) Bol 148 hta

This drug was donated by Sandoz Ag the manufacturers of Pyl08 68n (discussed p. 28). Bol 148 hta is not commercially available and no relevant information has been published in commercial or academic literature to date.

CALCIUM ANTAGONISTS

Calcium has an integral role in many normal cell functions, but in DMD there is evidence to suggest that excess calcium is associated with tissue necrosis and cell death (Leonard and Salpeter, 1979). For this reason, calcium antagonists were selected as being appropriate for trials.

In 1976, Wrogemann and Pena postulated that calcium overload in mitochondria was responsible for muscle cell necrosis. Excess of mitochondrial calcium in dystrophic hamsters was demonstrated to cause NAD^+ leakage and the disruption of oxidative-phosphorylation,

(Wrogemann et. al, 1979). Loss of magnesium induced an inability to sequester calcium and resulted in subsequent mitochondrial destruction. Observations, that syrian hamsters on calcium free diets expressed less cardiac and skeletal muscle necrosis, supported this view (Jasmin and Solymoss, 1975). Membrane defects similar to those observed in dystrophy were produced experimentally by treating normal frog muscle with the calcium specific ionophore A 23187 which by increasing calcium entry duplicates many changes seen in DMD (Duncan, 1978).

In DMD samples, some gross mitochondrial changes can be attributed to calcium but evidence suggests that oxidative-phosphorylation appears unaffected (Peter and Worsfold, 1969). Elevated levels of certain calcium-activated enzymes are observed in DMD (Kar and Pearson, 1978). One, a calcium-activated protease specifically attacks myofibrillar proteins (Sugita et. al, 1980). Hypercontracted muscle fibres, generally associated with excess calcium, are observed under light microscope preparations of biopsies taken from DMD muscle (Bodensteiner and Engel, 1973). X-ray fluorescence techniques have demonstrated total cell calcium to be elevated in dystrophy (Maunder-Sewry and Dubowitz, 1980). Maunder-Sewry and Dubowitz (1979) have also observed an increased calcium/phosphorous ratio in nuclei of cells from patients and carriers of DMD. Early in the disease elevated calcium levels were discovered in nuclei of cells shown by microscopy to be not yet affected by dystrophy (Sewry and Dubowitz, 1984). This nuclear calcium is involved in chromatin condensation and histone phosphorylation and thereby are implicated in cell division, RNA transcription and protein synthesis (Kanungo and Thakur, 1977). The full implications of calcium overload are not yet fully understood.

The calcium antagonists investigated all belong to a structurally diverse group of compounds which act at the voltage dependant slow calcium channels of muscle cells, (for review see Schwartz and Triggle, 1984). The drugs tested in this category were as follows:-

- (vii) Nifedipine
- (viii) Diltiazem
- (ix) Flunarizine
- (x) Cinnarizine
- (xi) Dantrolene
- (xii) Verapamil
- (xiii) Pyl08 68n

(vii) Nifedipine

Nifedipine, a dihydropyridine derivative, is selective potent calcium channel blocker (see Fig. 1I). Clinically it is prescribed for angina, hypertension and as an anti-arrhythmic drug (Henry, 1980). Radio-labelled derivatives have been also used for biochemical studies of calcium channels (Bolger et al, 1982; Norman et. al 1983). Experiments have demonstrated Nifedipine to bind to intact, isolated cells and also membranes purified from cardiac, skeletal and smooth muscles, and from brain samples (Schwartz and Triggle, 1984). In skeletal muscle the binding density is observed to be greatest at locations associated with the t-tubule system (Fosset et. al, 1983). The functional effects on skeletal muscle however are less pronounced than would be expected if compared to smooth muscle on a pro rata basis (Schwartz et. al 1985).

(viii) Diltiazem

The benzothiazepin derivative, Diltiazem whose structure is shown in Fig. 1I is used in the treatment of cardiac complaints (Check, 1981). It also acts to reduce calcium-dependent insulin secretion (Taniguchi et. al, 1977). Pharmacological data indicates that Diltiazem acts similarly to Verapamil, and may have potential advantages in exerting effects on fast calcium channels at high concentration and evoking fewer side effects (Henry, 1980). Previous studies of treatment of dystrophic hamsters resulted in 37% reduction of creatine kinase (CK) and improved muscle ultrastructure as shown by histological analyses (Bhattacharya et. al, 1982). This drug is favoured clinically because of the minimal side effects.

(ix),(x) Flunarizine and Cinnarizine

These two compounds are structurally related; Flunarizine being the difluoro derivative of Cinnarizine (structure shown Fig. 1II). They selectively block calcium channels in the vasculature but have no effect on myocardial tissue (Van Nueten et. al, 1978). Therapeutic trials of cinnarizine in the cardiomyopathic hamster improved heart muscle but not skeletal muscle lesion. Reductions of plasma CK (15%) were observed without loss of weight (Jasmin and Solymoss, 1975). Pharmacological research indicated that Flunarizine was the more potent antagonist of the two drugs (Van Neuten, et. al, 1978).

(xi) Dantrolene

Dantrolene (structure shown Fig. 1II) a muscle relaxant, inhibits calcium movements at intracellular storage sites thus depressing mechanical activity of muscles without effecting electrical properties or neuromuscular transmission (Hainault and Desmedt, 1974). Clinically it is used to counteract muscle spasticity (Putney and

Bianchi, 1974). Therapeutic trials on Storrs birds provided histological evidence of reduced muscle damage but no improvement in the ability to rise from supine (Cosmos and Butler, 1980).

(xii) Verapamil

Verapamil (structure shown Fig. 1II) binds to calcium channels on the cytosolic surface of muscle cells (Janis and Triggle, 1983). It acts in a dose dependent manner to inhibit transmembrane calcium efflux (Henry, 1980). Drug trials in the dystrophic hamster ameliorated muscle lesions in cardiac but not skeletal muscle (Jasmin and Solymoss, 1975). Verapamil treatment (dose range $0.2-2 \text{ mgkg}^{-1}\text{day}^{-1}$) in line 413 chickens significantly changed acetylcholinesterase patterns but did not improve other measured parameters (Entrikin et. al, 1981).

(xiii) Py108 68n

This compound structurally different to other dihydropyridine calcium antagonists (a benzoxadiazolyle dihydropyridine) is shown in Fig. 1I. It acts to lower blood pressure and heart rate and pharmacologically compares favourably to nifedipine (Hof et. al, 1982(a)). The observed potent non-competitive inhibition of contractions of rabbit aortic rings (induced by depolarisation) is typical of a channel blocker, and the mode of action has been demonstrated to be more selective than that of Verapamil (Hof et. al 1982(b)).

NEUROTROPHIC AGENTS

Neuromuscular transmission is not affected during the pathogenesis of DMD (Dubowitz, 1973). However neurotrophic agents were chosen for trial because of the regulatory influence of nerves on muscles. The type of neural transmission conditions the fibre-type composition of the receiving muscle cells (Buller et. al, 1960). Classical experiments demonstrated that the transfer of a nerve from a fast-twitch muscle to a slow twitch muscle (or vice versa) changed the fibre type composition of the muscle accordingly (Scott et. al 1984). It is possible therefore that drugs which modify nerves may be of benefit to patients by changing susceptible types to those more resistant to the dystrophic process.

Exercise of muscles also affords some protection against the progress of DMD. The life-span of patients has been extended by the use of prostheses to keep patients ambulant (Dubowitz and Heckmatt, 1980).

The drugs in this group exert diverse effects on nerves which in turn may be beneficial to the management of DMD.

(xiv) Isaxonine (IAPP)

(xv) BN1041-A, BN1041-B

(xvi) 4-aminopyridine (4-AP)

(xvii) 3, 4-diaminopyridine (DAP)

(xiv) Isaxonine

Isaxonine (IAPP) (N-isopropyl-amino-2-pyrimidine)

accumulates preferentially in nerves and promotes neural growth (Hantaz-Ambroise and Koenig, 1982; Bondour-Jahan, et. al, 1982). The addition of IAPP to nerve cells in culture promotes neurite clustering and increases collateral and terminal sprouting. In nerve-muscle co-culture, IAPP increases

nerve-muscle cell contact, elevates acetylcholinesterase levels and causes acetyl choline receptor clustering (Fardeaux, 1981). Analysis of plasma membrane proteins by SDS-gel electrophoresis shows IAPP to increase the number of protein bands in dystrophic samples. The relative proportions of proteins found are also altered by the drug (Lucas - Heron and Guiheneuc, 1982). Clinical trials on patients with leprous neuropathy show evidence of electromyographic improvements after IAPP treatment (Sebille and Hugelin, 1980). Hence the drug may delay dystrophic symptoms by increasing the regenerative capacity of the muscle fibres through the improvement of muscle innervation.

(xv) BN1041

Two derivatives of BN1041; BN1041-A (acid salt) and BN1041-B (basic salt) were investigated. These drugs were manufactured by Ipsen the manufacturers of IAPP but no information from literature currently available.

(xvi) 4-aminopyridine (4AP)

(xvii) 3, 4-diaminopyridine (DAP)

Both these compounds belong to a group of substances (2-; 3-; 4-aminopyridines: 2, 3-; 2,6-; 3,4-diamino pyridines) all of which possess novel effects on neuromuscular transmission (Thesslef, 1980). They are active at low concentrations (1 μ M) causing increased acetylcholinesterase (AChE) release which results in a muscle twitch independent of tetanic response. Prolonged AchE release occurs because the aminopyridines block the potassium channels responsible for nerve repolarisation. Thus the calcium efflux which directly activates transmitter release continues and the muscle twitch is prolonged (Molgo et. al, 1980).

Of the two chemicals, 4-AP has been most extensively investigated. Individually it exerts weak anti-curare effects, but when combined with standard anti-curare agents (neostigmine and pyridostigmine) evokes a positive synergistic response (Miller et. al, 1971). Experiments have also demonstrated that dantrolene-induced muscle relaxation can be antagonised by 4-AP (Bowman et. al, 1977). Mild improvements of muscle strength were demonstrated as a result of 4-AP treatment of myasthenia gravis and Eaton-Lambert myasthenic syndrome (Lundh et. al, 1977: 1979). Mental but not physical improvements were observed using this drug on Huntingdon's chorea. Neuromuscular blockage induced by botulinum toxin is antagonised by 4-AP but the reversal of paralysis is transient (Ball et. al, 1979).

DAP has a similar efficacy to 4AP but is more potent on cholinergic transmission (Thesslef, 1980). It produces less convulsant side effects than 4-AP and this is reflected in the doses selected for these trials. DAP and 4-AP may therefore improve the management of DMD by altering the twitch frequency of muscles leading to improved muscle exercise and changed fibre-type composition (Scott, et. al, 1984).

ANTI-PROTEASES

The destruction of muscle sarcoplasmic and contractile proteins observed in dystrophy is presumed to result from the elevated levels of cathepsins and other proteases found in these tissues (Weinstock et. al, 1958). The addition of the anti-proteases leupeptin and pepstatin to normal and dystrophic muscle cells in culture delays degeneration processes. (Stracher et. al, 1978). These inhibitors specifically attack cathepsins B and D respectively and elevated levels of both are observed in dystrophic tissues

(Pennington and Robinson, 1968; Libby and Goldberg 1978). However, trials of these and other anti-proteases on dystrophic mice (Bar harbour strain 129 Re dy dy) in various combinations had no clinical or histological effect (Enomoto and Bradley, 1977). Elevated levels of another enzyme, a calcium-activated protease have been observed in dystrophic tissues. The use of gel-electrophoretic techniques demonstrated that the addition of this protease to normal muscle homogenate resulted in the degradation of the myofibrillar proteins troponin-I, troponin-C and heavy myosin. A similar degradative pattern is found in dystrophic muscle preparations (Sugita, et. al, 1980). The compound Map-1 is investigated in this Section (Seglen and Gordon, 1982).

CHELATING AGENTS

Non protein bound iron is an important catalyst in reactions producing free oxide radicals and it is the balance between this and α -tocopherol (vitamin E) that determines whether or not oxidative tissue damage occurs (Mason and Horwitt, 1972). Similarities between skeletal muscle and erythrocyte membrane alterations in DMD and that induced in rats by vitamin E deprivation have been observed (Mason and Horwitt, 1972; Plishker and Appel, 1980; Rowland 1980). Other similarities between the 'nutritional myopathy' and DMD include creatinuria decreased muscle enzymes, membrane damage and degeneration. Replacement of vitamin E to rats on restricted diet resulted in a return to normal of DNA turn-over and healing and regeneration of muscles (Mason and Horwitt, 1972). In view of this, vitamin E replacement therapy was attempted in dystrophic mice (dy/dy) but with no beneficial effects (Tubis et al., 1959). Subsequently an alternative approach to treatment involving the use of an iron chelating agent was proposed. Desferal (desferioxamine) is used as a detoxifying agent in patients suffering

Figure 1I

Molecular structures of drugs.

A Nifedipine

5 Pyridine carboxylic acid, 1,4 dihydro-2,6-dimethyl-4-(2-nitrophenyl)-methyl ester.

B Dantrolene

1-[5-(p-nitrophenyl) furfurylidene amino] hydantoin

C Cyproheptadine

4(5H-dibenzo[a,d]cyprohepten-5-ylidene)-1-methyl piperidine hydrochloride mono hydrate

D Diltiazem

cis (+)-3-(acetyloxy)-5-[2-(dimethylamino) ethyl]-2,3-dihydro-2-(4 methoxyphenyl)-1,5-benzothiazepin-4 (5H-one).

E Pyl08 068n

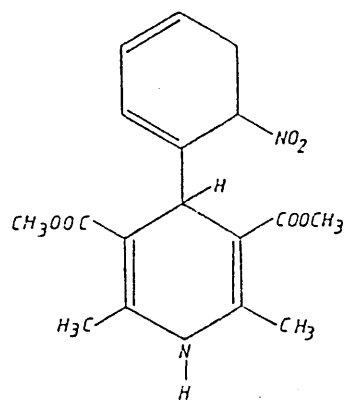
benzoxadiazolyle dihydropyridine derivative

F Minoxidil

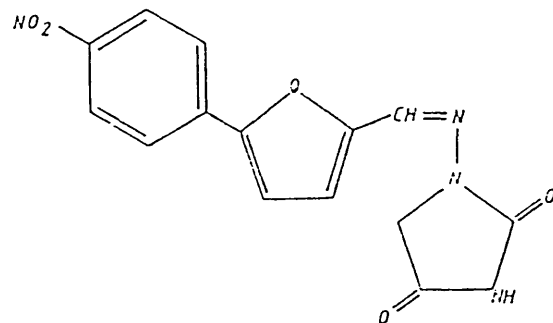
2,4-diamino-6-piperidino pyrimidine 3-oxide.

Figure 1I

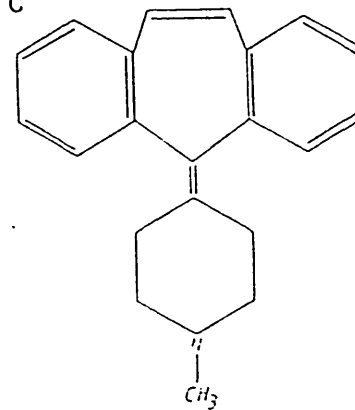
A



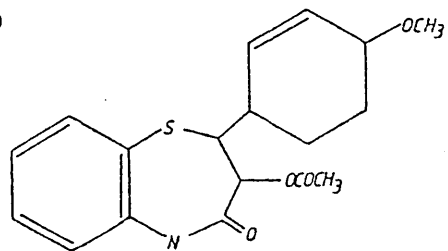
B



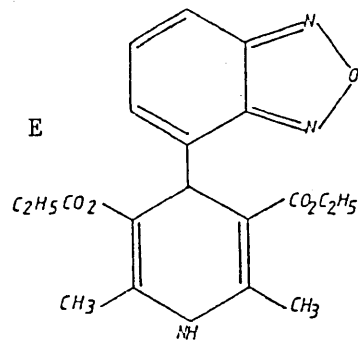
C



D



E



F

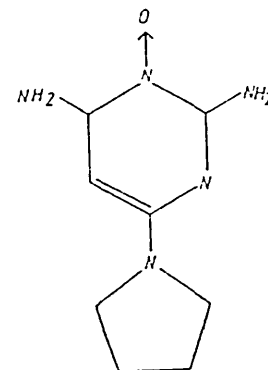


Figure 1II

Molecular structures of drugs.

A. Verapamil

α -[3-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]propyl]-3,4-methoxy-(1-methylethyl)-benzeneacetate.

B Cinnarizine (R=H)

(E)-1-bis(phenyl) methyl-4-(3-phenyl-2-propenyl) piperazine dihydrochloride

Flunarizine (R=F)

(E)-1-bis (4-fluorophenyl) methyl-4-(3-phenyl-2-propenyl) piperazine dihydrochloride

C Ketanserin

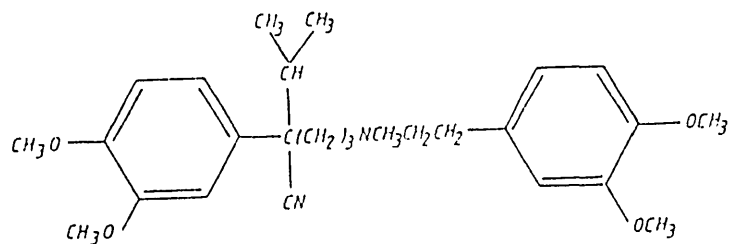
3- {2[4-(fluorobenzoyl)-1-piperidinyl] ethyl} 2,4(1H,3H) quinazolin-1-one.

D Desferoxamine (Desferal)

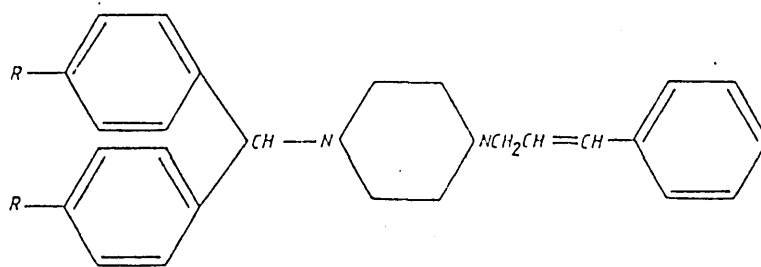
N-{5-[3[(5-aminopentyl)hydroxy carbamoyl] propionamido] pentyl}-3-[[5-(N-hydroxyacetamido) pentyl] carbamoyl] propionohydroxamic acid monomethanesulfonate salt

Figure 111

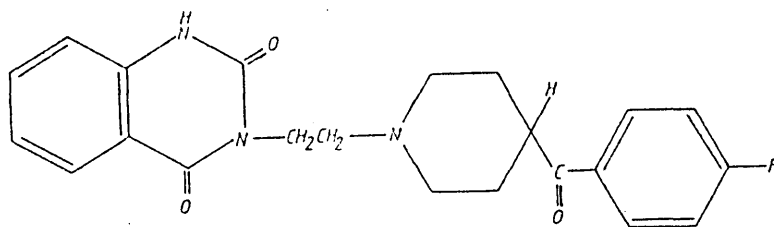
A



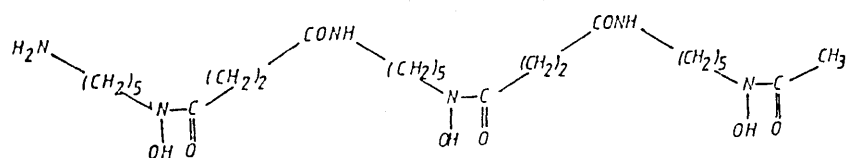
B



C



D



from iron overload and it was proposed that this drug would be a suitable candidate for therapeutic trials (Clark, 1984). Structure is shown in Fig. 111.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma, Poole, Dorset with the exception of sodium heparin (1000U/ml) from Duncan Flockhart Limited, CK (GSH-activated) and CK(NAC-activated) test kits from BCL, Lewes, Sussex, propylene glycol and disodium monophosphate, potassium dihydrogen phosphate from BDH, Poole, Dorset.

Test Kits

All enzyme test kits were purchased from BCL, Lewes, Sussex, U.K. The CK (GSH activated) test kit however has been deleted from the catalogue.

Drugs

Drugs were obtained from the following distinct sources:

Isaxonine (IAPP)	Ipsen, Paris, France.
BN1041-A	Ipsen, Paris, France.
BN1041-B	Ipsen, Paris, France.
Cyproheptadine	Merck, Sharp, Dohme, USA
Ketanserin (R41468)	Janssen Pharmaceuticals, Marlow, Bucks.
Flunarizine (R41 90)	Janssen Pharmaceuticals, Marlow, Bucks.
Cinnarizine (R1575 base)	Janssen Pharmaceuticals, Marlow, Bucks.
R55 667	Janssen Pharmaceuticals, Marlow, Bucks.
R49 945	Janssen Pharmaceuticals, Marlow, Bucks.
Bol 148 hta	Sandoz Ag. Basel, Switzerland
Py 108-068n	Sandoz Ag. Basel, Switzerland
Nifedipine	Bayer UK Limited, Haywards Heath, West Sussex, UK.

<u>Drug</u>	<u>Donor</u>
Verapamil	Abbot Laboratories Limited, Queensborough, Kent, U.K.
Dantrolene	Norwich Eaton Laboratories, Woking, Surrey, U.K.
Diltiazem	L.E.R.S., Paris, France
Minoxidil	UpJohn Limited, Crawley, West Sussex
Desferal	Ciba Laboratories, Horsham, West Sussex

Map-1 was a gift from Per O. Seglen, Norsk Hydro's Institute for Cancer research, The Norwegian Radium Hospital, Monebello, Oslo, Norway. 4-aminopyridine and 3,4-diaminopyridine was bought from Aldrich Chemical Company.

Methods

The dystrophic chickens used in the drug trials were New Hampshire line 413 (homozygous for dystrophy) and the genetically related normal New Hampshire line 412. These sub-lines of the original Davis stock were bred and maintained at Imperial College, London. Both flocks were maintained under constant conditions; temperature 25°C, relative humidity 40-50% and 12 hours light/dark cycle (Pizzey and Barnard, 1983). Food and water were available ad libitum and bird density was 10 birds per cage.

Each trial group and the accompanying dystrophic control group contained 10 birds randomly chosen from the same hatch. All were weighed on a day 2 ex ovo and every 7 days thereafter. Drugs were administered by daily intra-peritoneal (ip) injections commencing on day 3 ex ovo.

Administration of Drugs

The quantity of drug required for injection (x) was calculated from the bird mean weight according to the following equation:-

$$\frac{\text{BMW} \times \text{D}}{1000^2} = \text{X grams}$$

BMW = mean weight for 10 birds in grams

D = dosage in milligrams per kilogram mean body weight

The diluents used were:-

- (a) distilled de-ionised water (ddH₂O)
- (b) phosphate buffered saline (PBS)
- (c) 20% (v/v) propylene glycol in distilled de-ionised water
- (d) 35% (v/v) N-hydroxy ethyl lactamide in distilled de-ionised water

Drugs were made up in diluents according to their solubility and pH adjusted to physiological range where required (Table 1a). Drug solutions were stored at 0°C until required.

Flip Tests

All chickens included in drug trials were 'flipped' at 1600 hr on day 10 ex ovo and at weekly intervals thereafter until day 52. The results were expressed either as flip score (FS) or exhaustion score (ES) according to the method used.

TABLE 1a

Drugs and Diluents

<u>Drug</u>	<u>Diluent</u>
Ketanserin	distilled deionised water (ddH ₂ O)
R49 945	distilled deionised water
R55 667	35% (v/v) N-hydroxyethyl lactamide
cypheptadine	distilled deionised water (dd H ₂ O)
minoxidil	20% (v/v) propylene glycol/ddH ₂ O
Bol.148hta	distilled deionised water
nifedipine	20% (v/v) propylene glycol/dd H ₂ O
diltiazem	distilled deionised water
flunarizine	50% propylene glycol/PBS pH adjusted (~pH 7.00)
cinnarizine	50% propylene glycol/PBS (fine suspension)
dantrolene	20% (v/v) propylene glycol/dd H ₂ O
verapamil	distilled deionised water
Py108 068n	distilled deionised water
Isaxonine (IAPP)	20% (v/v) propylene glycol/PBS pH adjusted (~pH 7.00)
BN1041-A	20% (v/v) propylene glycol/PBS pH adjusted (~pH 7.00)
BN1041-B	20% (v/v) propylene glycol/PBS pH adjusted (~pH 7.00)
4-aminopyridine (4AP)	distilled deionised water
3,4-diaminopyridine	distilled deionised water
3-methyl adenine (MAP-1)	distilled deionised water
desferal (Desferioxamine)	35% (v/v) N-hydroxyethyl lactamide

FLIP SCORE (FS) was calculated as the number of times a chicken rose from a supine position, out of five successive attempts and was expressed flip/5.

EXHAUSTION SCORE (ES) was the number of times in rapid succession that a chicken rose from a supine position until exhausted or 31 flips were recorded (Hudecki and Entrikin, 1982). These were expressed directly as the number recorded FS or ES is stated for each trial.

Sample Preparation

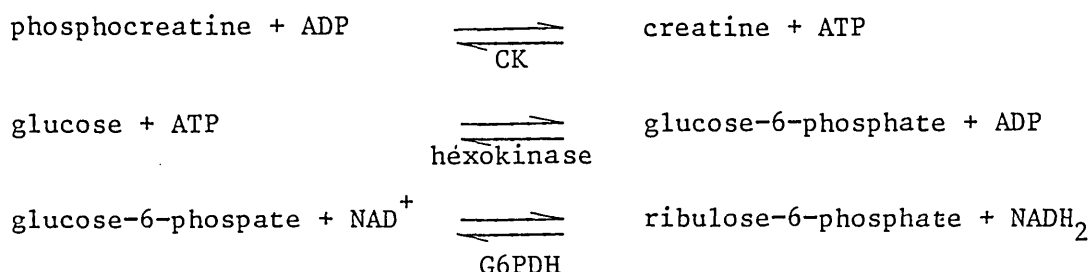
All experimental birds were bled on day 21 ex ovo and at weekly intervals until day 56. A sample (1 ml) of blood was withdrawn (without anaesthesia) from the jugular vein into centrifuge tubes containing sodium heparin (0.01 ml; 1000 Uml⁻¹), centrifuged in a bench top centrifuge (Haematocrit microfuge) for three minutes and the upper plasma layer retained for assay. Dilutions were carried out in 0.85% sodium chloride solution.

ENZYME ASSAYS

Enzyme assays were performed on LKB 8600 Mk II kinetic analyser linked to a Techman TE20 chart recorder. Set temperature for all assays and preincubations was 25°C. All samples in assay mixture were preincubated in the thermostatically controlled sample tunnel prior to addition of substrate.

Creatine Kinase (EC2.7.3.2)

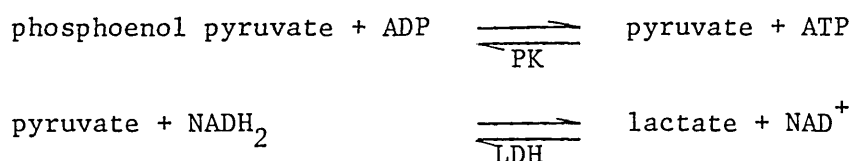
Creatine kinase (CK) activity was assayed using either the BCL CK (GSH-activated) test kit or BCL CK (NAC-activated) test kit, both utilising identical assay procedures but contain different activators, the former kit is no longer commercially available. Activity was determined in SI, UL^{-1} , from increase in absorbance at 340 nm resulting from NADH_2 production. Reactions involved are summarised below:-



Both methods use 0.5 ml of assay mixture but the GSH-activated method requires 0.025 ml of sample, whereas the NAC-activated method uses 0.02 ml. All other factors, preincubation at 25°C , addition of 0.05 ml substrate and 2 minutes incubation prior to reading were the same. Absorbance changes were monitored and enzyme activities calculated as above.

Pyruvate Kinase (EC2.7.1.40)

Pyruvate kinase (PK) activity was measured with the aid of BCL Pyruvate kinase (UV method) test kit. The manual assay was adapted to the automated system as described in Liu et. al (1980). Activity was calculated from the decrease in absorbance at 340 nm resulting from the NAD^+ production. Biochemical basis for this assay is described below:-



Samples (0.125 ml) were preincubated in assay mixture for 15 minutes at room temperature before addition of 0.025 ml of substrate. This was then incubated at 25°C for 2 minutes and absorbance read at 340 nm.

Phosphoglycerate mutase (EC 2.7.5.3)

Plasma phosphoglycerate mutase (PGAM) was assayed according to method described in detail in Section 3 p. 186.

Isocitrate dehydrogenase (EC 1.1.1.42)

Plasma isocitrate dehydrogenase (ICD) assay procedures followed the method described in detail in Section 2 p. 159.

CALCULATIONS

The enzyme activity was calculated from the volume activity (VA) of the sample and the change in absorbance per minute at 340 nm.

Thus

$$\text{mOD/min} \times \text{VA} = \text{U l}^{-1}$$

The change in absorbance was extrapolated from the gradient of the chart-recorder line and the volume activity calculated from the following equation:-

$$\frac{\text{total volume}}{\text{NAD}} \times \text{sample volume} = \text{VA}$$

The treated and untreated NAD plasma enzyme results were compared using the 'students' t-test. The distribution of both sample populations was assumed to be normal.

RESULTS

Serotonin antagonists

(i) Ketanserin was administered by standard trial procedures (see p. 38) at two dose levels 10 mgkg⁻¹day⁻¹ and 15 mgkg⁻¹day⁻¹ and in a subsequent trial orally, at 100 mgkg⁻¹day⁻¹. Results shown in Tables 1b, 1c indicate that under both ip dose regimes positive

beneficial effects were observed on plasma CK and PK levels. An increase of $5 \text{ mgkg}^{-1} \text{ day}^{-1}$ substantially improved and sustained the observed effects over the lower dose. Oral doses of $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ evoked no beneficial effects on levels of CK and PK.

Physical performance was improved under all three dose regimes (Fig. 1III). Again comparison of the ip doses demonstrated the higher dose to be more effective. Despite a lack of beneficial reductions of plasma enzymes with the oral dose, flip scores were greater for the treated group than for those of untreated control birds throughout the test period.

(ii) A single dose level of $40 \text{ mgkg}^{-1} \text{ day}^{-1}$ was selected for R49 945. Plasma enzyme levels (see Table 1d) were slightly reduced by this treatment. The greatest reduction from control values of CK levels monitored was 22% at d49 and for PK this was 12% also at d49, these effects however were lost by d56. The physical performance was monitored on days 46 and 56, at both time points the flip score of treated birds was substantially less (55%, 53%) than that of the untreated control birds. (Table 1d).

(iii) R55 667 was injected at $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ resulting in reductions of both CK and PK activity levels (Table 1e). The reduction of CK activity was greater than 20% at all time points except at d28. Variable reductions of PK activity were observed, the greatest reduction occurring at d56. This particular drug R55 667 generally benefited exhaustion scores and only at d31 were flip scores less than those of the control group (Fig. 1IV).

(iv) At $10 \text{ mgkg}^{-1} \text{ day}^{-1}$, cyproheptadine caused reductions in CK levels of 35% or greater throughout the trial period (see Table 1f). The reductions of PK activity were variable; the largest reduction was 45% at d35 and the smallest 9% at d4⁹. Improvements in flip score

were observed at all time points monitored (shown in Fig 1V).

(v) Minoxidil was evaluated at $10 \text{ mgkg}^{-1}\text{day}^{-1}$ and $20 \text{ mgkg}^{-1}\text{day}^{-1}$ dose levels. Initial beneficial effects observed on CK and PK activities under both dose regimes were not sustained to the end of the trial period (Tables 1g, 1h). Where beneficial effects were observed these were greater for CK than PK. Phosphoglycerate mutase (PGAM) activity was monitored (for method see p. 186) twice in both trials, at d28 observed reductions were comparable to those of CK. However at the lower dose, in contrast to CK and PK levels at d56, PGAM levels were reduced by 25%. In the second trial PGAM showed similar reductions to those of PK. Isocitrate dehydrogenase (ICD) activity was monitored (for method see p. 159) only at the lower dose trial where results complimented those of CK and PK.

Improvements in exhaustion score were observed under both dose regimes. At the $10 \text{ mgkg}^{-1}\text{day}^{-1}$ level, in contrast to the plasma enzymes results, the exhaustion score performance at early time-points was less than control values and improvements were only observed from d31 onwards. Higher dose levels improved initial exhaustion scores but the effect was not sustained (Fig. 1VI).

(vi) Bol 148 hta was initially administered at a dose of $5 \text{ mgkg}^{-1}\text{day}^{-1}$ increased to $10 \text{ mgkg}^{-1}\text{day}^{-1}$ at d37 and finally to $15 \text{ mgkg}^{-1}\text{day}^{-1}$ at d44 for the remainder of the trial. As a result, substantial reductions of both plasma enzyme levels were observed up to d49. At d56 reductions of activity were half those previously observed (Table 1 i). Physical performance was improved from d24 till d56 (Fig. 1VII).

In summary, the most effective reductions of plasma enzyme levels and improvements of flip performance were observed after treatment with cyproheptadine ($10 \text{ mgkg}^{-1}\text{day}^{-1}$), ketanserine

(15 mgkg⁻¹day⁻¹) and Bol 148 hta (5-15 mgkg⁻¹day⁻¹). The remainder of the drugs tested in this group did not show the same potential at the dosages selected for trial.

CALCIUM ANTAGONISTS

(vii) Two trials of nifedipine were undertaken: the first at a dose of 0.1 mgkg⁻¹day⁻¹ and the second increasing the dose from 0.1 -0.3 mgkg⁻¹day⁻¹. The low dosages were selected because of the observed acute and cumulative toxicity of this drug on chickens. An initial dose of 0.3 mgkg⁻¹day⁻¹ could not be sustained by the small birds therefore the dose levels were increased during the trial. This drug was also unstable in daylight ($t_{\frac{1}{2}} = 0.5$ minutes) and therefore precautions to exclude light were taken during preparation and handling procedures (Bayer handbook).

Results of the 0.1 mgkg⁻¹day⁻¹ trial were reductions of plasma enzyme levels (Table 1j). The initial good response at d28 was diminished in the following 14 days but regained the original reductions of activity at d56. Throughout this trial the exhaustion scores of treated birds were greater than those of the control birds (Fig. 1VIII).

In the second trial the initial effects on CK and PK levels were similar to those previously described but increased dosage did not improve efficacy and subsequently all initial gains were lost (Table 1k). Exhaustion scores for the treated group followed similar patterns to that of the plasma enzymes (Fig. 1VIII).

(viii) Diltiazem was evaluated at two dosages: 0.1 mgkg⁻¹day⁻¹ and 0.2 mgkg⁻¹day⁻¹. The lower dose trial was carried out as previously described (p. 39) however, the higher dose trial parameters were only measured at a limited number of time points. This drug was light

sensitive and suitable handling precautions were taken.

At $0.1 \text{ mgkg}^{-1}\text{day}^{-1}$ reductions in CK levels were greater than those observed for PK. The best effects on both enzymes were observed at d21, thereafter the reductions were diminished (Table 11). The physical performance of treated birds was improved between d17 and d38, at other time points the birds scored less than the control group (Fig. 11X).

Analysis of parameters measured during the $0.2 \text{ mgkg}^{-1}\text{day}^{-1}$ trial indicated that doubling the dose did not increase the beneficial effects on the plasma enzymes (Table 11). Exhaustion scores, however were improved at d31 and d41 (Fig. 1 IX).

(ix) The response to the administration of flunarizine at $10 \text{ mg kg}^{-1}\text{day}^{-1}$ and $15 \text{ mgkg}^{-1}\text{day}^{-1}$ was poor and variable especially at the lower dosage (Tables 1m & 1n). Examples of this variability are demonstrated by the CK results where at d28 there was a 37% increase in activity and at d42 a reduction of 40% was observed. The physical performance under the lower dose regime was consistently improved in control to the variability observed at the higher dose (Fig. 1X).

(x) Cinnarizine was administered at the same dosages ($10, 15 \text{ mgkg}^{-1}\text{day}^{-1}$) as the structurally related flunarizine (Fig. 11I). In this instance improvements in all parameters were observed. In general, the CK and PK response was a progressive reduction of plasma enzyme levels during the trial period which was diminished only at the final time-point (Tables 1m and 1n). The one exception to this was a PK result in the $15 \text{ mgkg}^{-1}\text{day}^{-1}$ trial at d56 where the observed reduction of activity was 45%. The dosage of $10 \text{ mgkg}^{-1}\text{day}^{-1}$ improved

the physical performance of treated birds throughout the trial, but the higher dose level evoked a positive response after d17 and this was reduced between d45 and d56 (Fig. 1 XI).

(xi) Dantrolene was analysed at a dosage of $10 \text{ mgkg}^{-1} \text{ day}^{-1}$.

At early time points both CK and PK levels were reduced, however, this effect was not sustained till d56 (Table 10). This loss of efficacy was more pronounced in the CK than PK results. PGAM activity was measured at d28 and d56; at both time-points reduction of activity were greater than those observed for CK or PK. Slight reductions of ICD activity were observed at d42, this was improved by d56 where the reduction of activity was greater than that measured for the other enzymes.

Improvements in exhaustion score were recorded at d24, d31 and d52. At other points measured no beneficial effects were observed (Fig. 1 XII).

(xii) The dose selected for the verapamil trial was $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$. At this concentration beneficial effects observed on initial analysis were accompanied by significant weight loss (shown in Table 1p), a phenomenon not observed with other drugs.

The improvements observed at d21 and d28 were not sustained by d56. Improvements of physical performance were observed only at d24 and d31, other scores were less than those of the untreated control birds (Fig. 1 XIII).

(xiii) The length of the Py 108-068n trial was restricted by the quantity of drug available and therefore $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ injections were administered daily for 28 days. Plasma enzyme levels were assayed on d28 and after a further 7 days without drug on d35.

Observed reductions of CK activity at d28 were greater than those of PK but at d35 the CK effect was lost while the PK reduction improved (Table 1q).

The physical performance followed a pattern similar to that of CK; the 43% improvement at d24 was not maintained at the d32 reading.

In summary, treatments of dystrophic chickens with nifedipine ($0.1 \text{ mgkg}^{-1}\text{day}^{-1}$) and diltiazem ($0.1 \text{ mgkg}^{-1}\text{day}^{-1}$) was effective at improving all parameters measured. Increasing dosages of these drugs did not improve these effects. Administration of cinnarizine demonstrated progressive beneficial effects which contrasted the results of the difluoro derivative flunarizine at equivalent dosages. Pyl08 068n also showed promise in the treatment of dystrophy but dantrolene despite initial results did not sustain the beneficial effect.

NEUROTROPHIC AGENTS

(xiv) IAPP was investigated over a range of dosages, administered in three separate trials. The largest series included 40, 80, 120 and $200 \text{ mgkg}^{-1}\text{day}^{-1}$ doses, the remaining dosages 150 and $300 \text{ mgkg}^{-1}\text{day}^{-1}$ were investigated in separate experiments. On consideration of results at all doses, no linear dose response was evident. Greatest reductions of CK activity were observed at 40, 200 and $300 \text{ mgkg}^{-1}\text{day}^{-1}$ (Tables 1r and 1s). At the intermediate dosages, 80 and $120 \text{ mgkg}^{-1}\text{day}^{-1}$, some slight reductions of enzyme activity were observed which were outweighed by the increased activities measured at d35 (80 and 120) and d42 (80). Results of the 150 $\text{mgkg}^{-1}\text{day}^{-1}$ trial were better than those at 80 and $120 \text{ mgkg}^{-1}\text{day}^{-1}$

but not as beneficial as those observed at $200 \text{ mgkg}^{-1} \text{ day}^{-1}$. In this instance the substantial reduction of CK at d28 was diminished by d42 and PK reductions, although following a similar pattern were not as great as those of the other enzyme. The greatest reductions of enzyme levels were observed at the two highest drug doses (Table 1rs).

At doses 40, 80, 120 and $200 \text{ mgkg}^{-1} \text{ day}^{-1}$, physical performance was monitored by the exhaustion score method (Fig. XIV, XV). On d10 none of these treated groups scored more flips than control birds, however, from d18 to d45 at 40, 120 and $200 \text{ mgkg}^{-1} \text{ day}^{-1}$ doses positive improvements were observed. The $80 \text{ mgkg}^{-1} \text{ day}^{-1}$ treatment scores closely followed the pattern of the plasma enzymes. The flip score method was applied to the 150 and $300 \text{ mgkg}^{-1} \text{ day}^{-1}$ trials. At the lower dosage all flip scores were greater than control values, but the higher dose did not have such a consistent effect (Fig. 1XVI).

(xv) Two salts of the compound BN1041 investigated were BN1041-A (tartarate) and BN1041-B (basic). Three trials of BN1041-A were carried out at 72, 100 and 144 and $288 \text{ mgkg}^{-1} \text{ day}^{-1}$. No reductions of PK levels were observed at the lowest dosage but CK was improved from d42 to d56. At the $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ dose level both plasma enzymes were reduced by more than 40% at d28 however subsequently this effect was diminished (Table 1u). Throughout the trial at 144 and $288 \text{ mgkg}^{-1} \text{ day}^{-1}$ CK and PK levels were reduced. The effect of the treatment was greater on CK than on PK at these doses (Table 1t).

The physical performance at 72 and $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ was monitored by the flip score method (Fig. 1XVII). At the lower dose positive improvements on the control values were observed whereas at higher level only d46 flip scores were improved. The exhaustion score method was employed to assess the effects of 144 and $288 \text{ mgkg}^{-1} \text{ day}^{-1}$

dosages. Positive scores were observed from d26 onwards at the lower dose and from d26 at the higher level (Fig. 1XVIII).

Two trials of BN1041-B were carried out; at $50 \text{ mgkg}^{-1} \text{ day}^{-1}$ throughout the trial and at doses varying from $300\text{--}100 \text{ mgkg}^{-1} \text{ day}^{-1}$ in a single trial. In the first trial no beneficial effects of treatment on CK, PK and flip score were observed. The PK values were greater than those of the control birds from d35 to d56 (Table 1v).

The starting dosage in the second trial of $200 \text{ mgkg}^{-1} \text{ day}^{-1}$ was subsequently raised to $300 \text{ mgkg}^{-1} \text{ day}^{-1}$ after d28 enzyme analysis demonstrated no improvements of activity levels. At the higher dose deleterious effects on bird weight were observed and the dosage was dropped first to $200 \text{ mgkg}^{-1} \text{ day}^{-1}$ and finally to $100 \text{ mgkg}^{-1} \text{ day}^{-1}$. Reductions of CK and PK activity were demonstrated from d42 to d56 (Table 1v). The reductions of CK levels observed were greater than those of PK. The flip scores measured during this trial were improved in comparison to control values (Fig. 1XIX).

(xvi) The 4-AP trial was undertaken at $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$; a low dosage that could be tolerated by the chickens. Improvements of CK, PK and flip score were observed as a result of this treatment. Greatest reductions of CK and PK were observed at days 28 and 35, after which the effect was diminished (Table 1w). Flip scores were monitored at days 30, 45 and 53 and at all times treated birds performed better than control birds (Fig. 1XX).

(xvii) Tolerance of DAP was greater than for 4-AP in the test chickens and doses for the trial were set at 7 and $7.5 \text{ mgkg}^{-1} \text{ day}^{-1}$. Both doses reduced CK and PK activity levels throughout the trial period with the higher dose being most effective at days 28 and 35 (Table 1w). The lower dose was constant in its effect with similar reductions

observed at d28 and d56, whereas by d56 the upper dose, the effect was half that of d28. Both dosages resulted in improvements in the flip scores, the effect corresponding to the doses (Fig. 1XX).

In summary, at certain dosages all the drugs in this group reduced plasma enzyme levels and improved flip performance. The best results of IAPP treatment were observed at the extremes of the dose range. BN1041-A treatment was more effective than that of BN1041-B. All aminopyridines tested were efficacious but limited by toxicity factors.

ANTI PROTEASES

(xviii) Three trials of MAP-1 were undertaken at $10 \text{ mgkg}^{-1} \text{ day}^{-1}$, $50\text{-}100 \text{ mgkg}^{-1} \text{ day}^{-1}$ and at $100 \text{ mgkg}^{-1} \text{ day}^{-1}$. At the lowest dose, improvements in exhaustion score alone were observed (Fig. 1XXI). In the second trial the initial reductions of CK and PK activities were not sustained by d28 and thus the dosage was subsequently doubled. At this higher level gradual reductions of CK and PK were observed (Table 1x). Physical performance was also improved under this regime (Fig. 1XXI). The final trial at $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ confirmed the previous results. Limited quantities of drug remaining restricted the number of birds to be injected and thus the treated group consisted of five birds. The results of this treatment were reductions of CK and PK which varied between 18% and 78% for the former and 24% and 65% for PK (Table 1y). Plasma PGAM and ICD were also monitored. In the instance of PGAM the observed effect at d56 was half that of d28. The 39% reduction of ICD observed at d42 was negated by d56. Positive improvements in the physical performance of treated birds were observed at all timepoints except those of d10 and d45 (Fig. 1XXII).

CHELATING AGENTS

(xix) A single therapeutic trial of desferal was carried out at $100 \text{ mgkg}^{-1} \text{ day}^{-1}$. Reductions of CK, PK and PGAM were observed during the course of treatment. The greatest reduction of CK was 50% at d28 which was then diminished to 38% by d49. PK, measured from d28 to d42, demonstrated increasing reductions of activity, the remaining time points were completed by PGAM assay. The reductions of PGAM activity increased from 23% at d42 to 33% at d56 (Table 12). Physical performance was improved at d17 and from d31 to d53. At these points the exhaustion score of the treated birds was greater than those of the controls (Fig. 1XXIII).

DISCUSSION

The results of these therapeutic trials indicate that for every category examined at least one drug showed promise as an anti-dystrophic agent. This conclusion is based on plasma enzymes and physical performance results and therefore would require confirmation from histological studies before recommending use in human clinical tests.

Analysis of nine separate trials in the anti-seritonerpic category demonstrated that at the doses chosen three drugs displayed sustained positive beneficial effects. These were cyproheptadine ($10 \text{ mgkg}^{-1} \text{ day}^{-1}$), ketanserin ($15 \text{ mgkg}^{-1} \text{ day}^{-1}$) and Bol 148 hta ($5\text{-}15 \text{ mgkg}^{-1} \text{ day}^{-1}$). Cyproheptadine at this dosage caused reductions of CK, PK and improvements of exhaustion score which compared favourably with previous trials at $20 \text{ mgkg}^{-1} \text{ day}^{-1}$ (Barnard and Barnard, 1980). In this trial at $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ the greatest reductions of CK and PK were 48% for both enzymes and at $20 \text{ mgkg}^{-1} \text{ day}^{-1}$ these were 84% and 60% respectively. Therefore by using cyproheptadine as a suitable

standard the other drugs in this category may be evaluated.

Ketanserin ($15 \text{ mgkg}^{-1} \text{ day}^{-1}$), when compared to cyproheptadine, showed similar improvements in all three parameters. At the lower dose however, poor results were observed and any beneficial effects were transient. Oral administration of this drug also had no effect but this may indicate that the dose was not sufficiently high.

The third drug from this group to show positive effects was Bol 148 hta which improved the levels of plasma enzymes to a greater extent than the physical performance. Results of this increasing dose regime ($5\text{-}15 \text{ mgkg}^{-1} \text{ day}^{-1}$) showed superior effects on the plasma enzymes than cyproheptadine.

Further experiments are required before a complete evaluation of these two drugs can be made. In the case of ketanserin at the highest dose in this trial the effects are no better than cyproheptadine and thus higher doses need to be evaluated to determine whether any further improvements could be achieved. Increased oral doses would also indicate whether this mode of administration was practicable. For Bol 148 hta increased single dose trials are required to elucidate the correct efficacy of this drug and also to demonstrate whether improvements of exhaustion score could be achieved. Other drugs in this category did not compare favourably with cyproheptadine. This does not exclude the possibility that at higher doses they would have beneficial effects. Ketanserin is an example of this for at $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ the effects were minimal, whereas an increase of $5 \text{ mgkg}^{-1} \text{ day}^{-1}$ produced improved results. R55 667 showed comparable results to ketanserin at the same dosage and therefore it would be profitable to investigate this drug at a higher dose level. In contrast, R49 945 demonstrated no effect at $40 \text{ mgkg}^{-1} \text{ day}^{-1}$ and it is doubtful whether increasing the dosage would be of any benefit.

Minoxidil presents a different case, at both dosages initial reductions of plasma enzymes were observed but were not sustained. It is possible that increased concentrations of this drug may have a more sustained effect, although weight loss resulting from higher doses administration would be a limiting factor.

Beneficial effects of treatment of birds with calcium antagonists were observed at most drug dosages evaluated. These improvements may be classified as follows:-

- (a) sustained beneficial effects on all parameters throughout trial period;
- (b) initial protective effect diminished as trial progressed;
- (c) improved efficacy with length of trial;
- (d) no effect at that particular dose.

Nifedipine at a dosage of $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$ fulfils the criteria of the first classification. The initial beneficial reductions of CK and PK and improved exhaustion scores were retained till the end of the trial. Four drugs were included in the second category. These were diltiazem ($0.1, 0.2 \text{ mgkg}^{-1} \text{ day}^{-1}$), dantrolene ($10 \text{ mgkg}^{-1} \text{ day}^{-1}$) and verapamil ($0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$). The diltiazem and nifedipine results demonstrate a characteristic of calcium antagonists previously noted in therapy accompanying heart surgery (Hearse, 1984), i.e. increased dosage often had deleterious rather than beneficial effects. The third category contains cinnarizine which resulted in increasing improvements of CK and PK reductions and improved physical performance at both doses selected. This progressive effect of cinnarizine was previously observed (Van Nueten et. al 1978).

In contrast, the final category contains the structurally related compound flunarizine which was administered at the same dosages as cinnarizine. These two drugs vary only in two fluorines

(Fig III) yet produce diverse results which may be useful in elucidating biochemical defects present in dystrophy.

Pyl08 68n was not included in these categories because the drug administration was limited to 28 days. Further extended experiments are required before the status of this drug can be determined.

The extreme toxic effects of calcium antagonists were considered as the limiting factor in the selection of doses. Nifedipine and verapamil were the most toxic and Pyl08 68n the most tolerated in the trials. The doses chosen reflect this and fortuitously nifedipine and verapamil were efficacious in this range. Previous trials of verapamil on Davis 413 birds at $2 \text{ mgkg}^{-1} \text{ day}^{-1}$ resulted in 75% fatality of the treated group. At lower dosages (0.5 and $1 \text{ mgkg}^{-1} \text{ day}^{-1}$) however no reductions of CK on d28 analysis were observed. Improvements in flip score however were similar to those described herein (Entrikin et. al, 1981). In contrast, Hudecki et. al (1984) found doses of $20 \text{ mgkg}^{-1} \text{ day}^{-1}$ of nifedipine, diltiazem and verapamil tolerable to chickens. The CK results reported were highly variable, but comparable in some instances to those described here. The variability may be attributed to problems of drug solubility at high concentrations.

The reductions of CK activity resulting from diltiazem treatment ($0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$) were in the same range as those observed after oral administration of the drug to dystrophic hamsters. (Bhattacherya et. al, 1982). Oral doses of diltiazem were tolerable to both normal and dystrophic hamsters. Further chicken trials of this drug should include the oral method of administration and if successful may minimise the toxic effects observed on ip injections of calcium antagonists. The pharmacology of oral administration

of nifedipine and diltiazem has been well documented (Henry, 1980). Nifedipine was shown to be a potent and long acting calcium antagonist with a capacity to relax serotonin induced contractions. Diltiazem was also found to be a potent antagonist but with minimal adverse side effects. At high concentrations, diltiazem may also exert an effect on fast calcium channels.

Four of these calcium antagonists, nifedipine, diltiazem, cinnarizine, and Pyl08 68n warrent further research either for their potential in therapy or as biochemical probes into the muscle defect.

Therapy with the neurotrophic agents resulted in beneficial effects at certain doses of IAPP, BN1041 and the aminopyridines. The IAPP results are a prime example of the care needed to select the drug dosage to give optimum efficacy with a minimum of detrimental side effects. IAPP at $40 \text{ mgkg}^{-1} \text{ day}^{-1}$ and above $150 \text{ mgkg}^{-1} \text{ day}^{-1}$ reduces plasma enzyme levels and improves flip scores however at $80 \text{ mgkg}^{-1} \text{ day}^{-1}$ and $120 \text{ mgkg}^{-1} \text{ day}^{-1}$ deleterious effects on CK and PK were observed. In contrast, neither of the BN1041 derivatives were effective below $100 \text{ mgkg}^{-1} \text{ day}^{-1}$. However at other doses tested for BN1041-A, progressive improvements were observed with increased dosage. Some evidence of this was observed in the BN1041-B trials also, although direct comparison with BN1041-A could not be made from the present study. They appear, however, to be effective in the same dose range. Any differences of effect may be attributed to pH of the drugs, which although important in ip injections may not be crucial on oral administration. At doses above $200 \text{ mgkg}^{-1} \text{ day}^{-1}$ no further improvements of effect were observed with either IAPP or BN1041-A.

The physical performance of all birds treated with IAPP was improved although at $300 \text{ mgkg}^{-1} \text{ day}^{-1}$ some weight loss effects may have countered the beneficial effect of treatment. The exhaustion

scores of birds treated with BN1041 derivatives responded in a dose dependant manner; the best effect was observed at the highest doses.

The results of IAPP treatment recorded in this thesis, although not directly contradicting published findings in line 413 birds, do not duplicate the original work (Barnard and Barnard, 1980). Only at a dose of $200 \text{ mgkg}^{-1} \text{ day}^{-1}$ were comparable reductions of plasma enzymes and improved flip scores shown in the present work. At other dosages, however smaller effects were observed in this work than previously recorded. The only difference between the two trial procedures was that in the present work the drug/diluent solution was pH adjusted to the physiological range and therefore loss of efficacy may be attributed to this factor.

The aminopyridine^s were also effective in reducing plasma enzyme levels and improving physical performance. At all doses tested beneficial results were observed, however, the toxicity of these compounds may preclude their use as therapeutic agents in children. In agreement with known pharmacological data DAP was as effective as 4-AP but considerably less toxic and may be more suited as an antidystrophic drug (Barnard et. al, 1982).

The single drug in the antiprotease category, Map-1 was effective at 50 and $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ dosages, a response which was dose dependant. Treatment improved all three parameters measured at the highest dose level. This compound, if these results are confirmed by histological research, may prove a useful drug in the treatment of DMD. The lack of deleterious weight effects in addition to its other properties would make Map-1 an ideal candidate for clinical trials.

Finally, desferal showed promise in the chelating agents category. The limited trial results demonstrated that alleviation of symptoms was possible with this drug. Further trials are required, however,

before conclusions can be drawn as to the suitability of this drug for the management of DMD.

In conclusion therapeutic trials of the various types of agents demonstrated that some drugs from each category were effective in the management of the dystrophic condition. Each drug was targeted at a known symptom of the disease but in absence of a known primary effector it is impossible to speculate why the drugs have these effects. One drug investigated, Cinnarizine, may be of use in elucidating this point because of the diverse results of treatment with it and its difluoro derivative flunarizine. The two fluorine groups in the latter compound remove the beneficial effects observed with cinnarizine. Toxicity was a great problem in the instances of some calcium antagonists and the aminopyridines, especially so in the latter case where it would be doubtful if these could be administered to children. Nifedipine, a calcium antagonist, with additional antiseritonergeric properties may be of such great benefit that this outweighs the toxic effect and with careful management may be used for clinical trials. Map-1 another exceptional drug should be advanced for clinical trials because of the positive benefits demonstrated at all levels measured. Further trials on chickens of oral administration of this drug may confirm this view and results of its use in combination with e.g. Nifedipine might, as well as being an effective treatment, help elucidate the primary myopathy of DMD. Until this primary myopathy has been elucidated, drugs trials such as described in this section are a positive method of aiding sufferers. When the causative agent has been determined and described, then the focus of therapy will turn to the synthesis of specific agents which will then be tested in dystrophic chickens prior to clinical trials.

In the meantime the emphasis of trials should be to continue this work, elaborating on the beneficial effects of drugs and experimenting with combinations of drugs which in addition to therapeutic efficacy may also shed light on the causative agents involved in the pathogenesis of DMD.

TABLE 1b

Plasma Creatine kinase (CK) activities in ketanserin-treated and untreated dystrophic chickens.

Ketanserin was administered ip at dosages of 10 and 15 mgkg⁻¹day⁻¹ and orally at 100 mgkg⁻¹day⁻¹.

Each treated group was accompanied by age matched dystrophic control birds. All groups consisted of 10 birds except the 10 mgkg⁻¹day group and controls which contained 8 birds each. Daily injections commenced on day 4 ex ovo and birds were bled at weekly intervals commencing from day 21.

+ significantly different from control at $P < 0.025$ by Student's t-test

* significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1b

	10 mgkg ⁻¹ day ⁻¹ (ip)	Dystrophic control	15 mgkg ⁻¹ day ⁻¹ (ip)	Dystrophic control	100 mgkg ⁻¹ day ⁻¹ (oral)	Dystrophic control
DAY (ex ovo)	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean SEM	Mean ± SEM % reduction from control	Mean ± SEM
21	593 ± 136 14%	689 ± 118			1225 ± 251 39%	883 ± 229
28	1059 ± 368 14%	1228 ± 249	552 ± 52* 55%	1156 ± 158	2100 ± 334 3%	2045 ± 520
35	2719 ± 782 21%	3456 ± 733	1721 ± 212 * 60%	4001 ± 611	4069 ± 806 +20%	3382 ± 770
42	4670 ± 1279 35%	7168 ± 1909	2142 ± 409* 50%	4257 ± 471		
49	6966 ± 1614 1%	7033 ± 1144	3150 ± 457 27%	4336 ± 817	7171 ± 1376 +5%	6846 ± 773
56	7214 ± 1496 21%	9170 ± 1734	6188 ± 890† 33%	9252 ± 1091	9760 ± 1959 +16%	8433 ± 1078

TABLE 1c

Plasma Pyruvate kinase (PK) activities in ketanserin-treated and untreated dystrophic chicken.

Details of trial drugs administration are as described for table 1b.

o significantly different from control at $P < 0.05$ by Student's t-test

* significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1c

Day (ex ovo)	10 mgkg ⁻¹ day ⁻¹ (ip)	Dystrophic control	15 mgkg ⁻¹ day ⁻¹ (ip)	Dystrophic control	100 mgkg ⁻¹ day ⁻¹ (oral)	Dystrophic control
	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean ± SEM
21					584 ± 244	655 ± 191
28	481 ± 119 10%	532 ± 107	325 ± 27* 51%	658 ± 113	919 ± 116 +6%	871 ± 154
35	1004 ± 237 24%	1326 ± 316	749 ± 88* 49%	1466 ± 188	1805 ± 310 +31%	1381 ± 275
42	1544 ± 368 28%	2145 ± 473	948 ± 155 40%	1570 ± 161		
49	2490 ± 631 27%	3432 ± 705	1375 ± 172 4%	1439 ± 178	2597 ± 396 +14%	2274 ± 296
56	2628 ± 558 11%	2947 ± 528	2451 ± 350 ^o 27%	3340 ± 390	3301 ± 617 +29%	2559 ± 281

TABLE 1d

Plasma enzyme activities and flip scores for R49 945-treated and untreated dystrophic chickens.

R49 945 was administered ip at a dosage of $40 \text{ mgkg}^{-1} \text{ day}^{-1}$ commencing on day 4 ex ovo. Weekly bleedings were made from day 28. Each group consisted of 10 birds.

TABLE 1d

DAY (ex ovo)	Creatine Kinase U ⁻¹		Pyruvate Kinase U ⁻¹		Flip Score	
	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM	Treated Mean $\pm$ SEM % increase from control	Dystrophic control Mean $\pm$ SEM
28	4957 $\pm$ 755 +17%	4209 $\pm$ 765	1392 $\pm$ 197 +17%	1197 $\pm$ 163		
35	5635 $\pm$ 935 7%	6036 $\pm$ 789	2434 $\pm$ 669 6%	2604 $\pm$ 646		
42	6835 $\pm$ 1081 17%	8273 $\pm$ 1087	2905 $\pm$ 613 7%	3133 $\pm$ 387		
49	8323 $\pm$ 1067 22%	10700 $\pm$ 1528	3119 $\pm$ 593 12%	3524 $\pm$ 416	0.7 $\pm$ 0.42 -53%	1.5 $\pm$ 0.58
56	9796 $\pm$ 2046 1.6%	9950 $\pm$ 1518	3941 $\pm$ 913 +7%	3686 $\pm$ 504	0.5 $\pm$ 0.26 -55%	1.1 $\pm$ 0.6

TABLE 1e

Plasma enzyme activities for R55 667-treated and untreated dystrophic chickens.

R55-667 was administered ip at $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Weekly bleedings commenced on day 21.

The treated group contained 7 birds and the dystrophic control group 10 birds.

TABLE 1e

DAY (ex ovo)	Creatine U l^{-1} Kinase		Pyruvate U l^{-1} Kinase		Phosphoglycerate Mutase U l^{-1}	
	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM
21	1377 $\pm$ 610 20%	1729 $\pm$ 264	580 $\pm$ 193 5%	609 $\pm$ 73		
28	3257 $\pm$ 951 3%	3354 $\pm$ 480	943 $\pm$ 217 3%	971 $\pm$ 142	197 $\pm$ 36 22%	252 $\pm$ 27
35	4778 $\pm$ 1170 20%	5989 $\pm$ 950	1309 $\pm$ 1619 19%	1619 $\pm$ 181		
42	5127 $\pm$ 1253 29%	7243 $\pm$ 840	1511 $\pm$ 376 24%	1976 $\pm$ 188	697 $\pm$ 144 9%	766 $\pm$ 69
49	7927 $\pm$ 1910 20%	9944 $\pm$ 1258	2094 $\pm$ 446 13%	2413 $\pm$ 265		
56	6111 $\pm$ 1124 43%	10652 $\pm$ 1646	1389 $\pm$ 263 24%	1822 $\pm$ 270		

TABLE 1f

Plasma enzyme activities from cyproheptadine-treated and untreated dystrophic chickens.

Cyproheptadine was administered ip at $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Birds were bled weekly from day 28. The dystrophic control group contained 10 birds throughout, however the treated group was reduced from 9 to 8 birds after day 28.

† significantly different from control at $P < 0.025$ by Student's t-test

x significantly different from control at $P < 0.01$ by Student's t-test

* significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1 f

DAY (ex ovo)	Creatine Kinase U ⁻¹		Pyruvate Kinase U ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	1914 ± 331 ^x 47%	3615 ± 529	1149 ± 162 18%	1405 ± 258
35	3712 ± 950* 50%	7397 ± 865	1606 ± 307 [†] 45%	2928 ± 521
42	2693 ± 569* 48%	5153 ± 410	1857 ± 455 23%	2422 ± 248
49	3731 ± 968 35%	5775 ± 925	2514 ± 536 9%	2772 ± 426
56	6665 ± 1162 [†] 40%	11181 ± 1590	3477 ± 652 22%	4480 ± 588

TABLE 1g

Plasma enzyme activities from Minoxidil-treated (10) and untreated dystrophic chickens.

Minoxidil was administered ip at a dosage of $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 24 ex ovo. Weekly bleedings commenced from day 21. Both treated and untreated groups contained 10 birds.

TABLE 1g

DAY (ex ovo)	Creatine Kinase U ¹ ⁻¹		Pyruvate Kinase U ¹ ⁻¹		Phosphoglycerate Mutase U ¹ ⁻¹		Isocitrate Dehydrogenase U ¹ ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
21	941 ± 239 37%	1493 ± 448	319 ± 43 21%	406 ± 89				
28	2109 ± 466 28%	2945 ± 673	691 ± 175 14%	806 ± 201	82.6 ± 12.5 30%	118 ± 25		
35	4348 ± 841 +9%	3997 ± 792	1299 ± 220 20%	1625 ± 484				
42	6093 ± 1365 +3%	5935 ± 1086	1859 ± 349 +18%	1570 ± 262			10.62 ± 0.64 +11%	9.59 ± 0.64
49	8595 ± 1786 1%	8687 ± 1823	1974 ± 624 +10%	1795 ± 286				
56	11618 ± 2549 +7%	10818 ± 2412	2827 ± 556 +7%	2625 ± 497	513 ± 70 25%	684 ± 93	10.45 ± 1.07 +11%	9.41 ± 0.99

TABLE 1h

Plasma enzyme activities from Minoxidil-treated (20) and untreated dystrophic chickens.

Minoxidil was administered ip at a dosage of $20 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Weekly bleedings commenced from day 21. The treated group consisted of 9 birds and the control group of 10 birds.

o significantly different from control at $P < 0.05$ by Student's t-test

TABLE 1h

DAY (ex ovo)	Creatine Kinase U _l ⁻¹		Pyruvate Kinase U _l ⁻¹		Phosphoglycerate Mutase U _l ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
21	1319 ± 176 24%	1729 ± 264	522 ± 61 14%	609 ± 73		
28	2502 ± 341 25%	3354 ± 480	872 ± 112 10%	935 ± 142	195 ± 36 23%	252 ± 27
35	5493 ± 708 8%	5989 ± 949	1518 ± 193 6%	1619 ± 181		
42	10185 ± 1451 ^o 41%	7243 ± 840	2426 ± 324 +23%	766 ± 188	991 ± 117 +29%	766 ± 69
49	11262 ± 1528 +13%	9944 ± 1258	2415 ± 322 +0.08%	2413 ± 265		

TABLE 1i

Plasma enzyme activities for Bol 148 hta-treated and untreated dystrophic chickens.

Bol 148 hta was administered ip at increasing dosages:-

day 4 - day 36 5 mgkg⁻¹day⁻¹

day 37 - day 43 10 mgkg⁻¹day⁻¹

day 44 - day 56 15 mgkg⁻¹day⁻¹

The treated group consisted of 10 birds and the control group of 8 birds.

† significantly different from control at P < 0.025 by Student's t-test

o significantly different from control at P < 0.05 by Student's t-test

* significantly different from control at P < 0.005 by Student's t-test

TABLE 1i

DAY (<u>ex ovo</u>)	Creatine Kinase U1 ⁻¹		Pyruvate Kinase U1 ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	1362 ± 171 ^o 55%	3002 ± 764	780 ± 117 42%	1354 ± 431
35	3046 ± 55 43%	5337 ± 1402	1097 ± 179 54%	2374 ± 724
42	4995 ± 624 [†] 48%	9554 ± 2082	2042 ± 206 48%	3964 ± 839
49	13997 ± 449* 65%	11317 ± 1844	1616 ± 178* 65%	4599 ± 768
56	9737 ± 1144 ^o 28%	13842 ± 1752	3099 ± 348 24%	4068 ± 783

TABLE 1j

Plasma enzyme activities for Nifedipine-treated (0.1) and untreated dystrophic chickens.

Nifedipine was administered ip at a dosage of $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Both treated and untreated groups contained 10 birds except where treated group was reduced to 9 birds from day 50 to day 56.

+ significantly different from control at $P < 0.025$ by Student's t-test

* significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1j

Day (ex ovo)	Creatine Kinase U ^l ⁻¹		Pyruvate Kinase U ^l ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	664 ± 117 [†] 43%	1156 ± 158	356 ± 69 [†] 46%	658 ± 113
35	3069 ± 412 23%	4001 ± 611	1127 ± 132 23%	1466 ± 188
42	2728 ± 424 36%	4257 ± 471	1283 ± 178 18%	1570 ± 161
49	2514 ± 350 [†] 42%	4336 ± 817	846 ± 100* 41%	1439 ± 178
56	5207 ± 467* 44%	9252 ± 1091	2070 ± 192* 38%	3340 ± 390

TABLE 1k

Plasma enzyme activities for Nifedipine-treated and untreated dystrophic chickens.

Nifedipine was administered ip at increasing dosages:-

day 4 - day 29	$0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$
day 30 - day 43	$0.2 \text{ mgkg}^{-1} \text{ day}^{-1}$
day 44 - day 56	$0.3 \text{ mgkg}^{-1} \text{ day}^{-1}$

Both treated and untreated groups consisted of 10 birds.

TABLE 1k

DAY (<u>ex ovo</u>)	Creatine Kinase U ⁻¹		Pyruvate Kinase U ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
21	567 ± 71 36%	883 ± 229	366 ± 43 44%	655 ± 191
28	1697 ± 258 17%	2045 ± 520	726 ± 130 17%	871 ± 154
35	2900 ± 366 14%	3382 ± 770	1269 ± 163 8%	1381 ± 275
49	6668 ± 1047 2%	6846 ± 773	3345 ± 194 3%	2274 ± 296
56	9146 ± 957 8%	8433 ± 1078	3104 ± 303 +22%	2559 ± 281

TABLE 11

Plasma enzyme activities for Diltiazem-treated and untreated dystrophic chickens.

Diltiazem was administered ip in two separate trials at 0.1 and 0.2 mgkg⁻¹day⁻¹ from day 4 ex ovo.

At the higher dosage bleeding was restricted to days 35 and 42.

For the 0.1 mgkg⁻¹day⁻¹ trial the control group contained 10 birds throughout but the treated group was reduced to 9 birds after day 35.

In the 0.2 mgkg⁻¹day⁻¹ trial both treated and untreated groups contained 9 birds.

TABLE 11

Day (ex ovo)	Creatine Kinase U ⁻¹				Pyruvate Kinase U ⁻¹			
	0.1 mgkg ⁻¹ day ⁻¹	Dystrophic	0.2mgkg ⁻¹ day ⁻¹	Dystrophic	0.1 mgkg ⁻¹ day ⁻¹	Dystrophic	0.2 mgkg ⁻¹ day ⁻¹	Dystrophic
	Mean ± SEM % reduction from control	control Mean ± SEM	Mean ± SEM % reduction from control	control Mean ± SEM	Mean ± SEM % reduction from control	control Mean ± SEM	Mean ± SEM % reduction from control	control Mean ± SEM
21	498 ± 57 46%	932 ± 260			199 ± 14 28%	276 ± 66		
28	889 ± 168 41%	1516 ± 276			467 ± 59 22%	599 ± 92		
35	3264 ± 541 31%	4740 ± 835	4544 ± 770 10%	5032 ± 1216	1130 ± 159 16%	1342 ± 177	1310 ± 253 4%	1351 ± 230
42	3411 ± 799 32%	5073 ± 904	10212 ± 1123 10%	11407 ± 1973	1173 ± 198 19%	1453 ± 182	2142 ± 317 9%	2351 ± 439
49	6447 ± 1312 8%	7037 ± 887			1760 ± 322 +2%	1718 ± 268		
56	8953 ± 1574 24%	11770 ± 1852			2004 ± 210 +7%	1867 ± 265		

TABLE 1^m

Plasma enzyme activities for Flunarizine-and-Cinnarizine-treated (10) and untreated dystrophic chickens.

Flunarizine and Cinnarizine were administered at a dosage of 10 mgkg⁻¹ day⁻¹ from day 4 ex ovo. Both treated groups and controls contained 10 birds.

TABLE 1m

DAY (ex ovo)	Creatine Kinase U ⁻¹			Pyruvate Kinase U ⁻¹		
	Flunarizing (10 mgkg ⁻¹ day ⁻¹) Mean ± SEM % reduction from control	Cinnarizine (10 mgkg ⁻¹ day ⁻¹) Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Flunarizing (10 mgkg ⁻¹ day ⁻¹) Mean ± SEM % reduction from control	Cinnarizine (10 mgkg ⁻¹ day ⁻¹) Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
21	341 ± 65 6%	316 ± 49 11%	363 ± 49	267 ± 22 3%	225 ± 34 18%	276 ± 34
28	1313 ± 354 +37%	726 ± 174 24%	956 ± 301	819 ± 253 +5%	454 ± 106 41%	780 ± 274
35	2657 ± 541 +13%	1796 ± 513 23%	2346 ± 709	1510 ± 219 0.7%	907 ± 196 40%	1500 ± 369
42	3278 ± 651 40%	3807 ± 1269 31%	5488 ± 1262	1929 ± 323 14%	1865 ± 628 17%	2179 ± 479
49	6835 ± 1578 +17%	5034 ± 1230 14%	5823 ± 1239	2749 ± 542 +20%	1670 ± 428 28%	2318 ± 478

TABLE 1n

Plasma enzyme activities for Flunarizine-and-Cinnarizine treated (15) and untreated dystrophic chickens.

Flunarizine and Cinnarizine were administered ip at a dosage of $15 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Both treated groups consisted of 10 birds each. The number of birds in the control group was reduced from 10 to 9 at day 42.

TABLE 1n

DAY (ex ovo)	Creatine Kinase U ^l - ¹			Pyruvate Kinase U ^l - ¹		
	Flunarizine (15 mgkg ⁻¹ day ⁻¹)	Cinnarizine (15 mgkg ⁻¹ day ⁻¹)	Dystrophic control	Flunarizine (15 mgkg ⁻¹ day ⁻¹)	Cinnarizine (15 mgkg ⁻¹ day ⁻¹)	Dystrophic control
	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean SEM	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean SEM
21	644 ± 82 +38%	400 ± 45 14%	464 ± 78	411 ± 34 +34%	254 ± 24 17%	307 ± 46
28	2046 ± 385 +2%	1616 ± 292 19%	2000 ± 48	856 ± 137 3%	693 ± 105 22%	883 ± 121
35	2550 ± 398 18%	1917 ± 430 38%	3095 ± 769	1248 ± 164 +1%	898 ± 165 27%	1234 ± 250
42	4678 ± 617 11%	3645 ± 1162 31%	5283 ± 1080	2138 ± 345 +11%	1550 ± 324 21%	1961 ± 359
49	5472 ± 1175 +21%	3508 ± 590 22%	4517 ± 811	2570 ± 386 +14%	1641 ± 240 27%	2261 ± 347
56	8012 ± 1378 +9%	5833 ± 1035 21%	7361 ± 1361	2783 ± 475 13%	1743 ± 271 45%	3189 ± 667

TABLE 10

Plasma enzyme activities for Dantrolene-treated and untreated dystrophic chickens.

Dantrolene was administered ip at a dosage of $10 \text{ mg kg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Both treated and control groups contained 10 birds.

TABLE 10

Day (ex ovo)	Creatine Kinase U1 ⁻¹		Pyruvate Kinase U1 ⁻¹		Phosphoglycerate Mutase U1 ⁻¹		Isocitrate Dehydrogenase U1 ⁻¹	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
21	831 ± 111 44%	1493 ± 448	301 ± 39 26%	406 ± 89				
28	1888 ± 282 36%	2945 ± 673	537 ± 85 33%	806 ± 201	57 ± 10 52%	118 ± 25		
35	3997 ± 610 0%	3997 ± 792	994 ± 109 39%	1625 ± 484				
42	5266 ± 626 11%	5935 ± 1086	1220 ± 211 22%	1570 ± 262			9.1 ± 0.6 5%	9.59 ± 0.65
49	8114 ± 745 7%	8687 ± 1823	1705 ± 198 5%	1795 ± 286				
56	9915 ± 2206 8%	10818 ± 2412	2546 ± 339 3%	2625 ± 479	593 ± 109 13%	684 ± 93	8.08 ± 0.83 14%	9.41 ± 0.99

TABLE 1p

Plasma enzyme activities and weight determinations for Verapamil-treated and untreated dystrophic chickens.

Verapamil was administered ip at a dosage of $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Whole bird weight measurements were taken during the course of treatment. The control group consisted 10 birds whilst the treated contained 8 birds.

- o significantly different from control at $P < 0.05$ by Student's t-test
- x significantly different from control at $P < 0.01$ by Student's t-test
- * significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1p

Day (ex ovo)	Creatine Kinase U ⁻¹		Pyruvate Kinase U ⁻¹		Phosphoglycerate Mutase U ⁻¹		Weight g	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % difference from control	Dystrophic control Mean ± SEM
21	602 ± 77 ^x 54%	1304 ± 233	324 ± 39 34%	429 ± 50			147 ± 7.5* -19%	179 ± 5.2
28	871 ± 186* 73%	3192 ± 446	393 ± 53* 56%	912 ± 166	34 ± 2.01* 63%	91 ± 11.4	229 ± 13 ^o -15%	267 ± 8.5
56	8615 ± 1901 6%	9190 ± 1276	1964 ± 342 9%	2168 ± 184			631 ± 41 -8%	683 ± 18

TABLE 1q

Plasma enzyme activities and flip scores for Pyl08 068n-treated and untreated dystrophic chickens.

Pyl08 068n was administered ip at a dosage of $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 to day 28 ex ovo. The control and treated groups contained 8 and 7 birds respectively.

† significantly different from control at $P < 0.025$ by Student's t-test

TABLE 1 q

Day (ex ovo)	Creatine Kinase U ^l ⁻¹		Pyruvate Kinase U ^l ⁻¹		Flip Score	
	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	Treated Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	1266 ± 170† 58%	3002 ± 764	998 ± 128 26%	1353 ± 431	4.75 ± 0.25 +58%	3.00 ± 0.8
35	2922 ± 785 45%	5337 ± 1402	1348 ± 305 43%	2374 ± 724	3.00 ± 0.85 -20%	3.75 ± 0.72

TABLE 1r

Plasma enzyme activities for IAPP-treated (40,80,120,200) and untreated dystrophic chickens.

Isaxonine (IAPP) was administered ip at 40, 80, 120 and 200 $\text{mgkg}^{-1}\text{day}^{-1}$ from day 4 ex ovo. The numbers of birds per group were as follows:- n = 10, dystrophic control; n = 8, 40 $\text{mgkg}^{-1}\text{day}^{-1}$; n = 7, 80 and 200 $\text{mgkg}^{-1}\text{day}^{-1}$.

- † significant difference from control at $P < 0.025$ by Student's t-test
- o significant difference from control at $P < 0.05$ by Student's t-test
- * significant difference from control at $P < 0.005$ by Student's t-test

TABLE 1 r

Day (ex ovo)	Creatine Kinase U ⁻¹					Pyruvate Kinase U ⁻¹				
	40	80	120	200	Dystrophic control Mean ± SEM	40	80	120	200	Dystrophic control Mean ± SEM
	mgkg ⁻¹ day ⁻¹	mgkg ⁻¹ day ⁻¹	mgkg ⁻¹ day ⁻¹	mgkg ⁻¹ day ⁻¹		mgkg ⁻¹ day ⁻¹	mgkg ⁻¹ day ⁻¹	mgkg ⁻¹ day ⁻¹	mgkg ⁻¹ day ⁻¹	
	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control		Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	
21	339 ± 24 26%	459 ± 8 +0.2%	413 ± 80 10%	377 ± 60 18%	458 ± 36	178 ± 9.7 10%	190 ± 14 4%	193 ± 28 3%	195 ± 19 2%	198 ± 13
28	737 ± 139 27%	829 ± 224 18%	891 ± 301 12%	463 ± 48* 54%	1008 ± 157	379 ± 54 +6%	351 ± 73 2%	423 ± 139 +18%	238 ± 16° 33%	357 ± 55
35	765 ± 152° 38%	921 ± 176 25%	1520 ± 871 +25%	544 ± 72* 56%	1228 ± 171	244 ± 46 4%	217 ± 31 15%	712 ± 272 +17%	193 ± 23 24%	255 ± 35
42	3302 ± 595 +3%	6194 ± 1207 +92%	4668 ± 1509 +45%	2444 ± 576 24%	3217 ± 436					
49	4807 ± 1172 31%	6204 ± 1060 11%	5520 ± 2601 21%	4734 ± 804† 32%	6944 ± 602	1687 ± 288 10%	1570 ± 232 16%	1899 ± 591 +1%	1319 ± 238 30%	1878 ± 176

TABLE 1s

Plasma enzyme activities for IAPP-treated (150,300) and untreated dystrophic chickens.

Isaxonine (IAPP) was administered ip at 150 and 300 mgkg⁻¹ day⁻¹ from day 4 ex ovo. Each treated group was paired with an age matched dystrophic control group. Both control groups and 150 mgkg⁻¹ day⁻¹ treated group contained 10 birds. At 300 mgkg⁻¹ day numbers were reduced from 10 to 9 at day 4 and further reduced to 8 at day 29.

- o significantly different from control at $P < 0.05$ by Student's t-test
- + significantly different from control at $P < 0.025$ by Student's t-test
- * significantly different from control at $P < 0.005$ by Student's t-test
- significantly different from control at $P < 0.001$ by Student's t-test

Table 1s

Day (ex ovo)	Creatine Kinase U ⁻¹				Pyruvate Kinase U ⁻¹			
	150 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	300 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	150 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	300 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	2727 ± 509* 51%	5568 ± 807	3423 ± 447* 43%	5989 ± 897	1282 ± 181 [□] 36%	2003 ± 218	1440 ± 163 [†] 51%	2934 ± 609
35	6033 ± 843 36%	9475 ± 1229	6033 ± 754 ^o 30%	8577 ± 1096	2300 ± 304 [†] 37%	3676 ± 474	2401 ± 286 26%	3236 ± 412
42	7665 ± 1283 16%	9071 ± 1303	7567 ± 1888 19%	9312 ± 1192	4192 ± 773 +4%	4013 ± 516	3250 ± 451 25%	4345 ± 540
49	7243 ± 1341 16%	8593 ± 1223	5451 ± 1080* 56%	12447 ± 1993	3500 ± 731 +4%	3354 ± 460	2530 ± 576 ^o 37%	4041 ± 647
56			14640 ± 2245 10%	16227 ± 1948			4977 ± 781 +1.6%	4898 ± 497

TABLE 1rs

Compilation of results from tables 1r and 1s.

The effects on plasma CK and PK resulting from IAPP treatment over dosage range from 40 to 300 mgkg⁻¹day⁻¹ expressed as percentage reduction from respective control values are shown in Table 1rs.

- o significantly different from control at $P < 0.05$ by Student's t-test
- † significantly different from control at $P < 0.025$ by Student's t-test
- * significantly different from control at $P < 0.005$ by Student's t-test
- significantly different from control at $P < 0.001$ by Student's t-test

Table 1 rs

Day (ex ovo)	Creatine Kinase % reduction from control						Pyruvate Kinase % reduction from control					
	40	80	120	150	200	300	40	80	120	150	200	300
21	26	0.2	10		18		10	4	3		2	
28	27	18	12	51*	54*	43*	+6	2	+18	36 [□]	33 [○]	51 [†]
35	38 [○]	25	+24	36	56*	30 [○]	4	15	+179	37 [†]	24	26
42	3	+92	+45	16	24	19				+4		25
49	31	11	2.1	16	32 [†]	56*	10	16	+1	+4	30	37 [○]
56						10						1.6

TABLE 1t

Plasma creatine kinase (CK) activities from BN1041-A treated and untreated dystrophic chickens.

BN1041-A was administered ip at dosages of 72, 100, 144 and 288 mgkg⁻¹day⁻¹ from day 4 ex ovo.

Each treated group was accompanied by age-matched dystrophic control birds. All groups consisted of 10 birds except the 100 and 288 mgkg⁻¹day⁻¹ groups which contained 9 birds each.

- o significantly different from control at $P < 0.05$ by Student's t-test
- * significantly different from control at $P < 0.025$ by Student's t-test
- x significantly different from control at $P < 0.01$ by Student's t-test

TABLE 1t

Day (ex ovo)	72 mgkg ⁻¹ day ⁻¹	Dystrophic control	100 mgkg ⁻¹ day ⁻¹	Dystrophic control	144 mgkg ⁻¹ day ⁻¹	228 mgkg ⁻¹ day ⁻¹	Dystrophic control
	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean ± SEM
28	4106 ± 547 +57%	2614 ± 470	3196 ± 625 ^x -47%	5989 ± 897			
35	5736 ± 770 +130%	2505 ± 353	7106 ± 1315 17%	8577 ± 1096	3310 ± 701 29%	2991 ± 694 ^o 38%	4818 ± 704
42	5847 ± 1145 20%	7278 ± 1259	10732 ± 2051 +15%	9312 ± 1192	3805 ± 830* 50%	4600 ± 1107* 49%	8947 ± 1130
49	6116 ± 1153 12%	6935 ± 1573	9078 ± 1770 27%	12447 ± 1993	2651 ± 838* 51%	3634 ± 1063* 57%	8488 ± 1624
56	7238 ± 1472 ^x 35%	11105 ± 2021	12883 ± 2365 21%	16227 ± 1948	3810 ± 525 ^x 56%	5242 ± 1644 38%	8459 ± 1716

TABLE 1u

Plasma Pyruvate kinase (PK) activities from BN1041-A treated and untreated dystrophic chickens.

Details of trial drugs administration are as described for Table 1t.

- o significantly different from control at $P < 0.05$ by Student's t-test
- + significantly different from control at $P < 0.025$ by Student's t-test

TABLE 1u

Day (ev ovo)	72 mgkg ⁻¹ day ⁻¹	Dystrophic control	100 mgkg ⁻¹ day ⁻¹	Dystrophic control	144 mgkg ⁻¹ day ⁻¹	288 mgkg ⁻¹ day ⁻¹	Dystrophic control
	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean ± SEM	Mean ± SEM % reduction from control	Mean ± SEM % reduction from control	Mean ± SEM
28	1937 ± 349 +63%	1170 ± 401	1676 ± 401 43%	2934 ± 609			
35	2777 ± 342 +99%	1391 ± 227	2928 ± 550 10%	3236 ± 412	1443 ± 298 19%	1371 ± 252 23%	1771 ± 187
42	3148 ± 560 +4.5%	3010 ± 552	5044 ± 976 +16%	4345 ± 540	1406 ± 265 [†] 47%	1938 ± 485 27%	2662 ± 420
49	3705 ± 637 +30%	2855 ± 626	3312 ± 694 27%	4041 ± 647	1745 ± 286 39%	1556 ± 289 ^o 46%	2864 ± 607
56	3566 ± 640 +0.4%	3579 ± 675	4718 ± 956 3.7%	4898 ± 497	1663 ± 168 35%	2276 ± 599 12%	2579 ± 531

TABLE 1v

Plasma enzyme activities from BN1041-B treated and untreated dystrophic chickens.

BN1041-B was administered ip at dosages of 50 and 200-100 mgkg⁻¹day⁻¹ from day 4 ex ovo. Both treated groups were accompanied by age-matched dystrophic control birds. All groups contained 10 birds except under the higher dose regime where numbers were reduced to 9 after day 49.

- o significantly different from control at $P < 0.05$ by Student's t-test
- * significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1v

Day (ex ovo)	Creatine Kinase U ⁻¹				Pyruvate Kinase U ⁻¹			
	50 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	200-100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	50 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	200-100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	3402 ± 500 4%	3556 ± 578	2837 ± 638 +8.5%	2614 ± 470	1725 ± 167 +2%	1689 ± 270	1351 ± 407 +15%	1170 ± 220
35	8290 ± 843 2%	8493 ± 1046	2311 ± 420 8%	2505 ± 353	3436 ± 456 +38%	2492 ± 309	1361 ± 263 2%	1391 ± 227
42	12653 ± 1249 14%	11098 ± 1296	4072 ± 974 ^o 44%	7278 ± 1259	3557 ± 902 +13%	3155 ± 385	2571 ± 658 15%	3010 ± 552
49	16398 ± 1984 +17%	14030 ± 1445	3897 ± 813 ^o 44%	6936 ± 1573	5472 ± 496 +36%	4037 ± 490	2366 ± 553 17%	2855 ± 626
56	17234 ± 1424 11%	19940 ± 1816	4377 ± 617* 61%	11105 ± 2021	5275 ± 536 +37%	3848 ± 461	2249 ± 344 ^o 37%	3579 ± 675

Table 1w

Plasma enzyme activities from 4-aminopyridine- and diaminopyridine-treated and untreated dystrophic chickens. Diaminopyridine (DAP) was administered ip in two separate trials at 7 and 7.5 mgkg⁻¹day⁻¹ from day 4 ex ovo. 4AP was administered ip in a single trial at a dosage of 0.1 mgkg⁻¹day⁻¹ from day 4 ex ovo. All groups contained 10 birds except in the 4AP trial where numbers were reduced from 8 to 7 at day 29.

- o significantly different from control at $P < 0.05$ by Student's t-test
- † significantly different from control at $P < 0.025$ by Student's t-test
- x significantly different from control at $P < 0.01$ by Student's t-test
- * significantly different from control at $P < 0.005$ by Student's t-test

TABLE 1_w

Day (ex ovo)	Creatine Kinase U _l ⁻¹					Pyruvate Kinase U _l ⁻¹				
	DAP 7 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	DAP 7.5 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	4AP 0.1 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	DAP 7 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM	DAP 7.5 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	4AP 0.1 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	Dystrophic control Mean ± SEM
28	4094 ± 666 ^x 37%	7815 ± 808	2156 ± 453 ^x 61%	2952 ± 411 [*] 47%	5568 ± 807	1660 ± 210 ^x 36%	2599 ± 423	1056 ± 208 [*] 47%	1316 ± 210 [†] 34%	2003 ± 218
35	6104 ± 905 [*] 38%	9783 ± 679	4187 ± 743 [*] 56%	4342 ± 660 [*] 54%	9475 ± 1229	2233 ± 336 [*] 44%	4003 ± 247	1772 ± 295 [*] 52%	1682 ± 276 [*] 54%	3676 ± 474
42	7175 ± 622 [*] 44%	12748 ± 1241	5439 ± 932 [†] 40%	5223 ± 925 [†] 42%	9071 ± 1303	2534 ± 344 [*] 37%	4042 ± 277	2442 ± 446 [†] 39%	2018 ± 346 [*] 49%	4013 ± 516
49	10829 ± 1418 21%	13660 ± 1520	5285 ± 1332 38%	6051 ± 632 ^o 29%	8593 ± 1223	3217 ± 504 [*] 23%	4171 ± 422	2452 ± 361 27%	2398 ± 481 ^x 29%	3354 ± 411
56	10241 ± 1339 [*] 37%	16290 ± 1231				2870 ± 288 [†] 30%	4090 ± 464			

Table 1x

Plasma enzyme activities from Map-1 treated and untreated dystrophic chickens.

Map-1 was administered ip at a dosage of $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ and at increasing dosages from $50 \text{ mgkg}^{-1} \text{ day}^{-1}$ (day 23 - day 56). Each treated group was accompanied by age-matched dystrophic control birds. Both the $10 \text{ mgkg}^{-1} \text{ day}^{-1}$ trial and control contained 10 birds however the higher dose group contained 9 birds and the accompanying control group 8 birds.

o significantly different from control at $P < 0.05$ by Student's t-test

† significantly different from control at $P < 0.025$ by Student's t-test

Table 1x

Day (ex ovo)	Creatine kinase U ⁻¹				Pyruvate kinase U ⁻¹			
	10 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM	50-100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM	10 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM	50-100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM
21			673 ± 127 ^o 54%	1455 ± 431			471 ± 63 35%	726 ± 195
28	2141 ± 612 +9%	1957 ± 361	2568 ± 559 14%	3002 ± 764	1307 ± 247 +31%	997 ± 204	1312 ± 237 2%	1353 ± 431
35	3339 ± 552 13%	3858 ± 769	3691 ± 690 31%	5337 ± 1402	1743 ± 282 10%	1937 ± 617	1895 ± 381 20%	2374 ± 724
42	5546 ± 789 +23%	4495 ± 824	5780 ± 1484 40%	9554 ± 2082	2134 ± 318 +23%	1703 ± 441	2092 ± 508 ^o 47%	3964 ± 839
49	7611 ± 949 +15%	6650 ± 1216	6073 ± 1018 ^o 46%	11317 ± 1812	3016 ± 413 +44%	2092 ± 456	2573 ± 435 [†] 44%	4599 ± 768
56	9338 ± 1050 12%	9168 ± 1567	7773 ± 2115 ^o 44%	13842 ± 1752	2263 ± 566 17%	2723 ± 498	3229 ± 784 21%	4068 ± 783

TABLE 1y

Plasma enzyme activities from Map 1-treated (100) and untreated dystrophic chickens.

Map-1 was administered ~~in~~ at a dosage of $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. The treated group contained 5 birds and the accompanying age-matched dystrophic control group contained 10 birds.

- o significantly different from control at $P < 0.05$ by Student's t-test
- * significantly different from control at $P < 0.005$ by Student's t-test

Table 1y

Day (ex ovo)	Creatine kinase U ⁻¹		Pyruvate kinase U ⁻¹		PGAM U ⁻¹		ICD U ⁻¹	
	100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM	100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM	100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM	100 mgkg ⁻¹ day ⁻¹ Mean ± SEM % reduction from control	dystrophic control Mean ± SEM
21	597 ± 185 ⁰ 60%	1493 ± 448	275 ± 123 32%	406 ± 89				
28	1577 ± 670 46%	2945 ± 673	596 ± 225 ⁰ 26%	806 ± 201	58 ± 18 ⁰ 51%	118 ± 25		
35	2525 ± 793 38%	3997 ± 792	758 ± 199 53%	1625 ± 484				
42	1614 ± 285* 78%	5935 ± 1086	686 ± 98* 56%	1570 ± 262			5.85 ± 0.26* 39%	9.59 ± 0.65
49	5883 ± 2016 32%	8687 ± 1823	1170 ± 132 ⁰ 35%	1795 ± 286				
56	8831 ± 2901 18%	10818 ± 2412	2002 ± 470 24%	2625 ± 479	546 ± 124 20%	648 ± 93	8.78 ± 1.36 7%	9.41 ± 0.99

Table 1z

Plasma enzyme activities from Desferal-treated and untreated dystrophic chickens.

Desferal was administered ip at a dosage of $100 \text{ mgkg}^{-1} \text{ day}^{-1}$ from day 4 ex ovo. Both treated and control groups contained 9 birds (day 4 to day 21) but were subsequently reduced to 8 birds (day 22 - day 56).

- o significantly different from control at $P < 0.05$ by student's t-test
- † significantly different from control at $P < 0.025$ by student's t-test
- * significantly different from control at $P < 0.005$ by student's t-test

TABLE 1z

Day (ex ovo)	Creatine Kinase U ⁻¹		Pyruvate Kinase U ⁻¹		Phosphoglycerate Mutase U ⁻¹	
	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM	Treated Mean $\pm$ SEM % reduction from control	Dystrophic control Mean $\pm$ SEM
21	1377 $\pm$ 333 23%	1782 $\pm$ 369	451 $\pm$ 107 19%	555 $\pm$ 90		
28	2126 $\pm$ 535* 50%	4282 $\pm$ 1049	738 $\pm$ 130 30%	1051 $\pm$ 186		
35	5031 $\pm$ 1054 34%	7597 $\pm$ 1349	1404 $\pm$ 261 25%	1861 $\pm$ 276		
42	4997 $\pm$ 806 ^o 35%	7636 $\pm$ 1266	965 $\pm$ 250 40%	1611 $\pm$ 310	1148 $\pm$ 160 23%	1483 $\pm$ 188
49	6157 $\pm$ 1045 [†] 38%	9998 $\pm$ 1492			889 $\pm$ 124 29%	1249 $\pm$ 128
56	8589 $\pm$ 1646 42%	14764 $\pm$ 3403			1143 $\pm$ 168 [†] 33%	1705 $\pm$ 172

Figure 1111

Exhaustion flip scores for Ketanserin-treated and untreated dystrophic chickens.

Exhaustion scores are shown for ketanserin-treated (■) and untreated (●) dystrophic chickens under three dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A dosage - $10 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and control groups contained 8 birds.
- B dosage - $15 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and control groups contained 10 birds.
- C dosage - $100 \text{ mgkg}^{-1} \text{ day}^{-1}$, orally. Both treated and control groups contained 10 birds.

EXHAUSTION FLIP

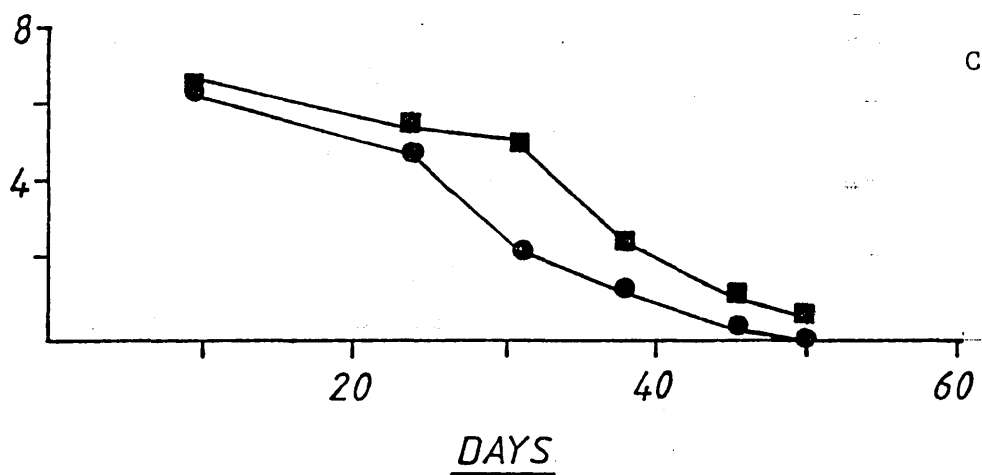
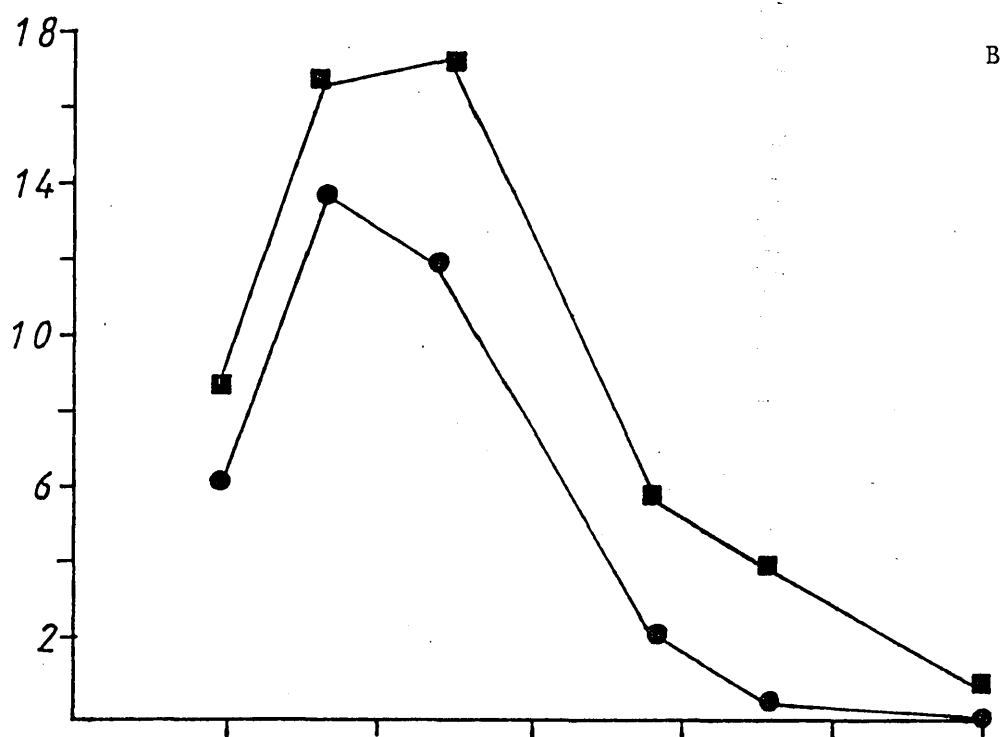
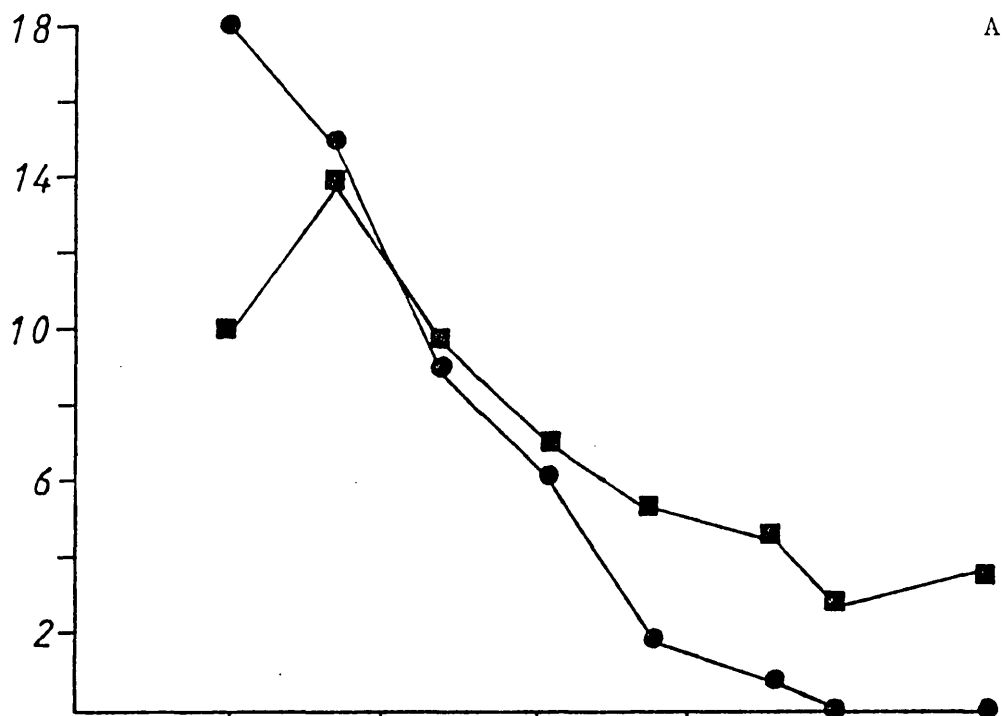


Figure 1IV

Exhaustion scores for R55 667-treated and untreated dystrophic chickens.

Exhaustion scores are shown for R55 667-treated (■) and untreated (●) dystrophic chickens. Treatment commenced from day 4 ex ovo. The treated and control groups contained 7 and 10 birds respectively.

Figure 1IV

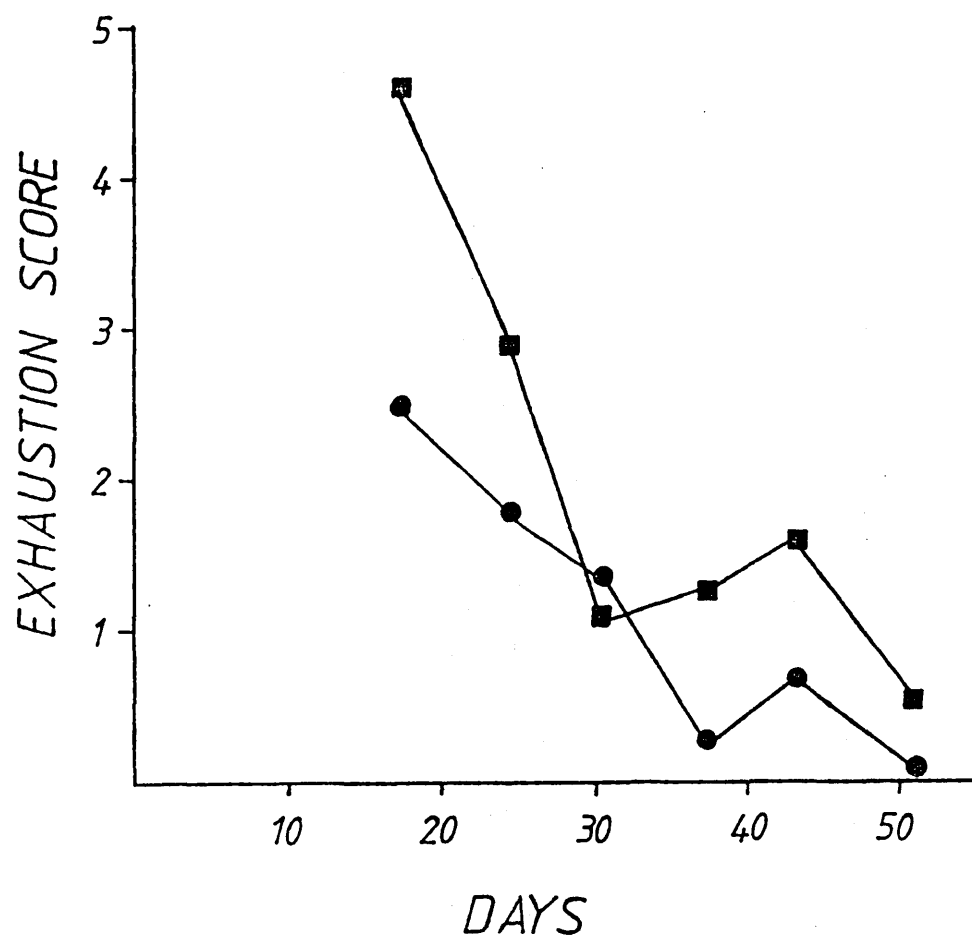


Figure 1V

Flip scores for Cyproheptadine -treated and untreated dystrophic chickens.

Flip scores are shown for cyproheptadine -treated (■) and untreated (●) dystrophic birds. Treatment commenced from day 4 ex ovo. The control group contained 10 birds throughout, the treated group was reduced from 9 to 8 birds at day 29.

Figure 1V

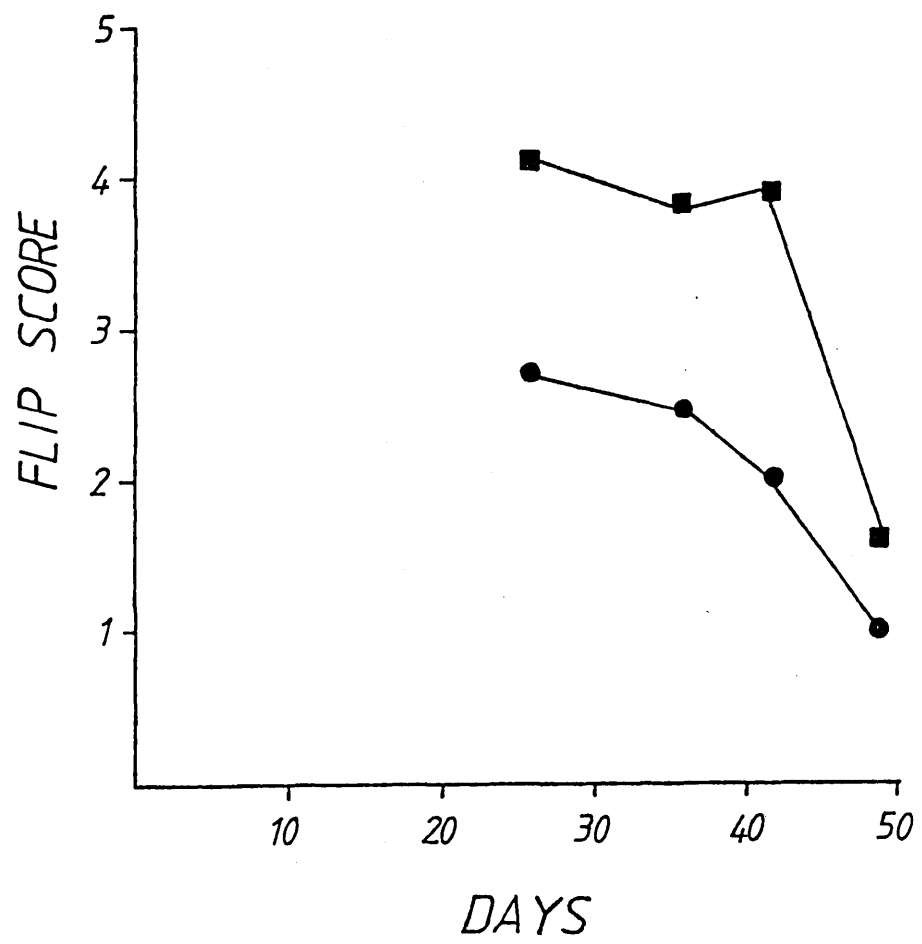


Figure 1VI

Exhaustion scores for Minoxidil-treated and untreated dystrophic chickens.

Exhaustion scores are shown for minoxidil-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage - $10 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Treated and control groups contain 10 birds each.
- B. dosage - $20 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Treated and control groups contain 9 and 10 birds respectively.

Figure lVI

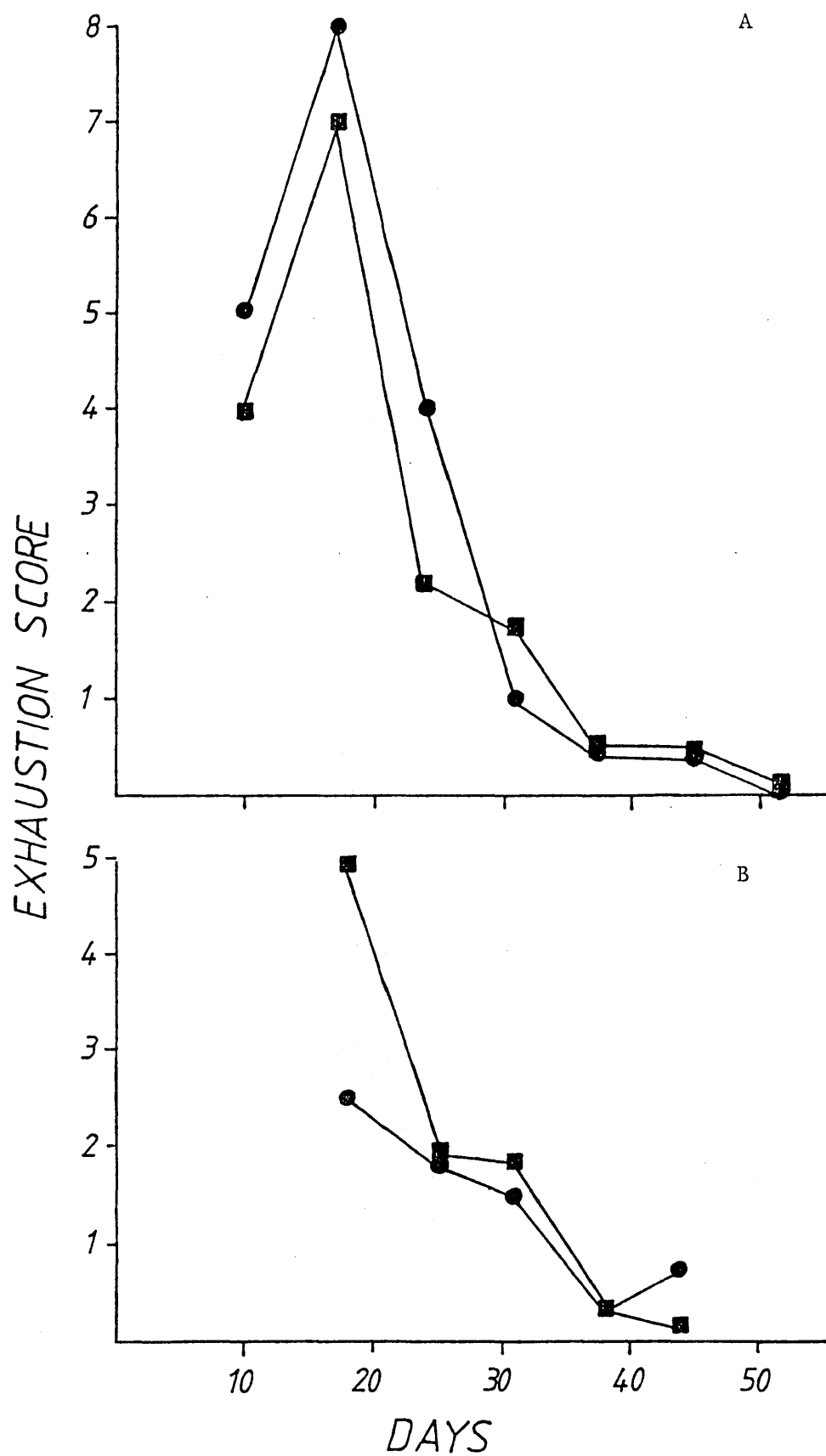


Figure 1VII

Exhaustion scores for Bol 148 hta-treated and untreated dystrophic chickens.

Exhaustion scores are shown for Bol 148 hta-treated (■) and untreated (●) dystrophic birds at increasing dosages:-

day 4 - day 36 dosage $5 \text{ mgkg}^{-1} \text{ day}^{-1}$.

day 37 - day 43 dosage $10 \text{ mgkg}^{-1} \text{ day}^{-1}$

day 44 - day 56 dosage $15 \text{ mgkg}^{-1} \text{ day}^{-1}$

The treated and control groups contained 10 and 8 birds respectively.

Figure 1VII

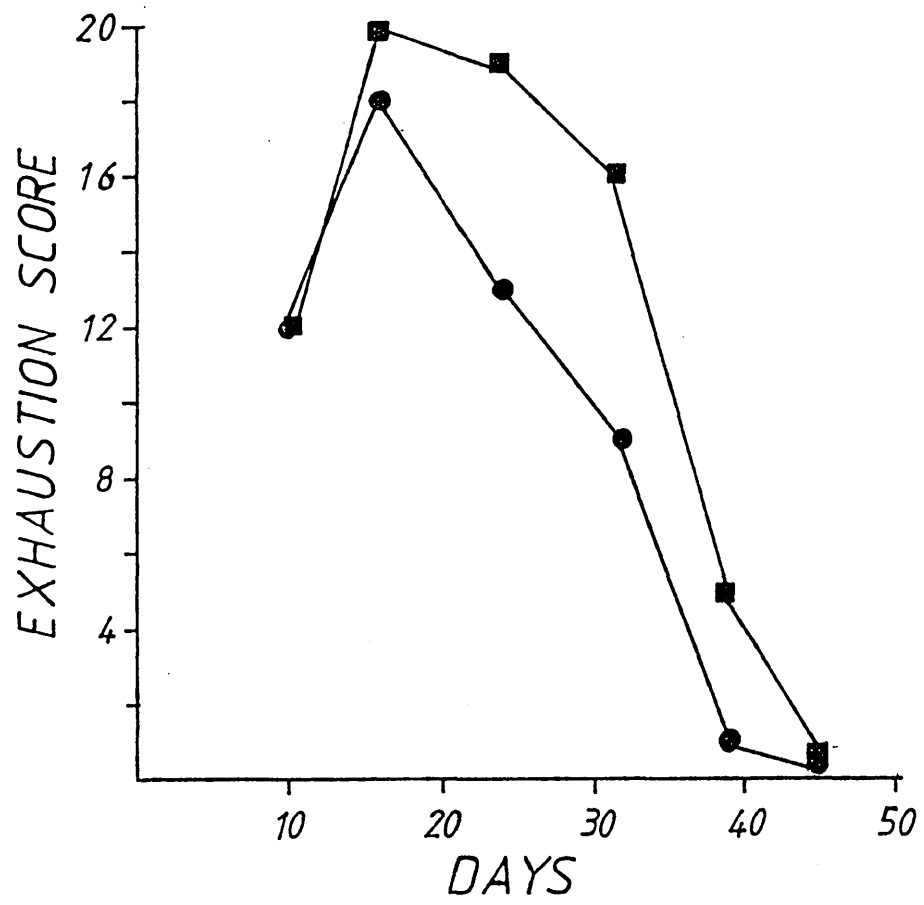


Figure 1VIII

Exhaustion scores for Nifedipine-treated and untreated dystrophic chickens.

Exhaustion scores are shown for nifedipine-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

A. dosage - $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and control groups contained 10 birds.

B. dosage - $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip; day 4 - day 29.
 $0.2 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip; day 30 - day 43.
 $0.3 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip; day 44 - day 56.

Both treated and control groups contained 10 birds.

Figure 1VIII

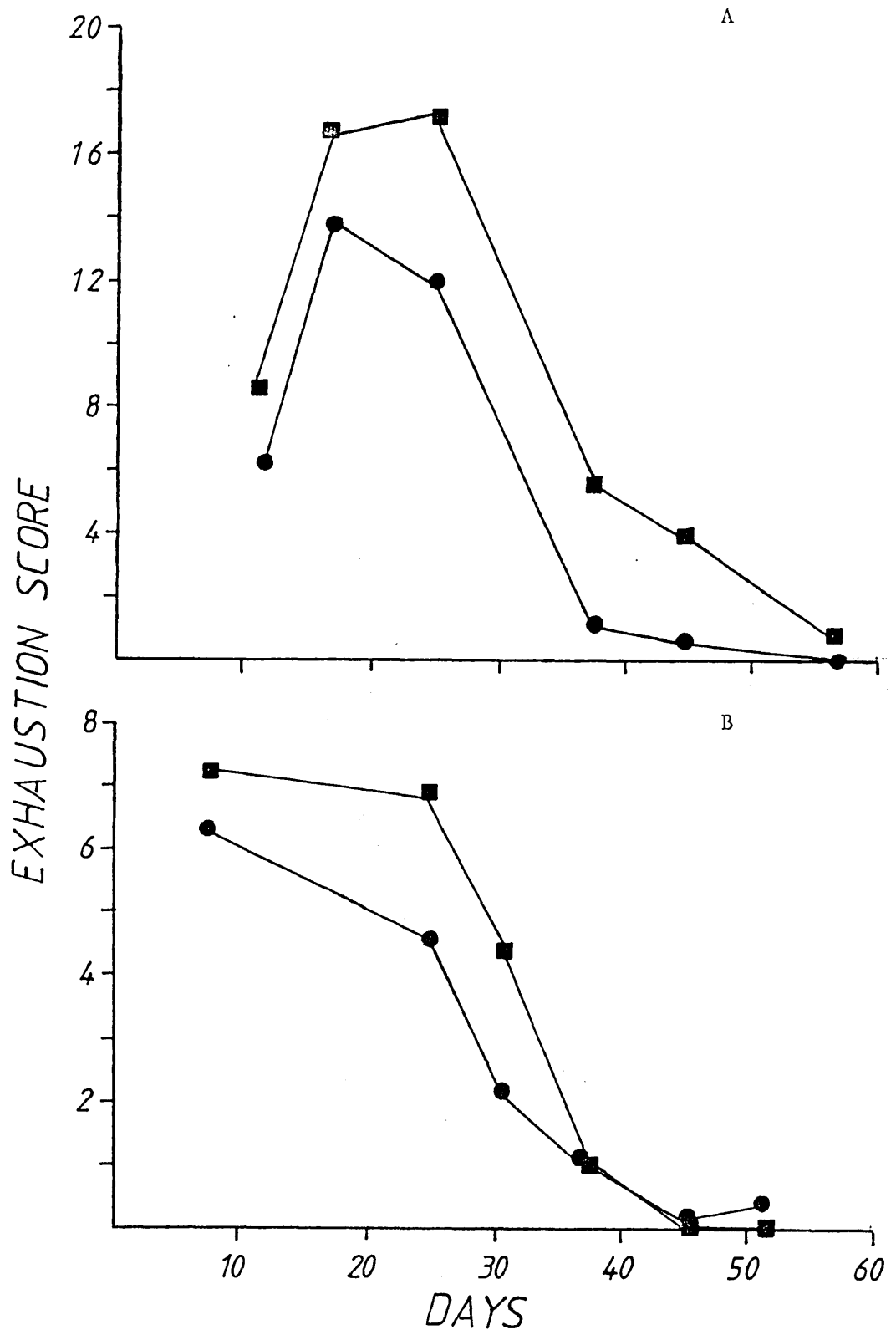


Figure 1IX

Exhaustion scores for Diltiazem-treated and untreated dystrophic chickens.

Exhaustion scores are shown for diltiazem-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$, i.p. Treated group was reduced from 10 to 9 birds at day 35. Control group contained 10 birds throughout.
- B. dosage $0.2 \text{ mgkg}^{-1} \text{ day}^{-1}$, i.p. Both treated and control groups contain 9 birds each.

Figure 1IX

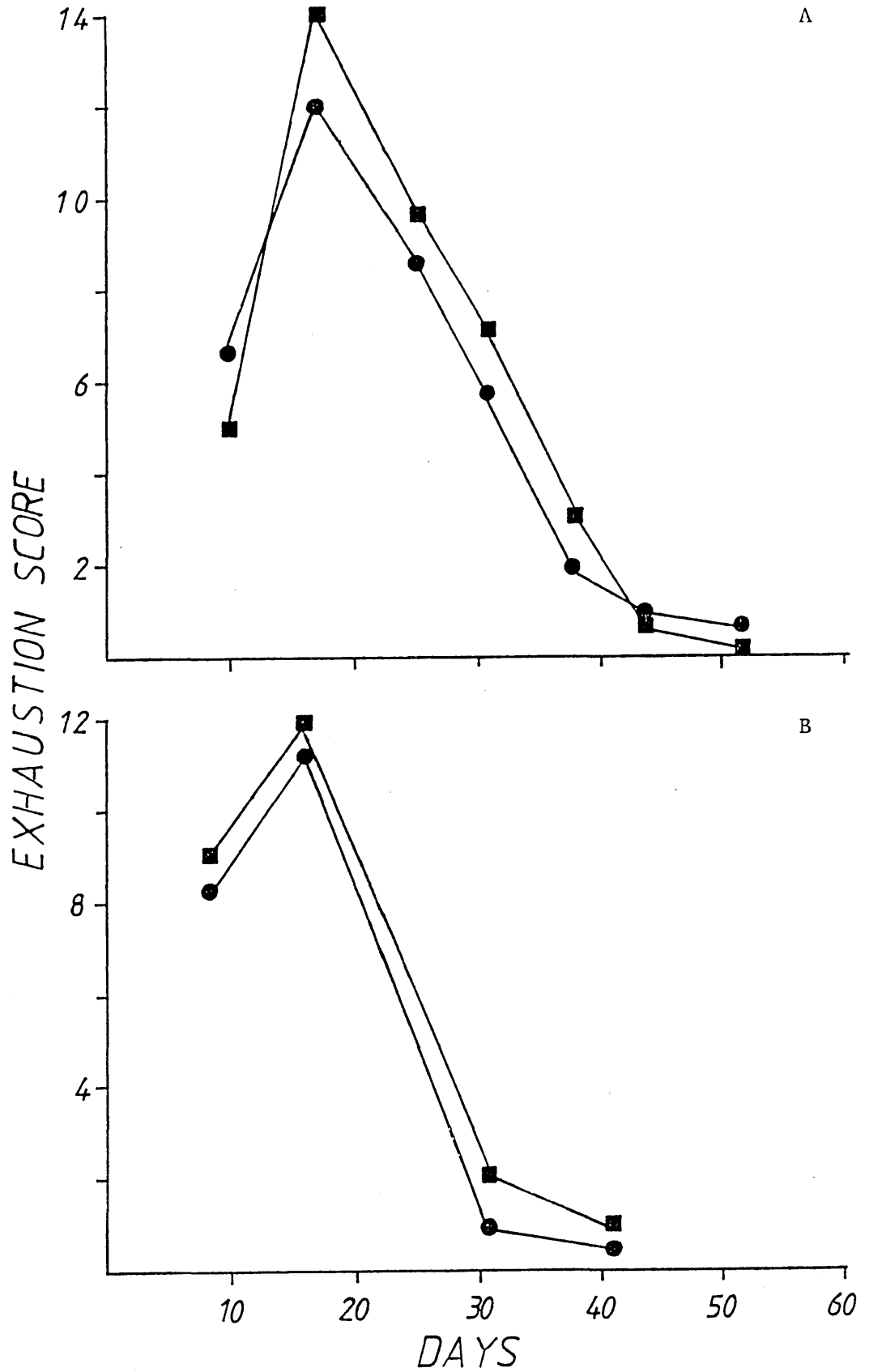


Figure 1X

Exhaustion scores for Flunarizine-treated and untreated dystrophic chickens.

Exhaustion scores are shown for flunarizine-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage - $10 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and untreated groups contained 10 birds.
- B. dosage - $15 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. The control group was reduced from 10 to 9 birds at day 42. The treated group contained 10 birds throughout.

Figure 1X

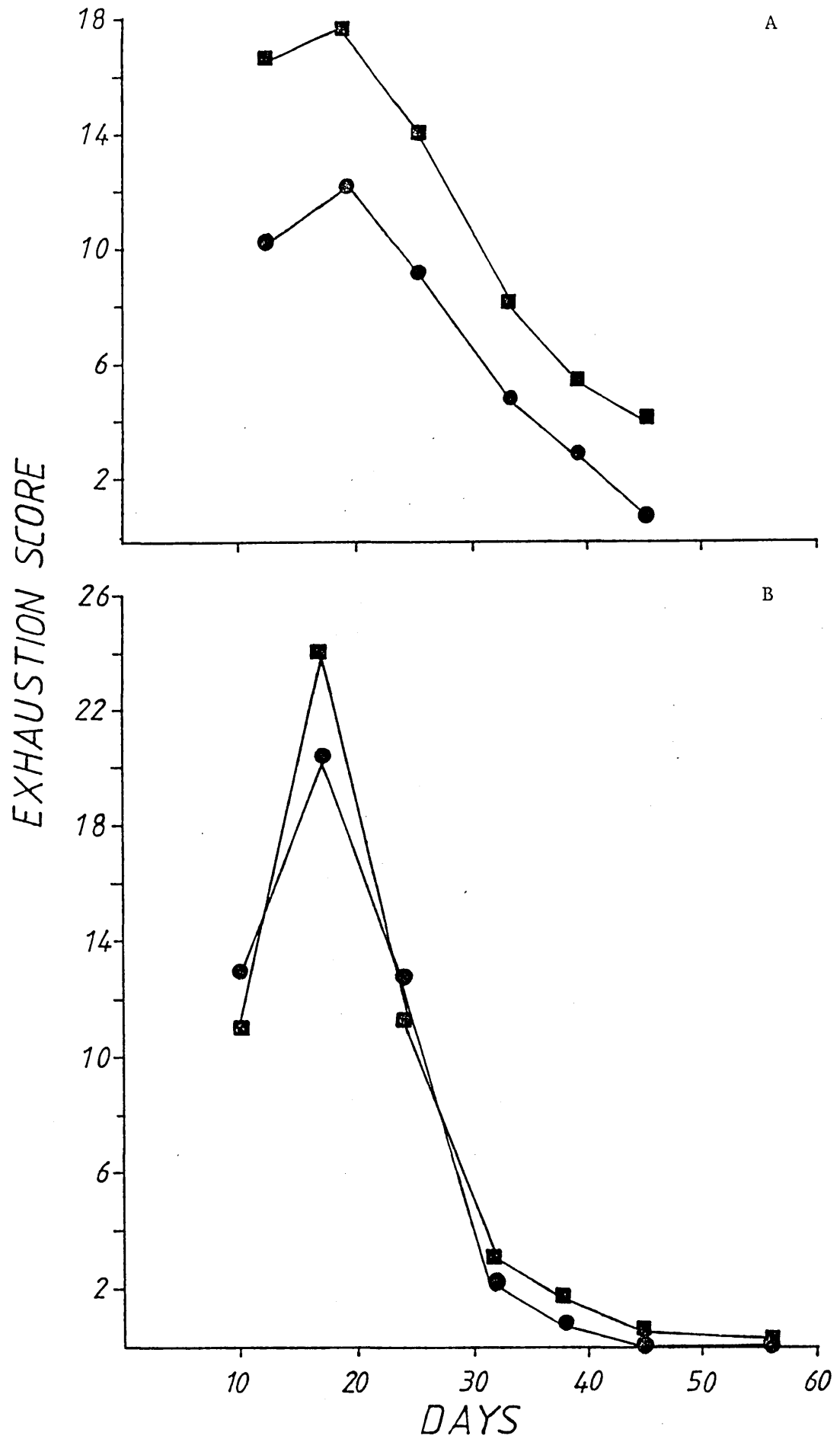


Figure LXI

Exhaustion scores for Cinnarizine-treated and untreated dystrophic chickens.

Exhaustion scores are shown for cinnarizine-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage $10 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and untreated groups contained 10 birds.
- B. dosage $15 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. The control group was reduced from 10 to 9 birds at day 42. The treated group contained 10 birds throughout.

Figure 1XI

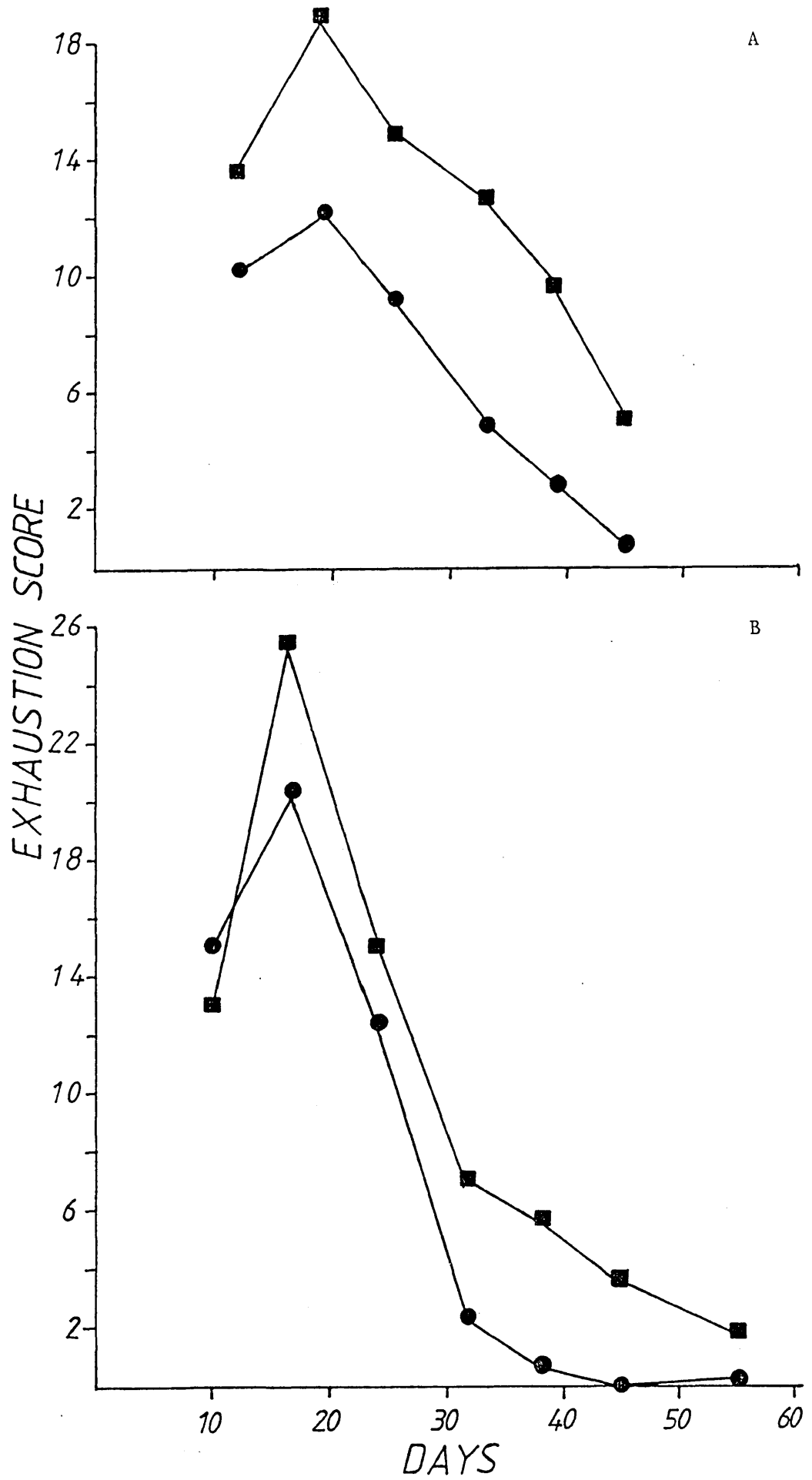


Figure LXII

Exhaustion scores for Dantrolene-treated and untreated dystrophic chickens.

Exhaustion scores are shown for dantrolene-treated (■) and untreated (●) dystrophic birds at a dosage of $10 \text{ mgkg}^{-1} \text{ day}^{-1}$. Treatment commenced from day 4 ex ovo. Both treated and untreated groups contained 10 birds.

Figure LXII

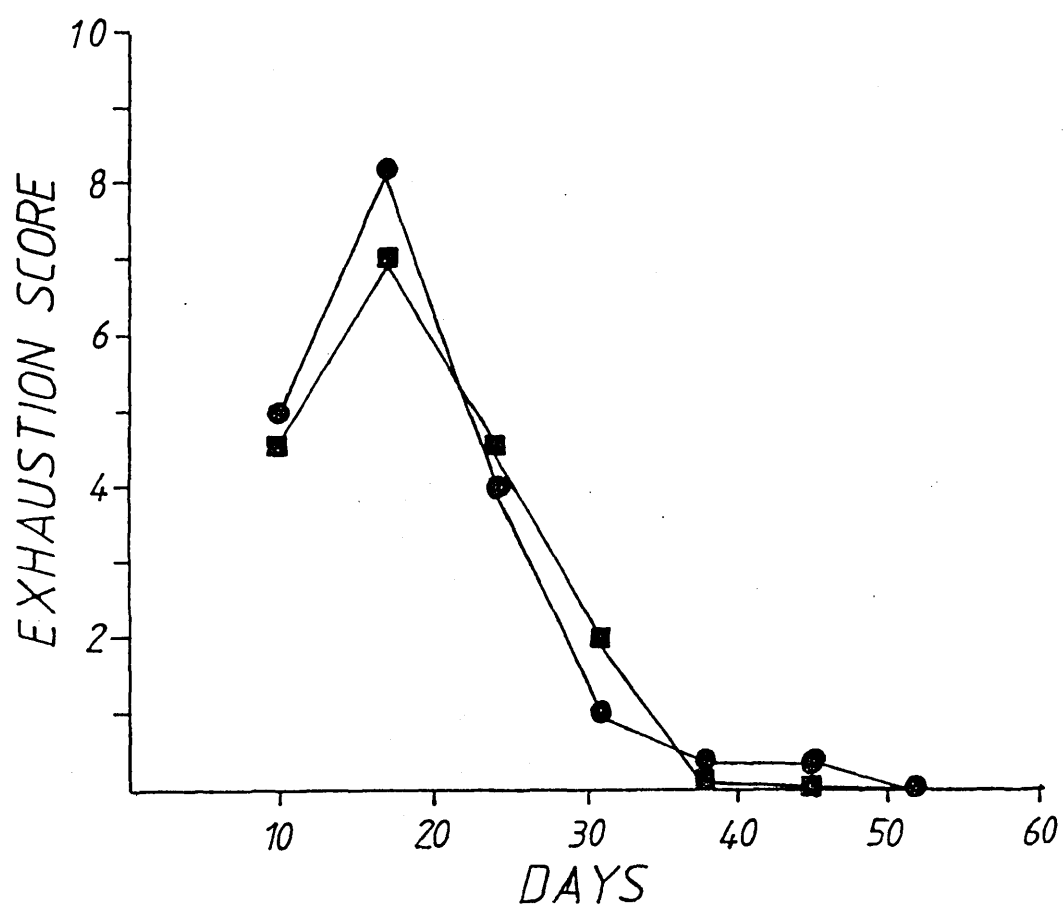


Figure 1 XIII

Exhaustion scores for Verapamil-treated and untreated dystrophic chickens.

Exhaustion scores are shown for verapamil-treated (■) and untreated (●) dystrophic birds at a dosage of $0.1\text{mgkg}^{-1}\text{day}^{-1}$ from day 4 ex ovo. The control and treated groups contained 10 and 8 birds respectively.

Figure LXIII

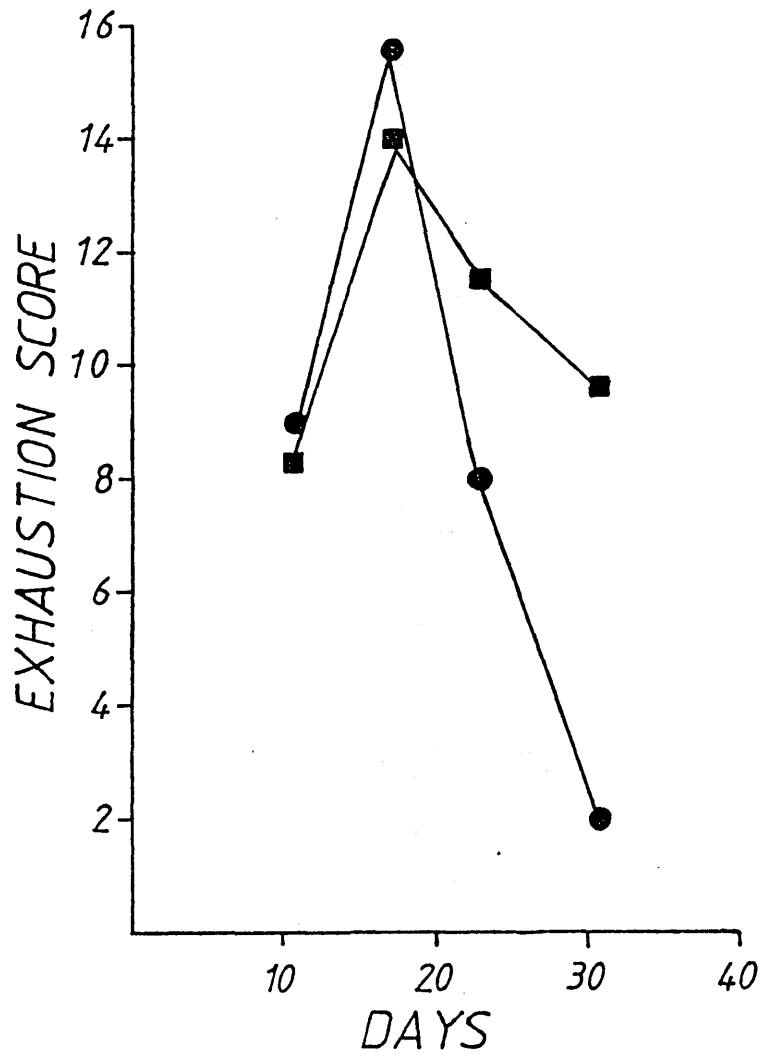


Figure 1XIV

Exhaustion scores for IAPP-treated (40,80) and untreated dystrophic chickens.

Exhaustion scores are shown for IAPP-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

A. dosage $40 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Treated and control groups contained 8 and 10 birds respectively.

B. dosage $80 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Treated and control groups contained 7 and 10 birds respectively.

Figure LXIV

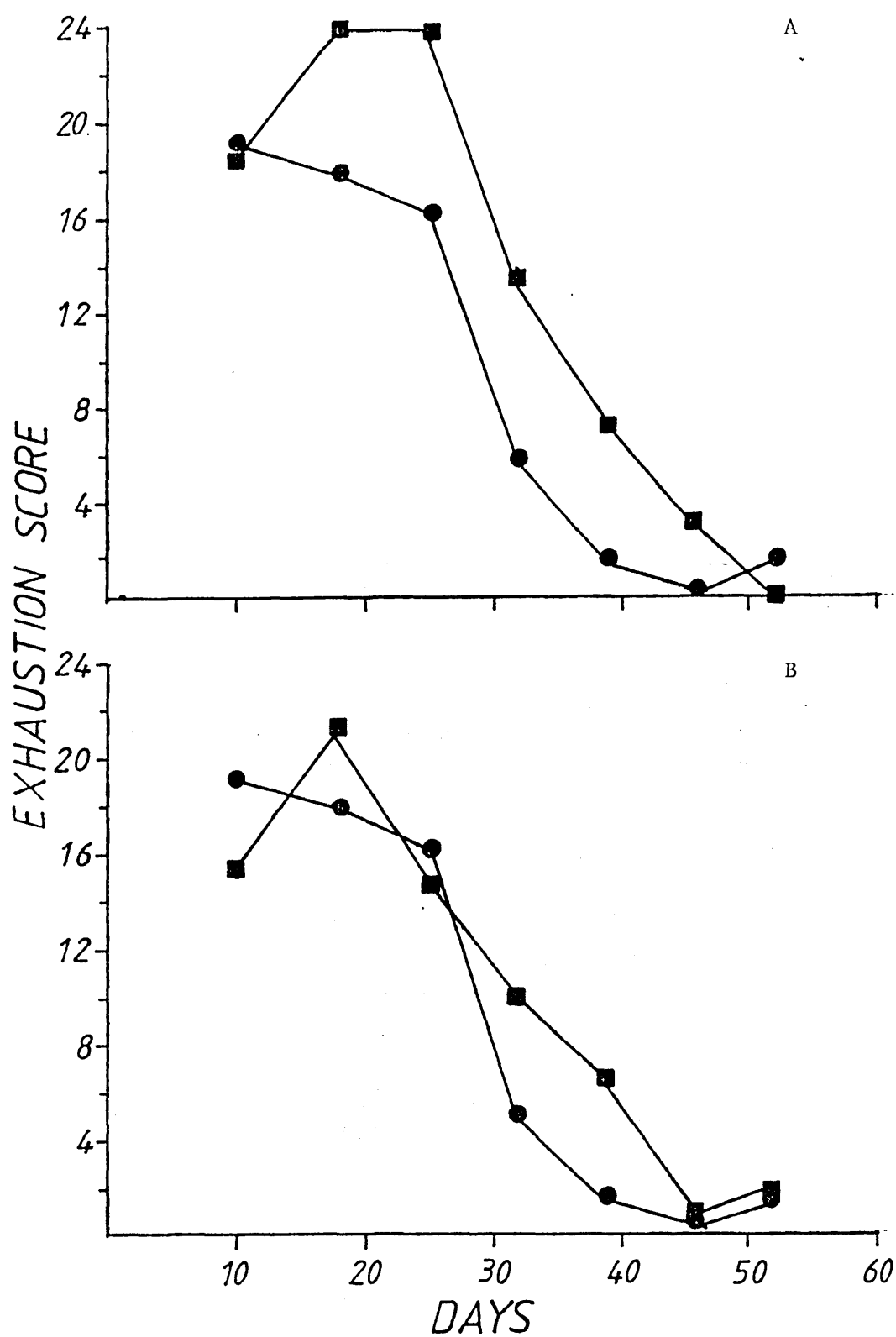


Figure 1XV

Exhaustion scores for IAPP-treated (120,200) and untreated dystrophic chickens.

Exhaustion scores are shown for IAPP-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage $120 \text{ mgkg}^{-1} \text{ day}^{-1}$, i.p. The treated and control groups contained 5 and 10 birds respectively.
- B. dosage $200 \text{ mgkg}^{-1} \text{ day}$, ip. The treated and control groups contained 7 and 10 birds respectively.

Figure LXV

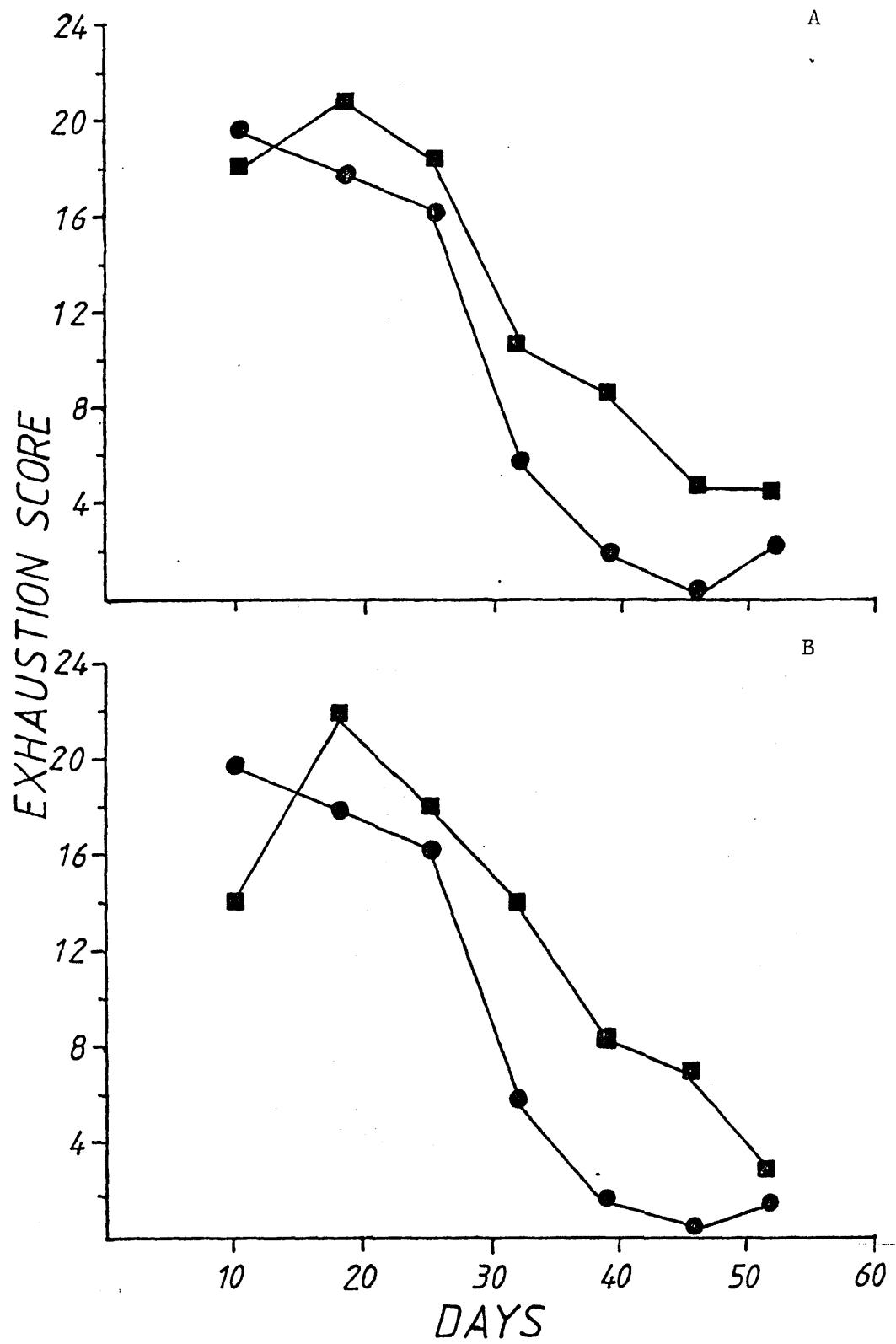


Figure 1XVI

Flip scores for IAPP-treated (150,300) and untreated dystrophic chickens.

Flip scores are shown for IAPP-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage $150 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and untreated groups contained 10 birds.
- B. dosage $300 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. The treated and untreated groups contained 8 and 10 birds respectively.

Figure LXVI

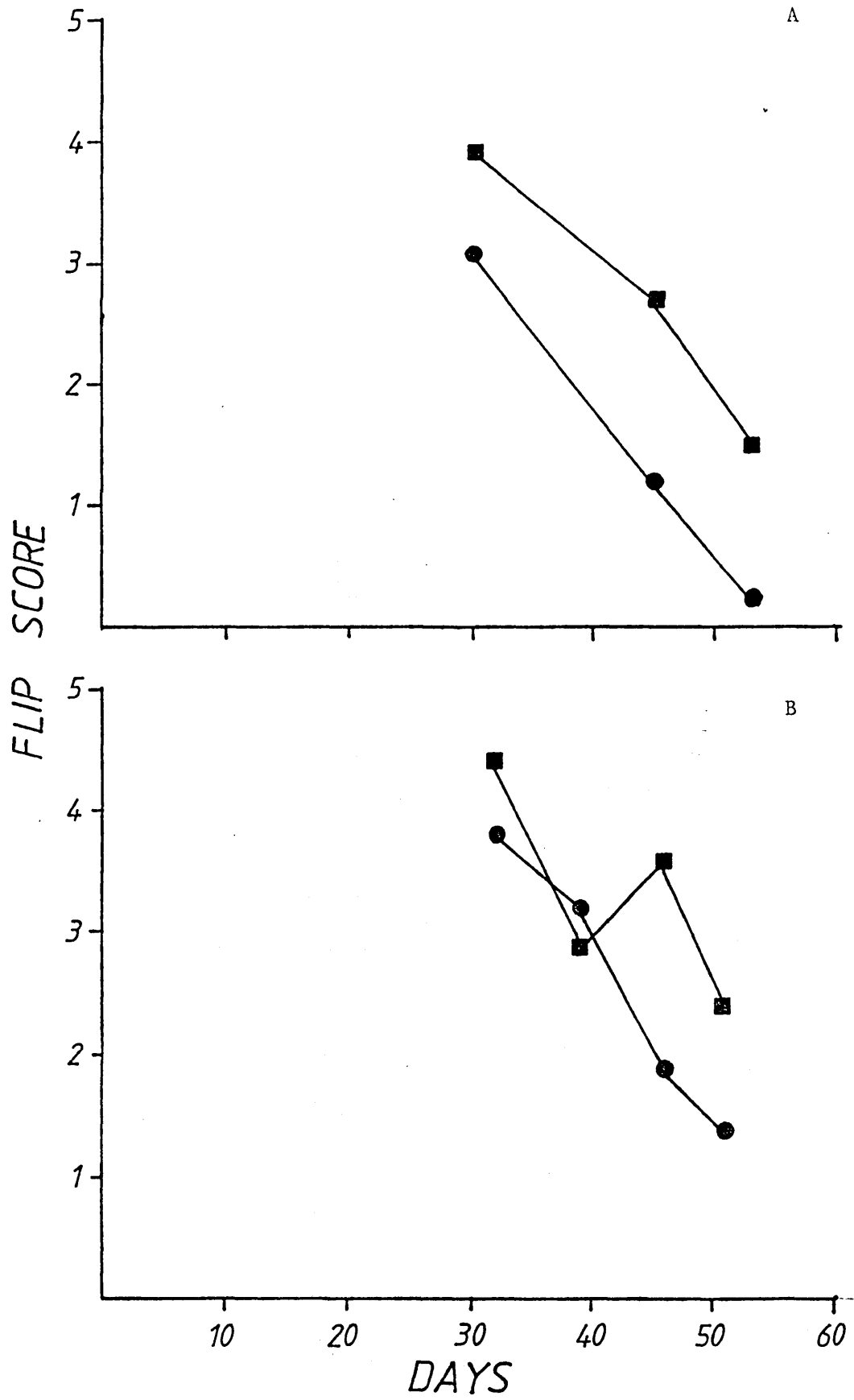


Figure 1XVII

Flip scores for BN1041-A-treated (72,100) and untreated dystrophic chickens.

Flip scores are shown for BN 1041 A-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage $72 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and control groups contained 10 birds.
- B. dosage $100 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. The treated and control groups contained 9 and 10 birds respectively.

Figure LXVII

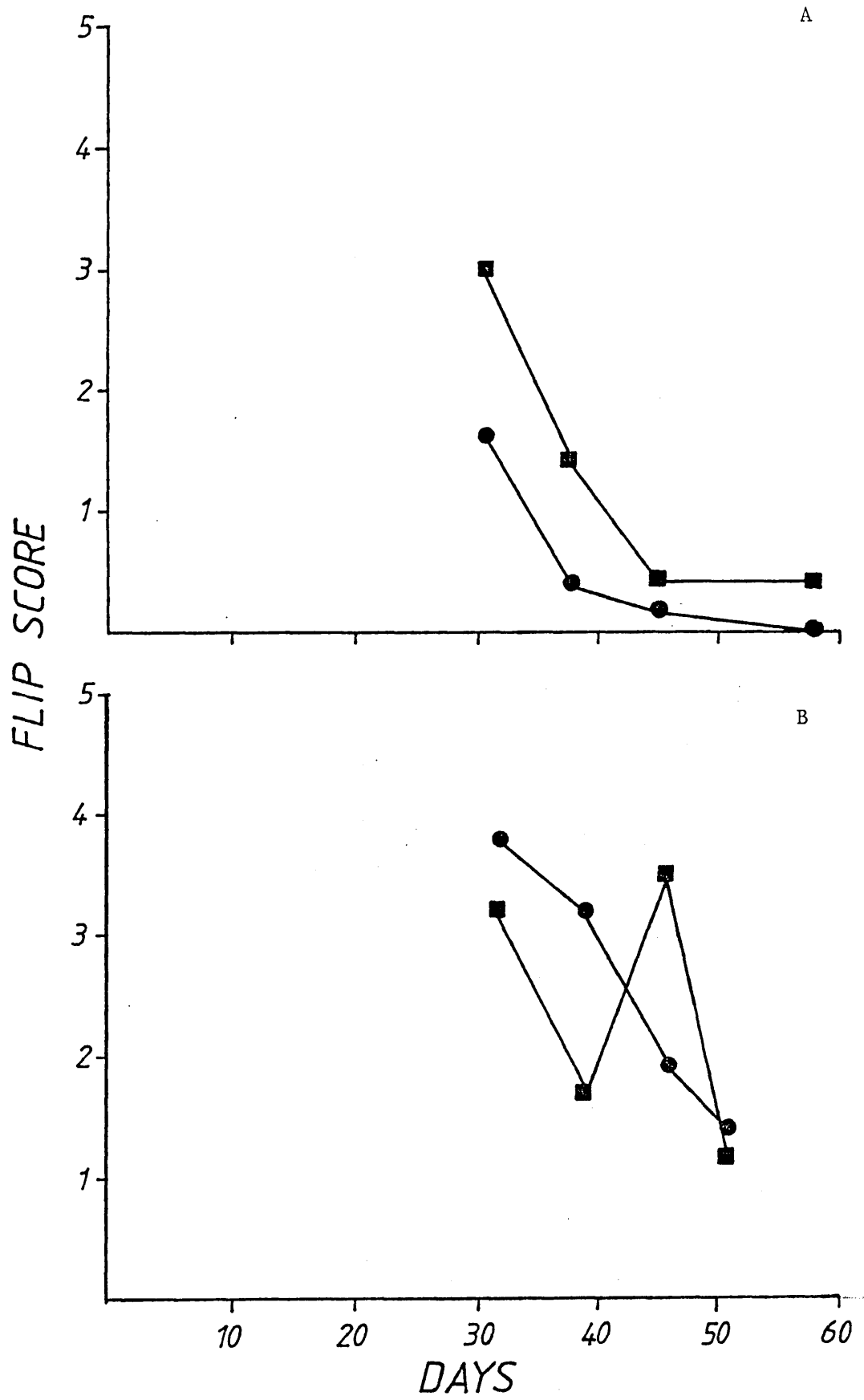


Figure 1XVIII

Exhaustion scores for BN1041-A-treated and untreated dystrophic chickens.

Exhaustion scores are shown for BN1041-A-treated (■) and untreated (●) dystrophic chickens under two dosage regimes. In both cases treatment commenced from day 4 ex ovo.

- A. dosage $144 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and untreated groups contained 10 birds.
- B. dosage $288 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. The treated and untreated groups contained 9 and 10 birds respectively.

Figure LXVIII

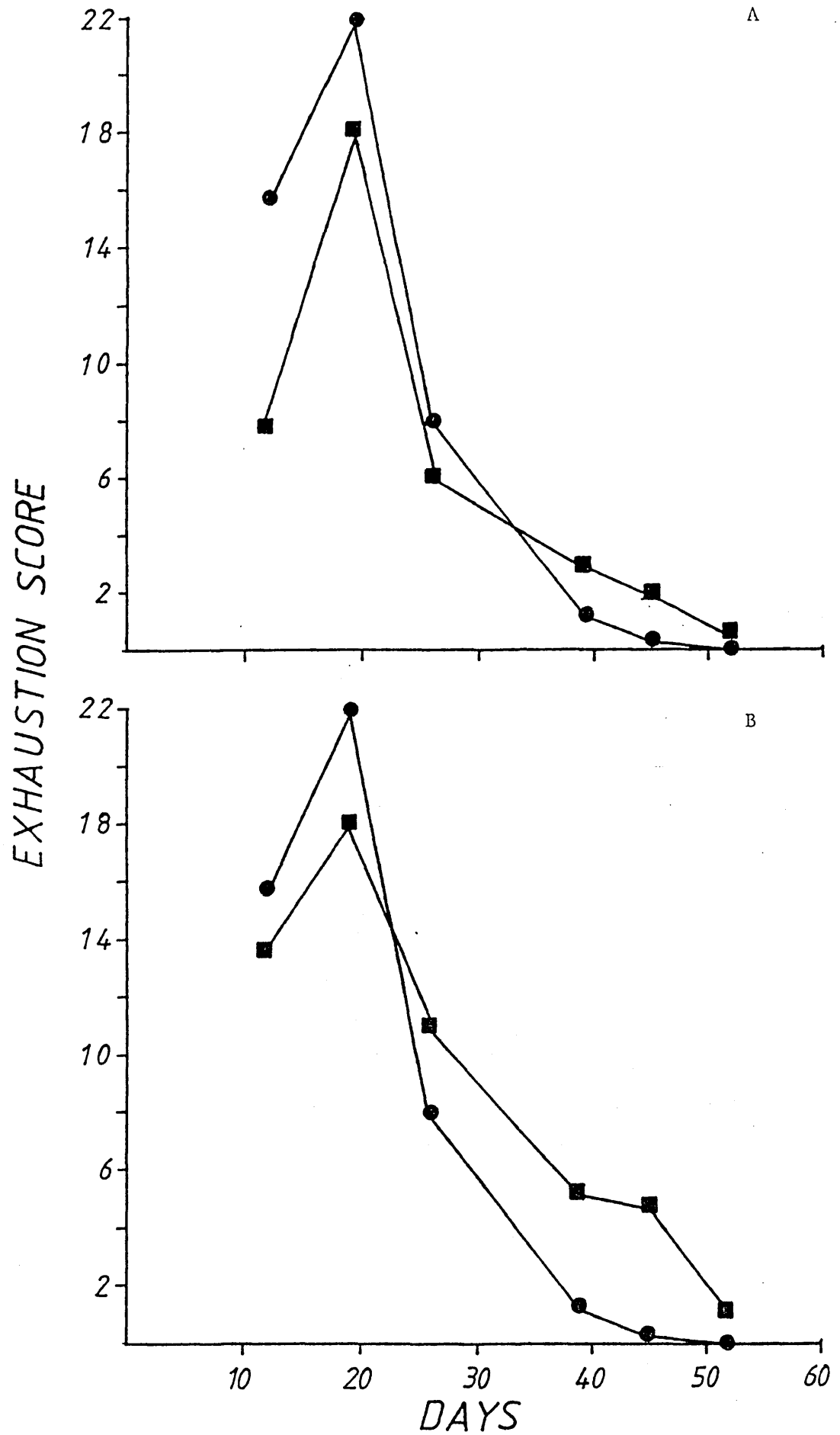


Figure LXIX

Flip scores for BN 1041-B-treated and untreated dystrophic chickens.

Flip scores are shown for BN 1041-B-treated (■) and untreated (●) dystrophic birds under two dosage regimes. In each case treatment commenced from day 4 ex ovo.

- A. dosage $50 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and untreated groups contained 10 birds.
- B. dosage $300\text{--}100 \text{ mgkg}^{-1} \text{ day}^{-1}$ ip. The treated and untreated groups contained 9 and 10 birds respectively.

Figure 1XIX

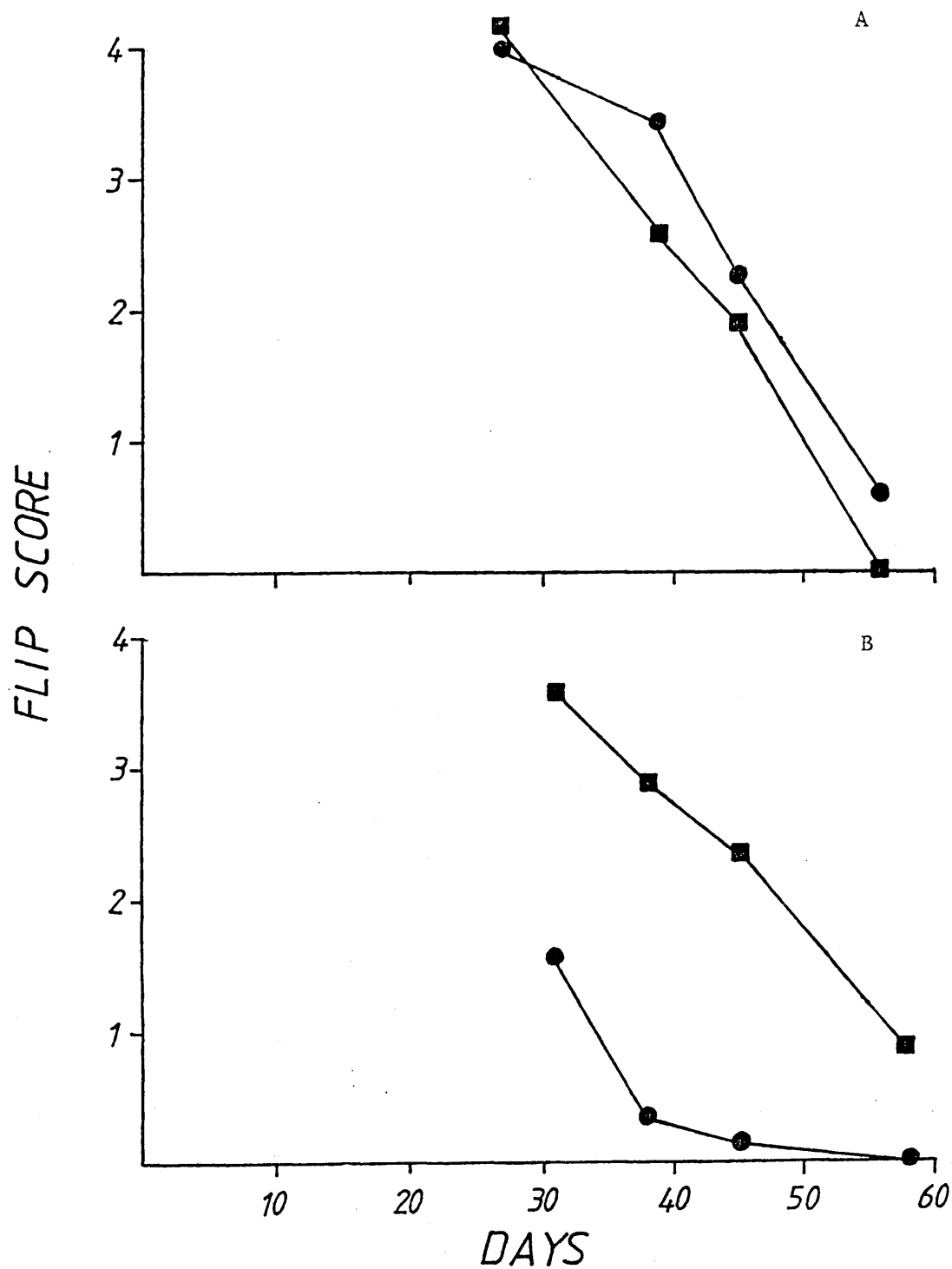


Figure 1XX

Flip scores for 4AP-and DAP-treated and untreated dystrophic chickens.

Flip scores are shown for 4AP and DAP-treated and untreated dystrophic chickens.

- A. dosage 4AP (□) $0.1 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip.
DAP (■) $7.5 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip.

The DAP-treated and untreated (●) groups contained 10 birds each and the 4AP treated groups contained 7 birds

- B. dosage DAP (■) $7 \text{ mgkg}^{-1} \text{ day}^{-1}$, ip. Both treated and untreated (●) groups contained 10 birds.

Figure LXX

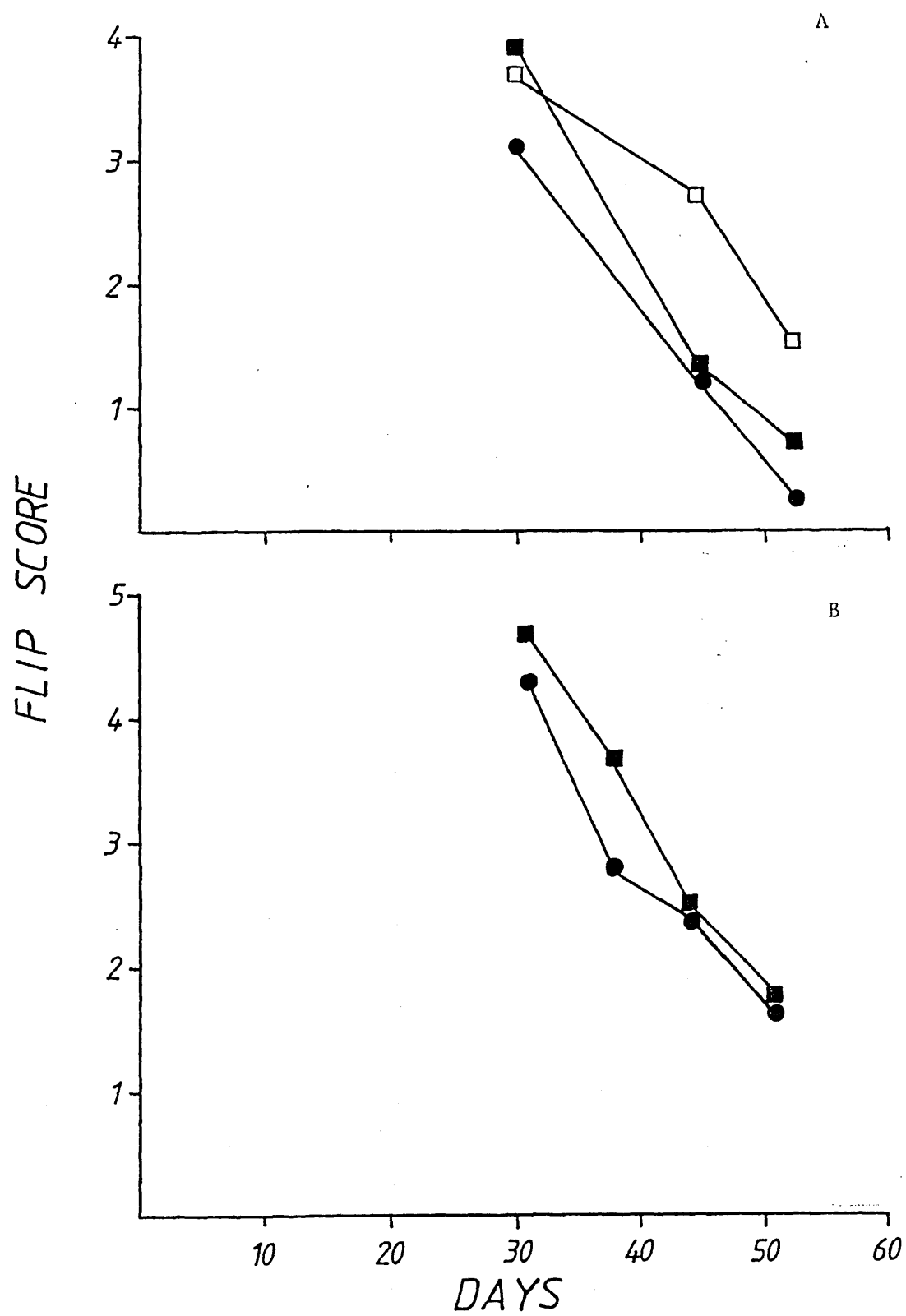


Figure 1XXI

Flip and exhaustion scores for Map ¹-treated and untreated dystrophic chickens.

Flip scores for 10 mgkg⁻¹day⁻¹ treated and exhaustion scores for varying dosages from 50 to 100 mgkg⁻¹day⁻¹ treated dystrophic birds. In both cases treatment commenced from day 4 ex ovo.

A. dosage 10 mgkg⁻¹day⁻¹, ip. Both treated (■) and untreated (●) groups contained 10 birds.

B. dosage 50 mgkg⁻¹day⁻¹, ip. day 4 - day 32.
100 mgkg⁻¹day⁻¹, ip. day 33 - day 52.

The treated (■) and untreated (●) groups contained 9 and 8 birds respectively.

Figure LXXI

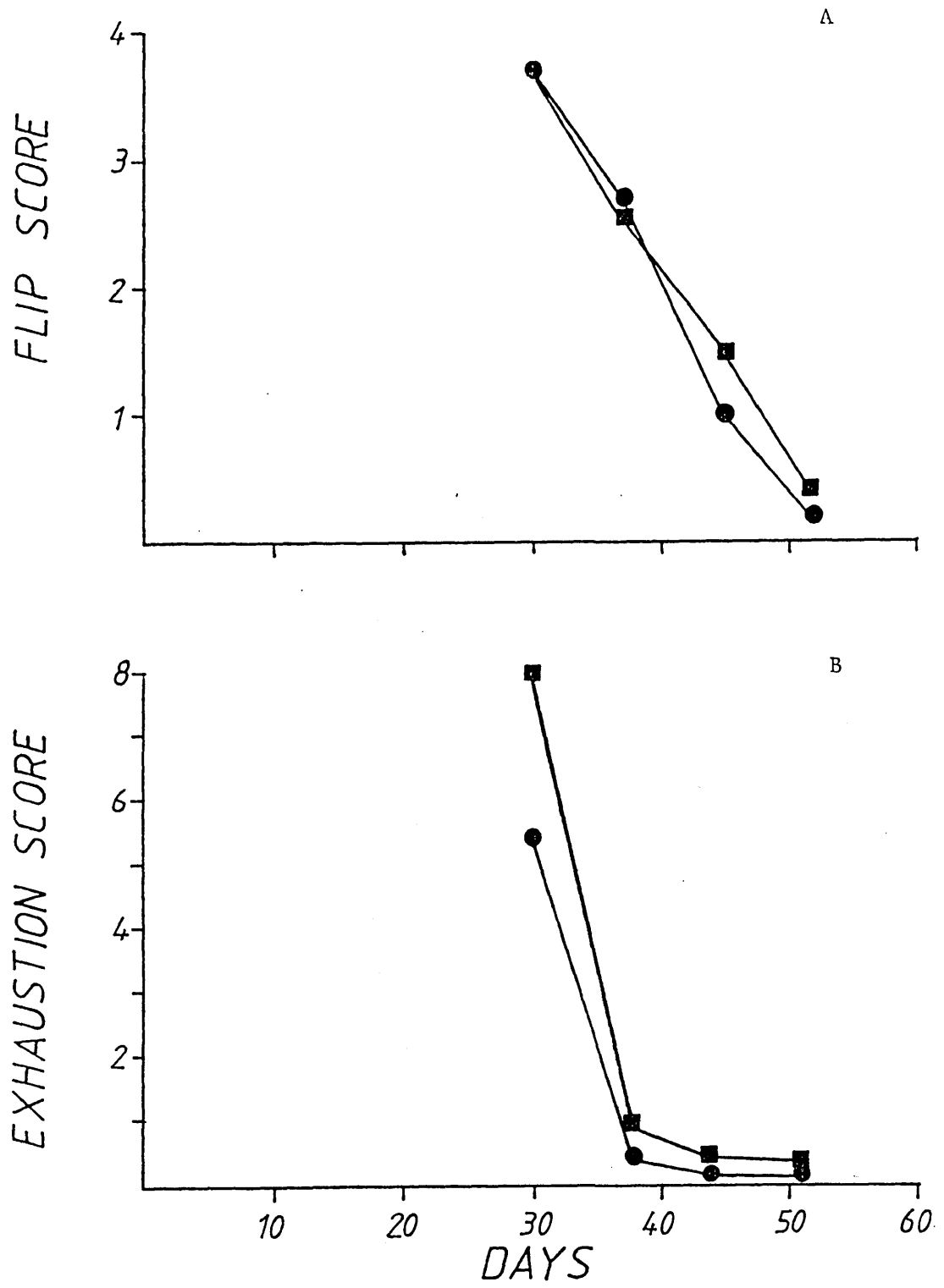


Figure 1XXII

Exhaustion scores for Map 1-treated and untreated dystrophic chickens.

Exhaustion scores for Map 1-treated (■) and untreated (●) dystrophic birds under a dosage regime of $100 \text{ mgkg}^{-1} \text{ day}^{-1}$.

Treatment commenced from day 4 ex ovo. The treated and untreated groups contained 5 and 10 birds respectively.

Figure 1XXII

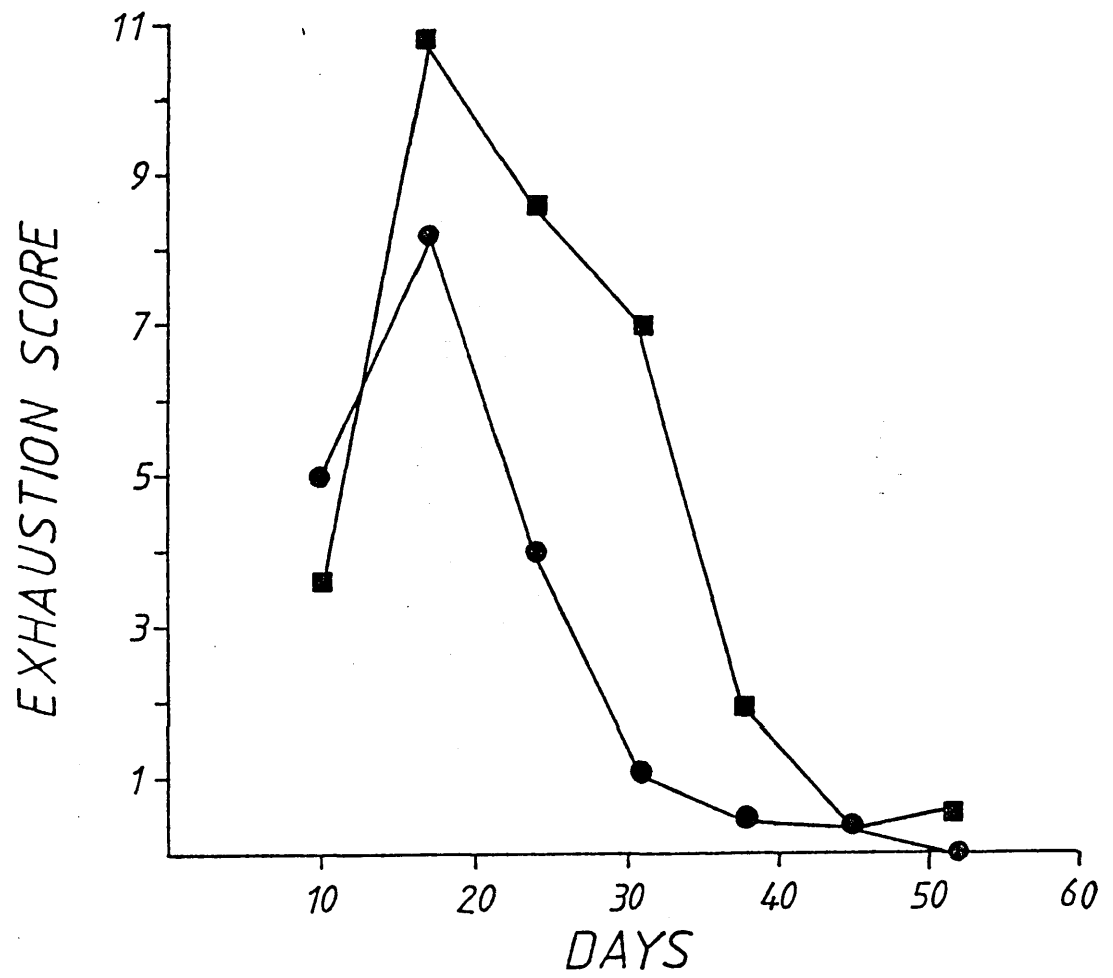
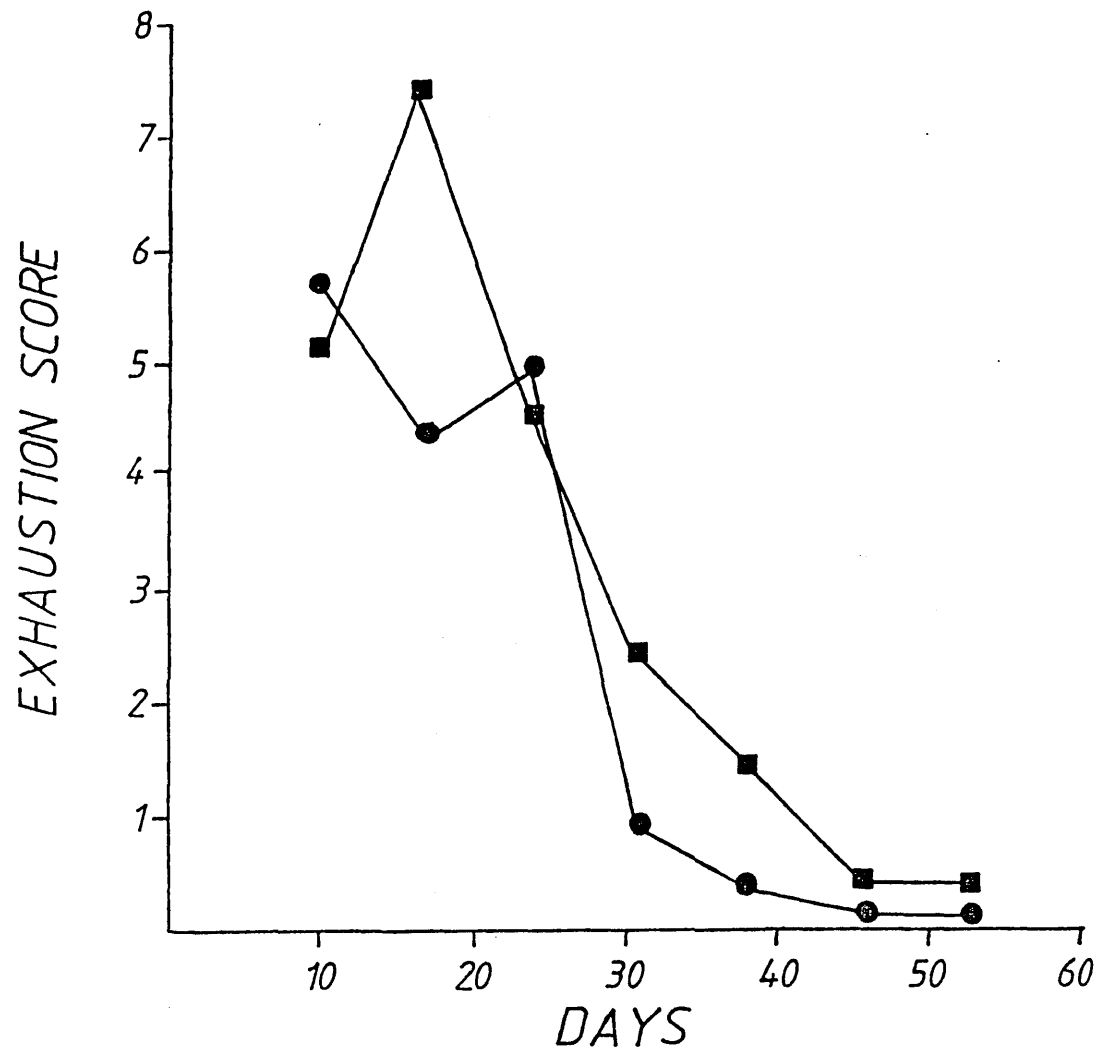


Figure LXXIII

Exhaustion scores for Desferal-treated and untreated dystrophic chickens.

Exhaustion scores for desferal-treated (■) and untreated (●) dystrophic birds under a dosage regime of $100 \text{ mgkg}^{-1} \text{ day}^{-1}$. Treatment commenced from day 4 ex ovo. Both treated and untreated groups were reduced from 9 to 8 birds at day 22.

Figure lXXIII



SECTION TWO

ISOCITRATE DEHYDROGENASE (NADP-LINKED)

INTRODUCTION

The project aims to investigate an enzyme for potential use in the diagnosis of Duchenne muscular dystrophy. For this purpose NADP-linked Isocitrate dehydrogenase (ICD) was analysed in dystrophic chickens (line 413) to ascertain it's potential as an enzyme marker. Interest in this particular enzyme arose from results found for a different dystrophic chicken (line 304), which indicated significant differences in ICD activity between normal (N) and dystrophic (D) birds at time of hatch. It was thought that if these results were confirmed, the enzyme might have potential use in foetal diagnosis and carrier detection.

In dystrophic New Hampshire chickens (line 304) it was shown by Cardinet et al. that the observed activity of the soluble form of ICD was significantly greater than that from normal birds (line 200). This reported increased activity, shown by enzyme assay of muscle extracts and direct histological studies was maintained during the period of hatch to six weeks ex ovo. (Cardinet et al., 1972).

Two forms of the enzyme are known, i.e. NAD-linked ICD (ec.1.1.1.41) and NADP-linked ICD (ec. 1.1.1.42). The former is located exclusively in the mitochondria while the latter is found both in the cytosol (extra-mitochondrial ICD) and in the mitochondria (intra-mitochondrial ICD). In 1953 Plaut isolated two forms of the enzyme from rat liver mitochondria. One form previously characterised required NADP for activity (Plaut and Sung, 1954). The second species was found to require NAD and ADP to catalyse the same reaction (Plaut, 1969). The metabolic roles of these two enzymes in the tricarboxylic acid cycle (TCA) was unclear but it was later demonstrated in mitochondrial preparations, that NAD-ICD/cytochrome-a

stoichiometry was constant in a number of species (Goebel and Klingenberg, 1963). In contrast, the NADP-ICD/cytochrome-a ratio fluctuated widely, suggesting no involvement of that enzyme in the TCA cycle. This view was confirmed by Nichols and Garland using intact mitochondria (Nichols and Garland, 1969).

It has been suggested that the extra-mitochondrial NADP-ICD supplies NADPH₂ for fatty acid synthesis (Baker and Newburgh, 1963). For example, increased levels of this enzyme in embryonic chick brain and liver was coincident with the observed occurrence of myelination and fat accumulation respectively. In contrast, Chabas has shown that there are no apparent differences in NADP-ICD activity between myelinated and unmyelinated areas of the central nervous system (Chabas et al., 1979). In addition, the development patterns of NADP-ICD activity in the rat heart do not correspond to those of the pentose phosphate pathway which are known to be active in fatty acid synthesis (Andres et al., 1980). In rat and chicken liver the intra- and extra-mitochondrial NADP-ICD activities have been shown to be immunologically distinct enzymes (Henderson, 1965). Lowenstein and Smith observed that antibodies raised against intra-mitochondrial NADP-ICD specifically inhibited this enzyme, but had no effect on extra-mitochondrial NADP-ICD (Lowenstein and Smith, 1962). Starch gel electrophoresis demonstrated that isozymic forms of extra-mitochondrial NADP-ICD from inbred mice displayed simple Mendelian inheritance (Bell and Baron, 1964). In this work no variation of intra-mitochondrial forms of the enzyme are observed, despite suggestions that they be coded by intra-mitochondrial genes (Henderson, 1968).

This study examines the plasma NADP-ICD activity in N and D chickens and also the activities of intra- and extra-mitochondrial NADP-ICD found in muscle homogenates.

MATERIALS

All chemicals were obtained from Sigma, Poole, Dorset except sodium hydroxide (BDH, Poole Dorset), sodium heparin (Duncan Flockhart Limited) and isocitrate dehydrogenase test Kit (BCL, Lewes, Sussex).

METHODS

New Hampshire chickens used for ICD analysis were Imperial College (I.C.) dystrophic line 413 and the genetically related New Hampshire normal I.C. line 412 (described in detail p. 38).

SAMPLE PREPARATION

Plasma

Blood (1ml) was withdrawn from the jugular vein without anaesthetic and mixed with 0.01ml sodium heparin (1000U/ml). This was centrifuged for 3 minutes in a haematocrit microfuge and the upper plasma layer retained for assay.

Muscle

Samples were taken from the pectoralis major (pectoral) muscle, care being taken to avoid the dorsal red stripe (Julian and Asmundson, 1963). Tissue was taken from the medial region of the anterior half of the muscle.

Extra-mitochondrial ICD

The soluble form of the enzyme was extracted by homogenisation in ice cold 50mM Tris-HCl pH 7.5 at 2°C in the ratio 100mg/ml

(wet weight/volume). This was ground in small volume glass/glass homogenisers. The homogenate was spun in a Sorval RC-5 (Dupont) centrifuge at 10691xg, 4°C for 30 minutes and the supernatant retained for assay.

Intra-mitochondrial ICD

The enzyme was extracted from pellets remaining from the extra-mitochondrial ICD extraction by resuspension in ice-cold 50mM Tris HCl, pH 7.5 containing 1% Triton X-100 for approximately 30-40 minutes (Husic and Suelter, 1980). The suspension was then centrifuged as for extra-mitochondrial ICD, the supernatant being retained for assay.

Total NADP-ICD

Muscle samples were homogenised in 50mM Tris HCl pH 7.5 containing 1% Triton for 30 minutes. Centrifugation procedures as described above.

Enzyme Assay

The assay was carried out using a kinetic analyser (LKB-8600 mk II) Assay conditions were 25°C and wavelength 340nm. Samples were preincubated at 25°C in the thermostated tunnel prior to assay.

Assay Principal

Enzyme activity was determined from increased absorbance at 340nm as a result of the formation of NADPH₂. Plasma activity was determined using ICD test kits (BCL) based on the same principle. Muscle supernatants were assayed in 0.1M Tris HCl pH 7.5 containing 0.001M NADP⁺, 0.01M MnCl₂ and 0.85% NaCl in a volume of 0.625ml. The samples (0.125ml) were preincubated at 25°C prior to addition of 0.025ml 0.1M dl-isocitric acid. The rate of reaction was monitored (Wolfson and Williams-Ashman, 1957). Activity in international units (SI) was calculated as described previously (p. 43).

Protein Determinations

Protein estimation were carried out using a modified method of Lowry adapted to the flow-cell Cecil spectrophotometer (Lowry et al., 1951). Readings at 750nm were recorded by Anadex recorder.

RESULTS

Plasma

Plasma activities were measured in normal and dystrophic chickens over an age range from 1 to 12 weeks (Fig. 2I). During weeks 1 to 5 there was no significant difference between activity levels, although from week 1 to week 4 normal levels were consistently higher than the dystrophic. Subsequently these normal levels fell to stabilise after week 7. A steady increase in the dystrophic activity was observed from week 1 to week 10 after which an even level was maintained. From week 6 to week 12 dystrophic activities were significantly higher than normal levels (student's t-test $p < 0.001$).

The range of ICD activities in both normal and dystrophic birds were lower than those of other established enzyme markers, creatine kinase (CK) and pyruvate kinase (PK). Normal plasma CK levels in this age range were 200 UL^{-1} , whereas dystrophic CK activities were 30x higher than this. In the case of plasma ICD levels the maximum increase observed was 1.5x that of comparable normal birds.

Muscle

Pectoral muscle homogenate intra- and extra-mitochondrial ICD activities were analysed over an age range from day 19 in ovo to 9 weeks ex ovo, (Figs. 2II, 2III). At d19 and d20 in ovo and at hatch no differences between normal and dystrophic samples, of either form, were observed. The pattern of intra- and extra-mitochondrial

ICD activities however, differ from d20 in ovo. Both normal and dystrophic intra-mitochondrial activities increase from d 19 to d20 in ovo then fall to lower than d19 levels at hatch. The normal levels drop further, to even out after week 1 whereas the dystrophic activity increases to week 2, then subsequently stabilises at significantly higher than normal levels for the remaining time points ($p < 0.005$). In contrast, normal and dystrophic extra-mitochondrial ICD activities continue to rise from d20 to week 1 and then fall in parallel till week 4. From week 4 to week 9 extra-mitochondrial ICD levels were significantly higher than the corresponding normal activities, ($p < 0.001$).

The ICD activities when compared as an extra-mitochondrial ICD/intra-mitochondrial ICD ratio show differences between normal and dystrophic birds at weeks 1 and 2. At both these time points the normal ratio is 62% greater than that of the dystrophic birds (Fig. 2IV).

DISCUSSION

Analysis of normal and dystrophic plasma ICD levels demonstrated significant differences in activity between them from week 6 to week 9. The extent of the differences and the age at which they occur preclude the application of ICD as an 'early' diagnostic marker. Such a marker would require that significant differences were observed during the first two weeks of life. When the differences of plasma ICD activity of normal and dystrophic birds were compared to those of CK and PK, they did not appear as substantial as the latter. Over this age range CK levels were 30x greater than the normal, whereas dystrophic ICD activities were 1.5x higher than control birds.

Therefore this enzyme does not appear as good an indicator of the dystrophic condition as other established markers. Although not significant on its own, the observed ICD differences might in conjunction with other marker enzymes provide confirmatory evidence during diagnostic procedures. However, since one cannot easily extrapolate from the chicken to the human, further investigations in the latter case might usefully be pursued.

It is of interest to note that this oxidative enzyme is found elevated in the plasma when no reduction in muscle activity is observed. However without isozymic analysis it would be impossible to determine whether this resulted from leakage from muscle or other tissues. The extent of the elevation in the chicken, however did not warrant further investigation by other techniques.

Although ICD is not an early marker of the disease in the dystrophic chicken, the plasma enzyme levels are still of use in therapeutic trials as an additional biochemical test (see p. 72,88,110) which could be used to measure the effects of treatment at the later stages of the disease progression.

The muscle homogenate results demonstrated in these experiments contrasts with the findings published by Cardinet, which originally stimulated interest in this work (Cardinet et al., 1972). These results showed dystrophic (line 304) activities to be significantly higher than normal (line 200) from hatch day to week 6 ex ovo. In line 413 this was not observed, significant differences were measured only from week 4 ex ovo. to week 14 (Table 2a). However, measurements were consistently higher from the day of hatch in the dystrophic muscle. The results in lines 413/412 appear consistent with known histological data on pectoral muscle in these lines

(Pizzey and Barnard, 1983). Barnard et al. demonstrated the uniform oxidative nature of normal and dystrophic fibres in vivo.

(Barnard et al., 1982). In normal muscle, post-hatch fibre-type changes occur from type IIA (oxidative/glycolytic) to 99% type IIB (glycolytic). The normal ICD activity coincides with this pattern, however, in dystrophic muscle 5% type IIIA fibres remain possibly accounting for higher extra-mitochondrial ICD levels observed (Barnard et al., 1982). Type IIB fibres also contain a lower density of mitochondria than type IIA, thus the decline in normal intra-mitochondrial ICD levels after hatch reflects this change and the remaining dystrophic activity may be accounted for by the 5% type IIA fibres (Barnard et al., 1982).

The small discrepancy between the results recorded here and those of Cardinet may be accounted for when line differences are taken into account. The dystrophic line 413 and normal line 412 have a common genetic background and were derived, by outcross, from line 304. Therefore there are many similarities between the lines but there also reported dissimilarities (Wilson et al., 1979). The major source of discrepancy may be the use of line 200 normal birds as controls; these birds do not share a genetic background with line 304 and thus differences could originate from their use (Wilson et al., 1979).

The results of the chicken muscle homogenate experiments might suggest that ICD does not fulfil the requirements of an early applicable to diagnosis of DMD, since the requirement is a significant difference of activity at or around birth. The differences found at subsequent ages in the chicken, as well as being interesting from an overall metabolic point of view, might also be demonstrated in dystrophic children and thus have a practical diagnostic application.

The differences of extra-mitochondrial ICD/intra-mitochondrial ICD ratio at weeks 1 and 2 in chicken, if confirmed in humans, might aid post-natal diagnosis. However confirming this result in DMD at the present time is problematical because of difficulties in obtaining age-matched muscle biopsy samples from children.

The activity differences observed at week 4 and thereafter in the chicken, although not directly equating to the same age in man, may be of use in analysis of biopsies of carriers and probable carriers should these findings be confirmed. This would need in depth analysis of banks of known carrier samples to confirm the validity of this hypothesis. A potential experimental use for ICD, in chickens, would be as an additional biochemical indicator of the metabolic status of muscle tissue where fibre type changes were anticipated.

In conclusion, ICD although not fulfilling its original promise as a potential early marker of dystrophy, may if these chicken results are confirmed in DMD, prove to be an extra confirmatory marker of use at other stages in the pathogenesis of dystrophy.

Table 2a

AGE (Days)	EXTRA-MITOCHONDRIAL ICD		INTRA-MITOCHONDRIAL ICD	
	Dystrophic U mg protein ⁻¹ ± SEM	Normal U mg protein ⁻¹ ± SEM	Dystrophic U mg protein ⁻¹ ± SEM	Normal U mg protein ⁻¹ ± SEM
19 (<u>in ovo</u>)	33.71 ±3.23	37.2 ±3.685	58.95 ±8.47	59.78 ±5.21
1 (<u>ex ovo</u>)	52.19 ±2.1	50.67 ±5.47	39.19 ±5.09	44.61 ±6.64
7 (<u>ex ovo</u>)	39.65 ±5.59	47.61 ±3.47	36.61 ±9.79	14.42 ±1.53
14 (<u>ex ovo</u>)	69.83 ^x ±6.52	46.34 ±5.82	81.01 [*] ±4.96	26.29 ±1.91

Normal and dystrophic muscle ICD activities expressed in (SI) units per mg protein from day 19 in ovo to day 14 ex ovo. Each time point is the mean value of 10 birds plus or minus the standard error of the mean (SEM).

x significantly different from normal at P < 0.01 by student's t-test

* significantly different from normal at P < 0.005 by student's t-test

Figure 2I

Plasma Isocitrate dehydrogenase activities from normal and dystrophic chickens.

Plasma isocitrate dehydrogenase (ICD) activities are shown for normal (■) and dystrophic (●) birds from 1 to 12 weeks ex ovo. Each point expressed in (SI) U l^{-1} is the mean of 10 or more bird determinations (except at week 10 where $n = 5$). The standard error of the mean (SEM) is indicated for each time point. Dystrophic values were significantly different from normal at $P < 0.001$ (by student's t -test) from week 6 to week 12.

Figure 2I

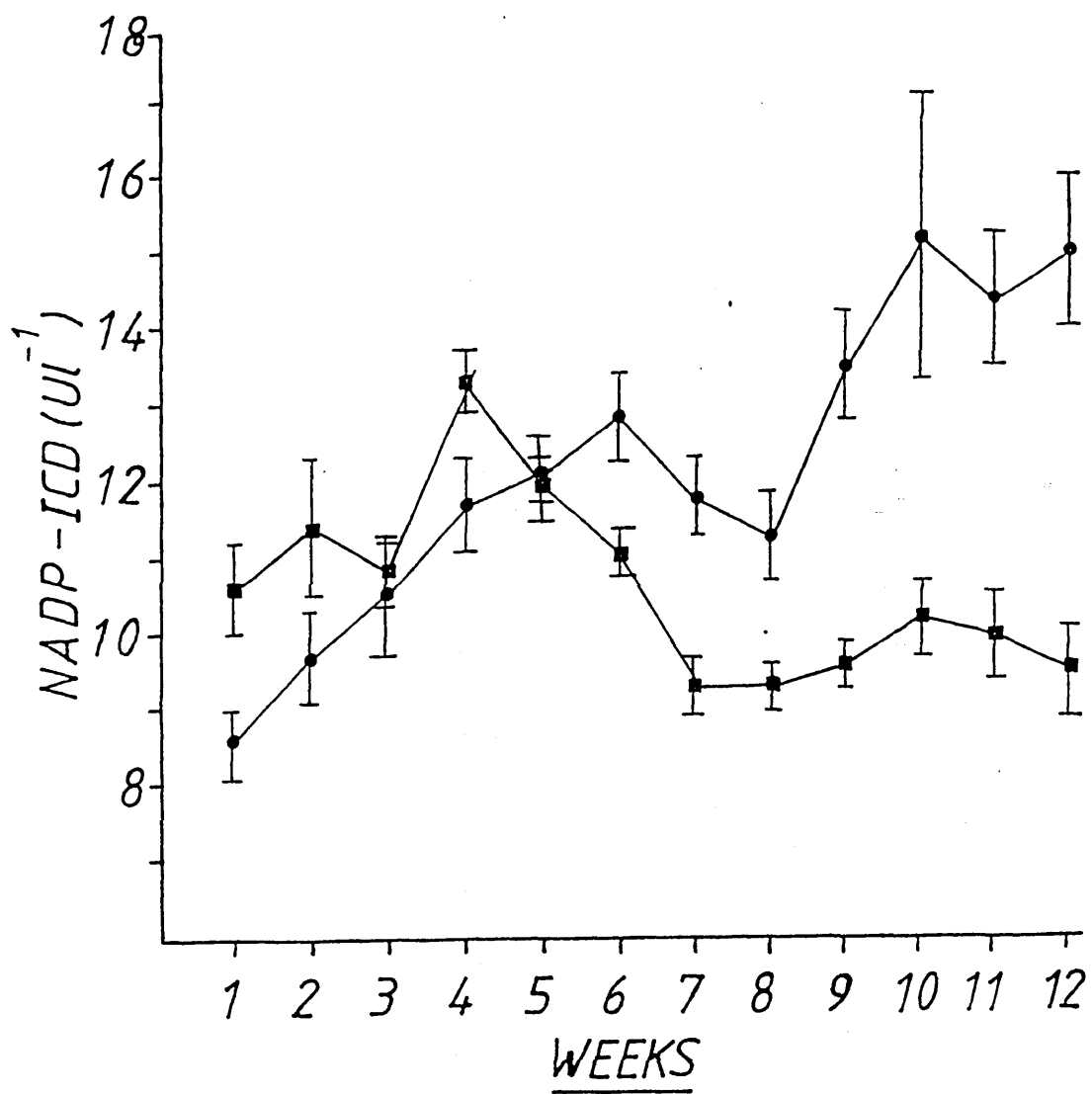


Figure 2II

Intra-mitochondrial Isocitrate dehydrogenase activities from normal and dystrophic chickens.

Enzyme activities (U.g wet weight tissue) for intra-mitochondrial isocitrate dehydrogenase extracted from pectoral muscle are shown for normal (■) and dystrophic (●) birds. Each point is the mean value for 10 or more birds (except day 19 in ovo, where n = 5) and standard error of the mean (SEM) is also indicated. Dystrophic values were significantly different (by student's t-test) normal at $P < 0.001$ from week 1 to week 9.

Figure 2II

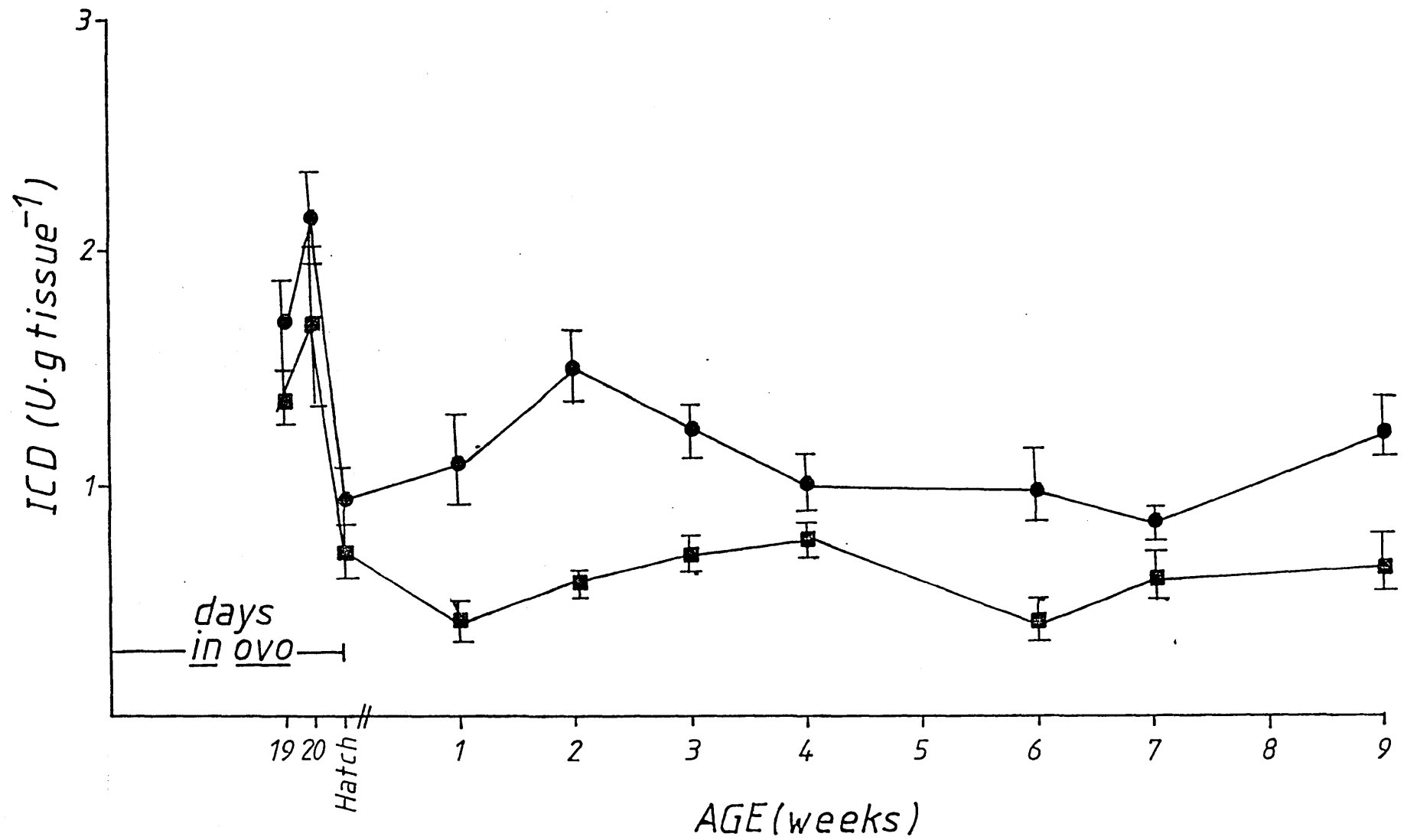


Figure 2III

Extra-mitochondrial Isocitrate dehydrogenase activities from normal and dystrophic chickens.

Enzyme activities (U.g wet weight tissue⁻¹) for extra-mitochondrial isocitrate dehydrogenase extracted from pectoral muscle are shown for normal (■) and dystrophic (●) chickens. Each point is the mean value for 10 or more birds (except day 19 in ovo where n = 5) and standard error of the mean (SEM) is also indicated. Dystrophic values were significantly different (by student's t-test) from normal at $P < 0.005$ from week 4 to week 9.

Figure 2III

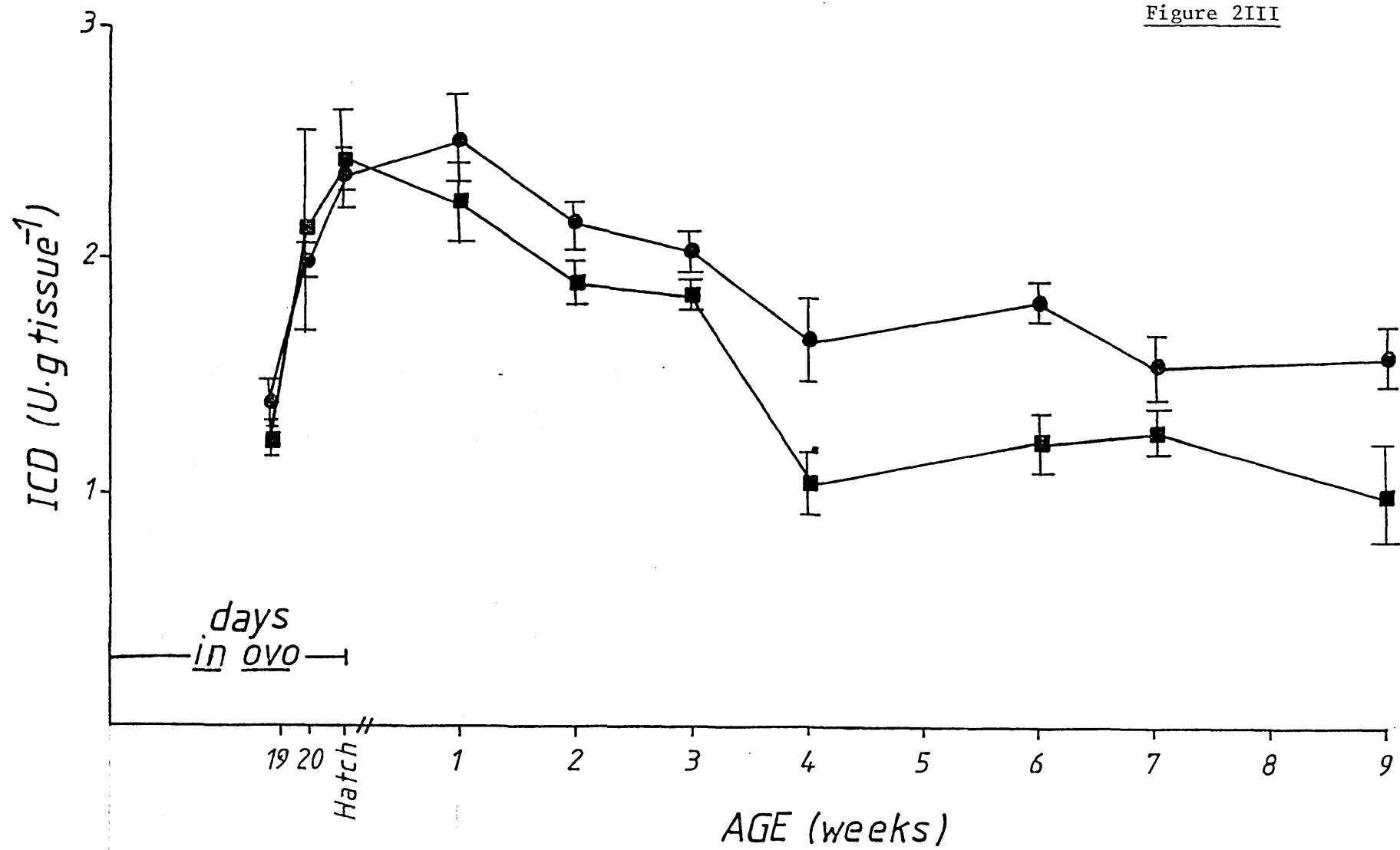
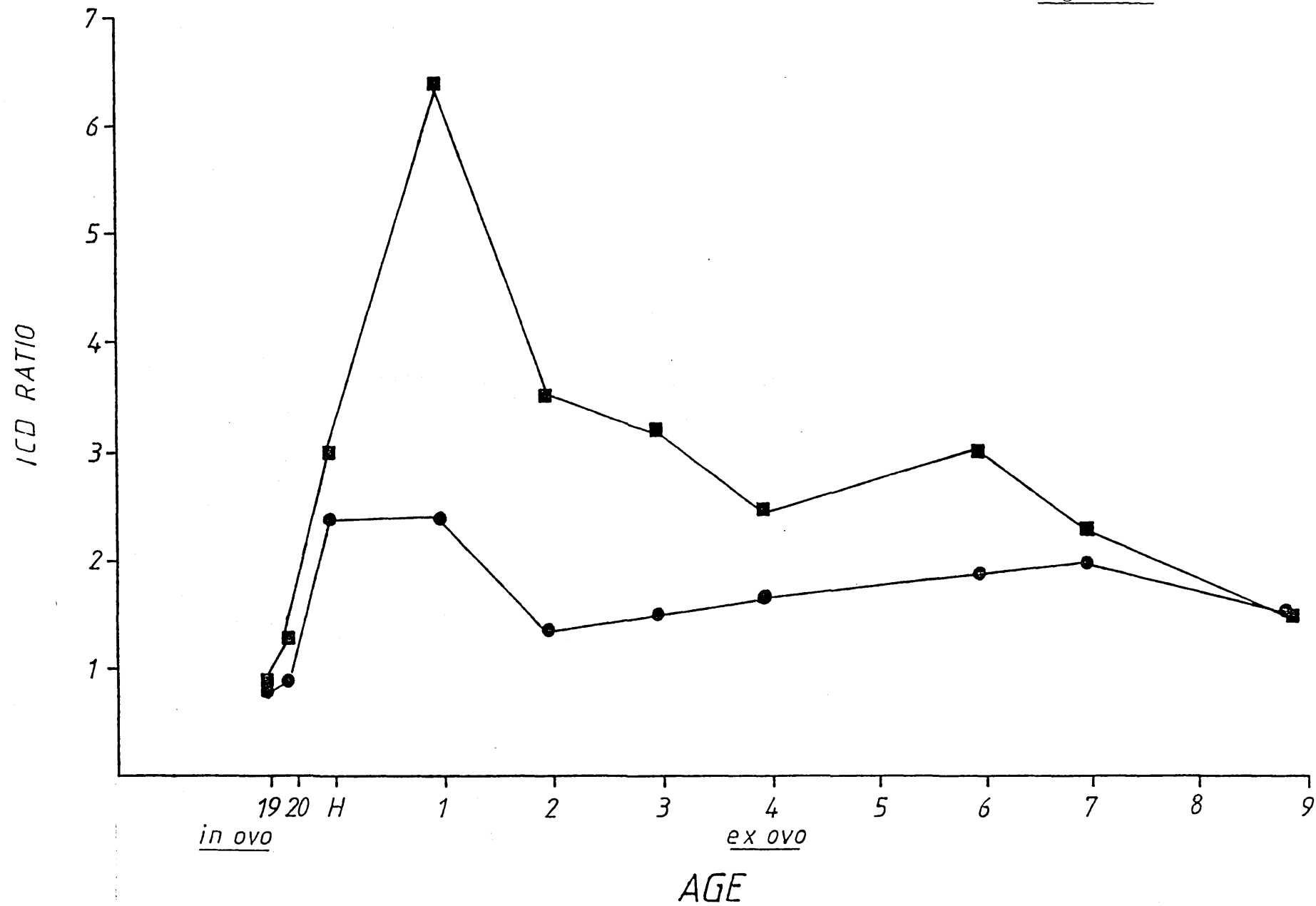


Figure 2IV

Isocitrate dehydrogenase ratios for normal and dystrophic chickens.

Isocitrate dehydrogenase activities expressed as the ratio extra-mitochondrial ICD/intra-mitochondrial ICD are shown for normal (■) and dystrophic (●) birds. The ICD ratio was calculated from data shown in Figures 2II and 2III.

Figure 2IV



SECTION THREE

PHOSPHOGLYCERATE MUTASE

INTRODUCTION

Enzyme analysis has provided a principal vehicle for research and diagnosis in DMD (Pennington, 1978; McCaman, 1963). No causative agent has been determined for this X-linked myopathy but the secondary symptoms have been well documented (for reviews Serratrice et. al, 1984; Walton and Mastaglia, 1980). Many of the abnormalities observed involved elevated enzyme levels and have been utilised as diagnostic tests (Pennington, 1980; Kiessling and Beckman, 1981; Zatz, 1984). A prime example is creatine kinase whose concentration is elevated in plasma Duchenne sufferers and some carriers (Ebashi et. al 1959, Zatz, et. al 1980). This has been a principal biochemical test in the UK but is subject to interference from several factors e.g. age of subject and physical activity prior to assay, which result in overlapping normal and pathological ranges and hence false positive results are recorded. Thus reports of sensitivity of carrier detection have varied from 70% to 45% of carriers detected (Emery, 1980; Griggs et. al, 1985). Therefore there is clearly a need for more accurate tests to replace CK and/or additional tests to increase the sensitivity of analysis.

This is especially pertinent in the case of early diagnosis of DMD in families with no previous history of the disease where the condition is assumed to arise as a result of spontaneous mutation. The spontaneous mutation rate (1/3000) for DMD is the highest known for an X-linked disease (Emery, 1980). The characterisation of the gene defect by genetic engineering techniques may reveal it to involve a directly expressed protein upon which a diagnostic test could be based (Roses, 1984; Emery, 1984). However should the characterised gene code for regulatory functions, then approaches to analysis based on enzymology would remain an important direction of study.

Phosphoglycerate mutase (PGAM), a glycolytic enzyme, has been observed to be a potential aid to diagnosis of DMD. Initial indications from enzyme analysis of dystrophic avian plasma revealed elevated levels of PGAM activity upto 400x that of the corresponding normal birds (Chown, et. al, 1984). Subsequent research on plasma from normal boys aged from 0 to 12 years demonstrated a range of activities from 15 to 57 U l^{-1} (mean $\pm$ SEM = $31.2 \pm 3.02 \text{U l}^{-1}$). The range of plasma PGAM activities for DMD patients of the same ages was from 39.5 to 145 U l^{-1} (mean $\pm$ SEM = $111 \pm 13.3 \text{U l}^{-1}$). These observed differences between normal and dystrophic PGAM levels, in both human and avian samples, are such that exploitation of the enzyme as a diagnostic test for DMD is worthy of further investigation (Chown, et. al, 1984). In support of this view, starch gel electrophoresis of muscle homogenates from DMD patients demonstrated abnormal electrophoretic banding pattern. This type of analysis showed PGAM, in common with CK, to display a foetal-type analytical banding pattern (Edwards, et. al, 1982). The starch gel isozyme banding pattern of PGAM from human samples, changes during foetal development from predominantly brain type (BB) at 62 days in ovo, through a combination of muscle and brain types (BB:MB:MM) at 164 days in ovo, to predominantly muscle type (MM) post partem (Omenn and Cheung, 1974). A combination of the starch gel electrophoresis data and plasma enzyme analysis indicate that further research would profitably be pursued in human samples.

The enzyme in question, phosphoglycerate mutase (ec.2.7.5.3) has an essential role in glycolysis, its obligatory function being to interconvert 2-phosphoglycerate and 3-phosphoglycerate (Meyerhoff and Kiessling, 1935). The reaction is dependent on a co-factor, 2,3-diphosphoglycerate, (Rodwell, et. al, 1957). Studies of various tissues demonstrated muscle to be a rich source for this

enzyme (Grisolia and Joyce, 1959). Previously there was confusion regarding the specific reactions catalysed by purified PGAM but the complexity of the kinetic and biochemical properties have now been elucidated (Rose, 1984). In pure preparations of PGAM, two other enzyme activities (bisphosphoglycerate synthase and bisphosphoglycerate phosphatase) were shown to co-purify with the mutase, thus resulting in confusion as to the principal function of the protein. It is now evident that the major function of mutase in muscle is accompanied by phosphorylase activity deriving from the same native protein species (Rose, 1984). In other tissues the exact mode of action observed is dependant on the physiological requirements of the cell (James et. al, 1972). The phosphoryl transfer involved in the mutase reaction involves the formation of a phospho-enzyme intermediate from 2,3-diphosphoglycerate. The kinetic mechanism has been described as following a ping-pong mechanism (Grisolia and Cleland 1968; Rose, 1984).

This research project addressed the potential diagnostic applications of PGAM in DMD by means of three distinct approaches.

- (i) Normal female plasma and serum PGAM values were determined by enzyme-linked assay (DiMauro, 1982). These mean values were subsequently used to calculate the upper limits of normal (95th percentile) and were used for comparison of samples 'at risk' for DMD.
- (ii) Development of a radioimmuno assay (RIA) for PGAM to replace the multiple enzyme linked spectrophotometric assay. The benefits of developing such a method would be a reduction in quantities of sample required (such as that available from infants) and a much higher sensitivity for the detection of low concentrations of PGAM. The major problems of the enzyme assay,

inaccuracies inherent in a system linking several enzymes and spurious elevation of activity induced by contaminants in enzyme preparations, would be eliminated by the use of RIA. RIA development was approached by raising antibodies directed against rabbit muscle PGAM plus the purification of the enzyme from human sources for use as standard. The purified human PGAM in turn could be used to raise specific antibodies for use in RIA.

(iii) Isoelectric focusing banding pattern of PGAM.

Given that the atypical banding pattern described by Omenn and Cheung (1974) could be confirmed by these methods, there would be sufficient potential for the development of this technique as a diagnostic tool. A full knowledge of the individual isoelectric point for each observed band would enable the separation of the particular isozymes from normal PGAM samples for use as antigens in raising PGAM isozyme-specific antibodies.

In summary, the aims of this project were to investigate the potential use of PGAM as a diagnostic aid in DMD and to develop more sensitive methods of detecting the enzyme.

MATERIALS AND METHODS

All chemicals were obtained from Sigma, Poole, Dorset, with the exception of those listed below:-

Glycerate 3 phosphate (G3P)

Enolase

Lactate dehydrogenase (LDH)

Nicotinamide adenosine (NADH₂)

Phosphoglycerate mutase (PGAM) from rabbit muscle
were obtained from BCL, Lewes, Sussex.

Butan-1-ol

Glycine

Hydrochloric acid (HCl)

Methanol

Acetic acid

were obtained from BDH, Poole, Dorset.

Pharmalyte (pH 3-10)

G75- Sephadex

were obtained from Pharmacia, Upsalla, Sweden.

Bolton-Hunter reagent (N-succinimidyl 3-(4-hydroxy, 5

¹²⁵Iodophenyl) propionate, 0.5 mCi)

Chloramine-T reagent

were obtained from Amersham International, Amersham, Buckinghamshire.

SAMPLE PREPARATION

Blood

Normal human female plasma and serum samples were a gift from Dr. N. Carter, Department of Child Health, St. Georges Hospital Medical School, St. Georges Hospital, Tooting, London. Blood samples were withdrawn from healthy donors into tubes containing lithium heparin or clotted as required. All plasma and serum was separated by standard procedures within one hour of sampling. These were then frozen and maintained at -80°C until required. Blood samples from women 'at risk' for carrying DMD were treated as described above and were donated by Dr. R. Watts, University College Hospital, London.

Muscle

Pectoral muscle was hand homogenised (glass/glass homogeniser 3ml maximum volume) in cold 0.005M tris-HCl buffer (pH 7.4) containing 0.001M EDTA (ratio 100 mg wet weight.mlbuffer⁻¹). The homogenate was centrifuged at 50,000 x g for 30 minutes at 4°C and the supernatant used immediately for assay and isoelectric focusing analyses. It was confirmed by assay of all fractions (supernatant and pellet) that the total PGAM content of the homogenised muscle was released into the soluble fraction by this procedure (Chown, et. al, 1984).

ENZYME PURIFICATION

The human muscle (adult and foetal) was a gift from Dr. N. Carter, Department of Child Health, St. Georges Hospital Medical School, Tooting, London. The samples (free of neuromuscular disease) were taken at post-mortem and frozen at -80°C until required.

The chicken muscle was taken from normal New Hampshire chickens line 412 (described previously page 158). Chicken samples were taken and used immediately for purification procedures.

ENZYME EXTRACTION

Frozen samples were thawed at room temperature. All muscles were blotted dry, weighed, chopped on ice and homogenised in 1.5 x volume of ice cold 0.01 M tris-HCl pH 7.4 (Grisolia and Carreras, 1975). The homogenate was centrifuged at 2000 x g for 30 minutes at 4°C and the resulting supernatant (crude fraction) retained for further purification.

HEAT TREATMENT

To the crude fraction, 0.1 volume of 60mM 2,3-diphosphoglycerate pH 7.00 was added and the mixture heated to 62°C for 10 minutes with constant stirring. The flask containing the mixture was chilled by plunging into ice for 5 minutes and the sample centrifuged at 2000 x g

for 15 minutes at 4°C. The supernatant was subsequently filtered through Watman N^o 1 paper and retained on ice (heat fraction).

AMMONIUM SULPHATE FRACTIONATION

Two ammonium sulphate fractionation steps were included. Solid ammonium sulphate was added to 35 percent saturation, calculated according to Scopes, (1982). The suspension was stirred for one hour and then centrifuged at 10690 x g for 30 minutes at 4°C. The supernatant was retained and the ammonium sulphate saturation increased to 60 percent for chicken muscle preparations and to 72 percent for human preparations. The optimal fractionations for the human and avian muscles were determined by small scale precipitation experiments at a range of ammonium sulphate saturations (shown for avian muscle in Fig. 3X). The mixture was stirred at 4°C for four hours and subsequently centrifuged at 18,000 x g for 30 minutes at 4°C. The pellet was retained and resuspended in 1.5 ml 0.01M tris-HCl pH 7.4 at 4°C.

AFFINITY CHROMATOGRAPHY

Lyophilised reactive 2-blue sepharose (Cibacron Blue^R) was washed on scintered glass with 200 ml distilled deionised water and expanded in tris-HCl pH 7.4 at 4°C. Packed columns (1cm x 11cm for column 1; 1cm x 1.5 cm for column 2) were washed with alternate high and low pH (pH 2 and 10) solutions and finally equilibrated with 0.01M tris-HCl pH 7.4. The flow rate was adjusted to 3 ml.minute⁻¹. Sample was applied to the column and washed into the bed with 4 column volumes of 0.01 M tris-HCl pH 7.4 buffer. Fractions (3ml) were collected and monitored spectrophotometrically at 280nm. The specific elution of PGAM was effected by the application of 5mM 2,3-DPG(pH 7.4) dissolved

in 2 column volumes of running buffer. The fraction size was reduced to 1 ml during elution and fractions with maximal absorbance were retained for enzyme assay and subsequently pooled. The column was washed and regenerated by application of 2 column volumes of running buffer containing 1M NaCl followed by alternate high and low pH buffer solutions. The columns were stored in running buffer containing 0.1 percent sodium azide as bacteriostat (Price and Stevens, 1983).

ULTRAFILTRATION

Pooled fractions were concentrated using an Amicon^R ultrafiltration unit fitted with 5000 MW cut off filter, at 70 psi N₂ at 4°C. The PGAM thus concentrated was stored at -20°C prior to use.

POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The purity of preparations was assessed by SDS-PAGE using a modified Laemmli discontinuous system (Laemmli, 1970).

Gel casting

Gels were cast vertically by hand between glass plates of dimensions 15 cm x 19 cm separated by 2mm spacer. The lower (separating) gel was poured first and air excluded to facilitate polymerisation. After this was complete the upper (stacking) gel was applied. Interdigitations were introduced by placing a 'comb' between the top edge of the plates. The gel was completely polymerised at room temperature.

Separating gel

A 10 percent polyacrylamide (9.75% acrylamide, 0.25% bisacrylamide) gel was prepared in 1.5M tris-HCl (pH 8.8) containing 0.1 percent SDS. The polymerisation process was facilitated by ammonium persulphate (0.02%) and N,N,N^1,N -tetramethyl ethylenediamine (60 μ l TEMED). After pouring (30ml) air was excluded by the addition of a butan-1-ol overlay and left to polymerise at room temperature.

Stacking gel

The upper gel layer of 3.1 percent polyacrylamide (3% acrylamide, 0.1% bis acrylamide) was dissolved in 1.5M tris-HCl (pH 6.8) containing 0.1 percent SDS. Cross-linking was facilitated by the addition of 1 percent ammonium persulphate and 30 μ l TEMED to the mixture. This was then overlayed (20 ml) on the separating gel and the 'comb' inserted (well size 0.2 ml).

Sample preparation

Samples (5 μ g protein) were heated to 95°C for 10 minutes in a 62mM tris buffer solution (pH 6.8) containing 1 percent SDS, 5 percent mercaptoethanol, 10 percent glycerol and bromophenol blue before loading into wells.

Electrophoresis Buffer

The upper and lower reservoirs contained the same 2.9 mM Tris buffer containing 0.01 percent SDS and 0.14 percent glycine.

Operating procedures

Once the gel was positioned on the electrophoresis equipment and the samples applied to individual wells the electrophoretic process was undertaken at 25 amperes constant current (8-9 volts. cm^{-1}). Current was removed from the gel when the blue dye marker was within 1 cm of the bottom edge.

Sample detection

The lower portion of the gel was immersed in a fixing: staining solution of 28 percent methanol, 10 percent acetic acid, 62 percent water containing 0.1 percent coomasie blue (R250). The gel was then heated to 62°C for 30 minutes in this solution. The stain solution was then exchanged for a destain solution of 8 percent acetic acid, 25 percent ethanol, 67 percent water and the heat process repeated. Subsequently protein bands appeared as sharp blue bands on a clear background (Righetti, 1983).

ISOELECTRIC FOCUSING

Flat-bed isoelectric focusing (IEF) techniques were employed to separate the isoenzyme form of PGAM.

Gel casting

A 2mm thick gel was cast onto a thin glass support plate (1.5 mm) and after initial polymerisation at room temperature was stored at 4°C overnight prior to use.

Gel preparation

A 5.15 percent polyacrylamide (5% acrylamide, 0.15% bis acrylamide) solution containing 1.25 ml Pharmalyte^R (pH range 3-10) was degassed. Polymerisation was facilitated by the addition of 2 percent ammonium persulphate. Once poured the gel was overlayed with butan-1-ol to exclude air.

Sample Application

Samples were applied to the gel on 3mm x 5mm watman number 1 paper (5 μ l per applicator). Where necessary samples were dialysed against 0.001M tris-HCl pH 7.4 overnight to remove excess salt.

Focusing procedures

The gel and glass support were placed on the cooling plate of the electrofocusing equipment (4°C) and wicks laid along opposite

edges of the gel (anode solution-1M phosphoric acid; cathode solution-1M NaOH). Focusing was carried out at 15 Watts constant power and 15 kilovolts. The sample applicators were removed after 30 minutes. The extent of focusing was estimated from the sharpness and position of myoglobin marker tracks.

pH gradient determination

The pH gradient established in the gel was measured using a calibrated surface pH electrode (Pye Unicam). The power was then reapplied to the gel to ensure sharp banding pattern.

The pH was also measured after samples were eluted from gel slices. Each tube was measured using a calibrated pH electrode prior to processing for enzyme assay.

Sample detection

(a) Protein stain

The portion of the gel to be stained for protein content was immediately placed in a solution containing 28 percent methanol, 11 percent TCA, 3.5 percent sulphosalicylic acid and 0.1 percent coomassie blue and heated to 60°C for 15 minutes. This fixing/staining solution was exchanged for the destain solution of 25 percent ethanol, 8 percent acetic acid and 67 percent water and heated as above (Righetti, 1983).

(b) Enzyme activity

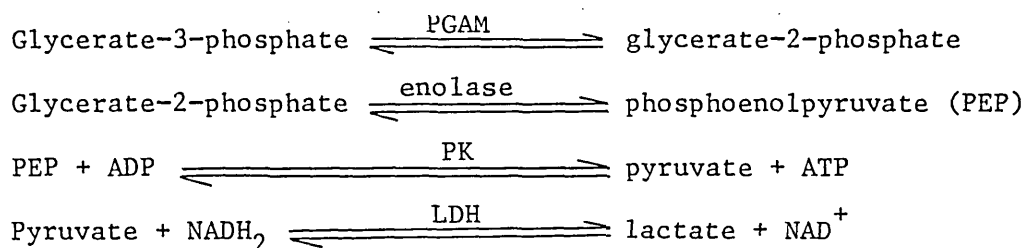
Individual gel tracks were sliced into 2mm segments while kept cold (4°C) and immediately placed in eppendorf tubes containing 0.3 ml distilled deionised water. Elution proceeded overnight at 4°C. Prior to assay the pH of the solution was determined (described above) and 1/20 volume of Dithiothreitol (DTT) added. Samples were then incubated at room temperature (23°C) for 30 minutes after which aliquots could be removed for assay.

ENZYME ASSAY

PGAM activity was monitored spectrophotometrically at 340 nm on LKB 8600 Mk II kinetic analyser. All assays were performed at 25°C and changes in absorbance due to NAD^+ production were monitored.

Assay principal

The enzyme activity was calculated from observed decreases in absorbance resulting from the oxidation of NADH_2 . The assay utilised the method described by DiMauro et. al (1982) adapted for semiautomated analysis (Chown, et. al, 1984).



PGAM = phosphoglycerate mutase

PK = pyruvate kinase

LDH = lactate dehydrogenase

Samples (15 μl -50 μl) were preincubated for 30 minutes at 25°C in degassed 0.1M tris-HCl buffer (pH 7.6) containing 1.7 mM MgSO_4 , 0.2mM NADH_2 , 0.6mM ADP, 0.1mM 2,3-DPG, 3.4 Uml^{-1} LDH, 1.4 Uml^{-1} PK and 1.5 U.ml^{-1} enolase. The addition of 137 mM G3P was followed by a further 3 minutes incubation at 25°C then spectrophotometric reading progressed (Chown, et. al, 1984).

The purity of G3P and LDH are critical, some contaminants in certain commercial preparations caused spurious activity values (Chown, et. al, 1984).

Enzyme activity was calculated as previously described in Section 1 p. 43.

RADIOIMMUNO ASSAY

Antisera production

Antisera to pure PGAM (rabbit muscle) was raised in guinea pigs. The initial intra-peritoneal injections of protein in Freund's complete adjuvant was followed at 2 week intervals by further ip injections of enzyme in incomplete adjuvant. The serum was separated from clotted red blood cells after overnight incubation at 4°C and stored frozen (-20°C) without preservatives. Sera from different bleedings were tested for the presence of antibodies using a gel double diffusion method (Oudin, 1952). Subsequently one guinea pig received one injection of PGAM (1mg) in distilled deionised water by intravenous injection.

Iodination of PGAM

Two methods of iodination were attempted: (a) Bolton-Hunter (Bolton and Hunter, 1973) and (b) Chloramine-T (Greenwood et. al, 1963).

(a) Bolton-Hunter method

This method was based on that used by Rosano and Kenny (1977). Rabbit muscle PGAM, 10µg in 10µl of 0.1M phosphate buffer pH 7.5 was added to 0.5mCi of dried iodinated ester (N-succinimidyl 3-(4-hydroxy, (¹²⁵I) iodophenyl propionate)). The reaction was allowed to proceed for 1 hour at room temperature (23°C) with occasional mixing. Comparative iodination for 1.25 hour at 4°C followed the same procedure except that 0.125mCi of dried iodinated ester was used. For both procedures incubation with 0.5ml of 0.2M glycine in reaction buffer for 2 minutes terminated the reaction. The products of iodination were separated at room temperature by immediate gel filtration through a sephadex G75 column (30 x 0.9 cm) equilibrated with 0.1M phosphate buffer pH 7.0 containing 2.5g.l⁻¹ gelatin. After addition of 60gl⁻¹ BSA fractions containing label were snap frozen in liquid nitrogen and stored at -70°C.

(b) Chloramine-T method

Rabbit muscle PGAM, 25 μ g was incubated for 2 minutes at room temperature (23°C) with 500 μ Ci of ^{125} I, 10 μ l of 0.5M sodium phosphate pH 7.5, 10 μ l Chloramine-T (2mg.ml⁻¹) in 0.05M sodium phosphate pH 7.5. After 2 minutes the reaction was stopped by addition of 25 μ l of sodium metabisulphite (400 μ g.ml⁻¹ in 0.05M sodium phosphate pH 7.5). Products were separated from free radiolabel by gel filtration as described for the Bolton-Hunter method.

Cellogel electrophoresis

Electrophoresis was performed using a tris-EDTA/borate buffer at pH 9.6 as described by Hopkinson et. al (1974). Electrophoresis was carried out for 30 minutes at 100 volts. The isolated sample track was then cut into 1cm strips and radioactivity counted on a gamma counter (LKB Wallac Rackgamma II).

Radioimmuno assay procedures

A PGAM standard curve was constructed as follows:- to 100 μ l of PGAM standard, 100 μ l of 1/100 dilution of guinea pig antiserum in 0.05M tris-HCl (pH 7.5) containing 2.5g.l⁻¹ bovine serum albumin was added and incubated together for 24 hours before addition of 100 μ l ^{125} I-PGAM. A further incubation of 48 hours was completed before the addition of 100 μ l second antibody in 0.05M tris-HCl (pH 7.5) containing 0.03M EDTA and 1g.l⁻¹ BSA. The second antibody in this instance a 1/20 dilution of donkey anti-guinea pig gammaglobulin was accompanied by 1/100 dilution of normal guinea pig serum. After an overnight incubation the tubes were centrifuged at 2000 x g and 4°C for 30 minutes. The supernatants were then carefully aspirated and discarded and the precipitates carefully counted for 100 seconds per tube using an LKB Wallac rackgamma II. All RIA's were performed in triplicate using LP3 tubes at 4°C throughout.

The standard curve was plotted as B/B_0 versus $\log_{10} \text{PGAM}$; where B was ^{125}I -PGAM bound to antisera and B_0 was the ^{125}I -PGAM bound to antisera in the absence of unlabelled PGAM. Prior to calculation a mean non-specific binding value in counts per minute was automatically subtracted from all of the tubes excepting the tubes representing 'total' radioactivity added to each tube.

RESULTS

Normal female plasma and serum PGM activity

Plasma and serum PGAM activities (U l^{-1}), determined by the method of DiMauro et. al (1982) are shown in Tables 3a and 3b. Mean plasma and serum activities for normal women aged between 15 and 74 years were $66.57 \pm 2.8 \text{ U l}^{-1}$ (n=71) and $55.05 \pm 2.31 \text{ U l}^{-1}$ (n=65) respectively. In Tables 3a and 3b the results were also segregated by date of birth into units of ten years and mean activity values calculated. Thus for plasma PGAM comparison of group means with that of the whole sample (n=71) revealed no significant differences in the age ranges 1931-1940, 1941-1950, and 1951-1960. Serum PGAM (Table 3b) was treated similarly to the plasma and in age groupings 1921-1930, 1931-1940, 1941-1950, 1951-1960 and 1961-1970 no significant differences between individual mean values and that of the complete sample (n=65) were observed.

Comparison of plasma and serum PGAM activities for the age range 1911-1970, indicated a significant difference between these mean values ($p < 0.005$), the plasma activity being the greater. Analysis of PGAM from plasma and serum separated from the same blood samples (Table 3c) did not confirm this result. There were however, variable differences between individual plasma and serum values within this group (n=9).

'At risk' carrier samples

Plasma PGAM, CK and CaIII activities from 23 women 'at risk' as carriers of DMD are shown in Table 3d. Creatine kinase (determined by BCL NAC-activated Test kit) and CaIII (determined by the method of Heath et. al, 1982) activities were provided by Dr. N. Carter, Department of Child Health, St. Georges Hospital Medical School, Tooting, London. All determinations in triplicate were conducted in 'blind' trials and matched with names only after calculations were carried out. A percentage

'carrier risk' value was also included in Table 3d. The risk assessment was performed by Dr. R. Watts, University College Hospital, London, who carefully studied family pedigrees and combined this data with plasma CK activities by a method based on Bayes theory (detailed by Emery, 1980). In this way both prior (based on family history) and conditional (based on phenotype, offspring and test results) probabilities were taken into account. Therefore 100% assessment indicates a known carrier (two affected children) or suspected carrier (one affected child). Subsequently M.J.S. was revealed to be a confirmed carrier.

Six individuals were also sampled at time intervals. Three of these showed no variation with time but the remainder differed by 10-16 activity units (Table 3e).

The application of creatine kinase (CK) analyses alone has been reported to detect between 45 and 70 percent of DMD carriers, but this may be increased to 83 percent when used in conjunction with assay for myoglobin (Emery, 1980; Grigg et. al, 1985). Therefore comparisons of pairs of markers may be a more sensitive method of detection. In view of this, PGAM was compared to two established markers CK and CaIII (Figs. 3I, 3II). CK plotted against CaIII is shown in Fig. 3III. On each figure the upper limit of normal (95th percentile) is shown for each enzyme. In the case of PGAM this was calculated from the mean plasma (n=71) value described above. For any pair of markers to be considered suitable for diagnosis of DMD carriers both should express greater than 95th percentile levels and thus lie in the upper right quarter in Figures 3I, 3II and 3III. Therefore it would be expected that those samples designated 100 percent carrier risk (O) would lie in this quarter. Although these samples (O) lie near the upper limit for CaIII and CK in all three figures, only in the comparison CK:CaIII does one sample fulfil the requirement. The one known carrier included in analysis demonstrated

only elevated CaIII levels. In all the figures (3I, II, III) the majority of samples with less than 100 percent carrier assessment were enclosed within both upper limits of normal.

RADIOIMUNO ASSAY

Antiserum preparation

Antibodies (ab) were raised to a commercial preparation (Sigma) of rabbit muscle PGAM. The purity of the PGAM was demonstrated by SDS-PAGE and is shown in Fig. 3IV where a single band indicated that no contaminant proteins were present in the preparation. The antibodies were raised in guinea pigs and after the third booster injection ab:ag complex formation could be visualised by Ochterlony double diffusion procedures (Fig. 3V). In order to increase antibody titre, one guinea pig was given an intravenous injection of PGAM (1 mgml^{-1}) in sterile water. The resulting antiserum reacted with both rabbit and human foetal muscle homogenate PGAM (Fig. 3VI). Antiserum from this guinea pig contained the highest titre of antibodies raised and was subsequently used for RIA development.

Radio-labelling of PGAM

Rabbit muscle PGAM of the same purity as that used to raise antibodies was iodinated initially by the Bolton-Hunter method at 23°C and subsequently by Bolton-Hunter at 4°C and by Chloramine-T (Rosano and Kenny, 1977; Greenwood *et. al* 1963). When iodinated PGAM from Bolton-Hunter iodination at 23°C was introduced to antiserum, low binding with antibody was observed. Therefore a further two iodinations were performed to determine whether the low ab:ag binding was due to iodination damage or low antibody titre. The column (sephadex G75) elution profiles for all three iodinations are procedures as shown in Fig. 3VII, VIII. In each case

^{125}I -PGAM was the first peak eluted. Incorporation of label by the Bolton-Hunter methods at 23°C and 4°C was 45 percent and 34 percent respectively. Radiolabel incorporation by Chloramine-T method was 60 percent. All three iodinations gave poor reaction with antibody. The antiserum bound 5,000 counts per minute (cpm) at 1/5 dilution, out of a total of 22,000 cpm of ^{125}I -PGAM.

Evaluation of ^{125}I -PGAM

The iodinated protein was evaluated by TCA precipitation and cellogel^R electrophoresis. In the first case precipitation of iodinated PGAM by an equal volume of 10 percent TCA gave 100 percent precipitation of radioactivity indicating that no protein cleavage was induced on iodination. Under the conditions used for cellogel electrophoresis native unlabelled enzyme remains at the origin while iodinated products move towards the anode, as a result of $^{125}\text{I}^{-}$ incorporation. These results shown in Table 3f indicate that the integrity of PGAM was retained after iodination. It was concluded that the low assay binding was due to the low antibody titre.

Standard curve

Despite the low antibody:antigen binding it was possible to demonstrate a standard curve using 1/100 dilution of antiserum (Fig. 3 IX). This was the greatest dilution of antiserum that enabled the construction of a standard curve. The linear portion, applicable for estimation of unknowns, covers the range $200\text{--}1080\text{ ngml}^{-1}$ PGAM. This was not sufficiently sensitive for use as a clinical RIA but demonstrated that given a higher antibody titre a more sensitive anti PGAM RIA would be feasible.

PROTEIN PURIFICATION

Purification procedures for PGAM were developed using a readily available muscle source i.e. that of chicken pectoral muscle. Having established the practical feasibility of the protocol it was then applied, with minor adjustments, to samples of human muscle.

Enzyme extraction and heat treatment

High levels of PGAM activity were extracted from muscle by homogenisation procedures. This activity remained in the supernatant after centrifugation (50,000 xg, 4°C, 30 min) and was called the crude fraction. The addition of 2,3-diphosphoglycerate (2,3 DPG) prior to heating the sample at 62°C protected the enzyme from denaturation and loss of enzymatic activity thus:-

	Protein μgml ⁻¹	PGAM mUml ⁻¹	SA
Crude Fraction	21.2	20,670	975
Heat Fraction	11.2	19,637	1753

The result of the heat procedure was a 47 percent reduction of protein in the sample and a 44 percent increase in specific activity (SA).

Ammonium sulphate precipitation

Ammonium sulphate precipitation was employed to further reduce the amount of protein impurities in the sample. The effects of a range 0 to 100 percent saturation of ammonium sulphate on protein content and enzyme activity can be observed in Fig. 3X. In the range 0-40 percent saturation PGAM remained in solution. From this a procedure using two successive fractionations with ammonium sulphate was developed. In the first instance ammonium sulphate saturation was raised from 0 to 35 percent saturation by the addition of solid. Observation of Fig. 3X shows that 50 percent of the protein in the sample was precipitated by

centrifugation leaving PGAM in solution. After centrifugation the supernatant containing PGAM was retained and the ammonium sulphate content raised to 70 percent saturation. PGAM was precipitated by centrifugation and the pellet resuspended in a minimal volume of buffer.

Affinity chromatography

Batch binding experiments were performed in eppendorf tubes to determine optimal times of incubation for adsorption and desorption. Tables 3g and 3h indicate that binding equilibrium was approached within 1 hour of sample-medium contact. Similarly, specific elution was accomplished within 1 hour of addition of 2,3 DGP. Elution products were analysed by SDS-PAGE and these results are shown in Fig. 3XI. The single band in track 4 is a protein of molecular weight 30K as calculated from the standard proteins in track 1. This compares favourably with the known molecular weight for the two subunits of PGAM.

These small scale purification procedures were applied to adult human muscle the results of which are shown in Fig. 3IV. However, examination of analyses on SDS-PAGE revealed a second species of higher molecular weight which apparently copurified with the PGAM. Analysis of the ammonium sulphate precipitation procedures by SDS-PAGE indicated that this second protein coprecipitated at 70 percent saturation with PGAM. However it was considered that the operation of a larger scale affinity column (11cm x 1cm) with controlled adsorption and desorption procedures would ensure a pure preparation of PGAM.

Binding of PGAM to and specific elution from Cibacron-blue column 1 (11cm x 1cm) was tested using pure rabbit muscle PGAM (Sigma). The elution profile for the protein, monitored spectrophotometrically as increases in absorbance at A_{280} , is shown in Fig. 3XII. In this instance the enzyme was specifically eluted after the application of

5mM 2,3 DGP to the column. Fig. 3XIII demonstrates the interference to binding resulting from high salt concentration (0.3M ammonium sulphate). Thus it is essential to remove salt by dialysis prior to sample application to the column. After elution was completed the column was then cleaned, regenerated and equilibrated with buffer. The binding and elution profile of a chicken preparation applied to the column, as monitored by increased absorbance at A_{280} , is shown in Fig. 3XIV. The peak of absorbance observed after application of 5mM 2,3 DGP coincides with that of PGAM activity.

Human muscle PGAM

Human adult and feotal muscle was partially purified as described previously with the exception that for feotal muscle, the heat step was omitted due to the heat sensitivity of the immature enzyme form. Both enzymes were applied to Cibacron-blue column 1 and specifically eluted as described above. Fractions with optimum enzyme activity were combined and concentrated by ultrafiltration procedures. The final results of purification are shown by SDS-PAGE in Fig. 3XV. In the instances of both adult and feotal muscle preparations impurities observed as additional protein bands, were present. Further purification was attempted by reapplying samples to a further cibacron-blue column (1.5cm x 1cm) and eluted fractions were taken for analysis. The result of the 'second pass', reductions of bands present in SDS-PAGE, is shown in Fig. 3XVI.

ISOELECTRIC FOCUSING

All isoelectric focusing (IEF) was undertaken by standard flat-bed procedures. Visualisation of PGAM banding patterns was obtained by slicing (0.1cm) and eluting in distilled H₂O and subsequently assaying for enzyme activity. Alternatively tracks were stained for protein.

pH gradient

The pH gradient was determined using a surface electrode (Pye Unicam), 15 minutes before the completion of focusing. At this time, a stable pH gradient should have been formed and established. A second determination from the same gel was taken on the eluate from the gel slices. The results of the two methods are shown in Fig. 3XVII. In both methods the linear phase of the pH gradient spanned the range from 2 to 8cm.

Rabbit and mouse PGAM isozymic patterns

The isozyme banding pattern for native enzyme from rabbit muscle (Sigma) and mouse pectoral muscle and brain are shown in Figs. 3 XVIII and XIX. For each muscle sample 3 major bands of enzyme activity were observed as compared to two major bands for mouse brain. The lowest pH peak of enzyme activity was assumed, from comparison of mouse muscle and brain activity patterns, to correspond to the brain specific (BB) isotype.

Human muscle PGAM isozymes

The isozyme banding patterns from adult and foetal muscles are shown in Figs. 3XX and XXI. In common with the homogenates from rabbit and mouse muscle, the adult human muscle preparation demonstrated three major peaks of enzyme activity after isoelectric focusing. In the latter instance, however, subsidiary peaks were also noted. IEF of human foetal muscle revealed only one major peak of activity between pH 5.00 and 6.00. This was assumed to correspond to the brain (BB)

isotype previously noted from mouse brain preparations. Fig. 3XXII shows IEF tracks corresponding to the stages of partial purification, first pass column elution and second pass column elution which have been stained for protein. Comparison of the banding patterns demonstrates the removal of contaminants with purification procedures. Loss of the brain band however may result from the final column elution.

DISCUSSION

For an enzyme to be applicable to carrier detection in DMD it must occur at elevated levels ideally in the plasma of female carriers. Generally an enzyme level is considered to be elevated when activity exceeds the 95th percentile of the normal mean value (upper limit of normal). The enzyme investigated in this section, PGAM, was assayed in normal female plasma and serum to establish a normal baseline level and also in those 'at risk' of carrying DMD.

The normal PGAM values thus established for serum and plasma were 66.57 U l^{-1} and 55.05 U l^{-1} respectively over an age range from 14 to 74 years. The observed difference between these two values of 17 percent warranted further investigation and thus blood samples from which both serum and plasma was obtained were studied. Six out of the nine available cases showed higher plasma than serum values, however, comparison of the means by student's t-test showed no significant difference ($p < 0.1$). Therefore care should be taken when assessing 'at risk' samples to compare them with the upper limit of normal for the corresponding plasma or serum. There is some possibility that the higher plasma values may be attributed to interference from the anti-coagulant used.

Simultaneous comparison of two enzymes may increase the percentage chance of correct diagnosis by the elimination of false positive results. In this study PGAM was compared to CK and CaIII (data supplied by Dr. N. Carter). In each figure (3I, 3II, 3III) the 'at risk' samples with calculated 100 percent probability of being a DMD carrier were indicated by the symbol $\circ$ and the one definite carrier by the symbol $\square$. An enzyme of diagnostic significance must demonstrate greater activity levels than the upper limit of normal for the particular enzyme and in simultaneous comparison studies be greater than this limit for both enzymes studied (upper right quarter in Figs. 3I, 3II, 3III). In both comparisons of PGAM with CK and CaIII no such samples were found. In the instance of CK and CaIII, 1 and 3 samples respectively were found to lie above their upper limit of normal and thus simultaneous comparison of these two enzymes resulted in one positive result. PGAM, however, from these analyses, does not appear to be of use in detection. This may be attributed to the insensitivity of the assay used carrier and development of a sensitive PGAM specific RIA may reveal differences not detectable by the enzyme linked assay system. It must be noted that the one known carrier did not fall in the upper right quarter in the CK/CaIII comparison although it lay above the upper limit of normal for CaIII, thus highlighting the problems inherent in this type of analysis where the ranges of carrier sample values tend to overlap those of the normal samples. It also demonstrates the necessity of multiple enzyme analysis for definitive carrier detection.

Development of RIA for PGAM was necessitated by the need for a more sensitive assay which could utilise small sample volumes (such as those available from babies and infants) and also eliminate the inaccuracies inherent in a multiple enzyme linked assay system. Thus

although analysis of plasma PGAM by standard assay procedures was negative, it would be informative to re-investigate plasma enzymes by RIA. Determination of inactive enzyme content or presence of specific isotypes could aid diagnosis.

Guinea pig anti-rabbit PGAM antibodies were successfully raised as shown by precipitation lines in Ochterlony double diffusion procedures. It was also demonstrated that PGAM could be iodinated successfully without damage (by cellogel electrophoresis and TCA precipitation). When the ^{125}I -PGAM was addressed by antiserum, binding could only be observed at a maximum dilution of 1/100. At this dilution of antiserum, a standard curve was obtained, however the useful range of PGAM detection extended only from 200 to 700 ngml⁻¹. This dilution of antiserum was not considered sufficiently high enough for a sensitive clinical RIA where dilutions in the range 1/1000 to 1/5000 would be considered the norm. Combination of these results with the observed recognition by double diffusion procedure of human foetal muscle PGAM, indicated that further development of a sensitive PGAM specific RIA would profitably be pursued.

Progress towards this development may be accomplished with the complete purification of human muscle PGAM. This purified enzyme would then be employed as antigen to raise specific antibodies in a larger mammal than that previously used e.g. sheep, goat or donkey which would provide larger quantities of antiserum. The pure PGAM would also be of use as 'standard' in the established RIA. The purification procedures employed in this project although not purifying PGAM to homogeneity did demonstrate that high enzyme activity could be extracted from frozen human (adult and foetal) muscle sources and contaminants removed to a great extent. The addition of a further purification step e.g. ion exchange, may be sufficient to refine the purification procedures such

that all contaminants were removed. The insertion of this step prior to affinity chromatography may also eliminate the need for a second 'pass' affinity column and remove those contaminating proteins observed on SDS-PAGE (Fig. 3XXII).

Isoelectric focusing procedures were undertaken to visualise the isoenzyme banding pattern of PGAM from several muscle sources. Rabbit, mouse and human muscle PGAM gave a 3 peak activity pattern which was in agreement with starch gel electrophoresis data already available (Omenn and Cheung, 1974). These three peaks were designated MM, MB, BB. Analysis of mouse muscle and brain indicated that the peak activity focused at the most acidic pH was that associated with the brain form (BB). Comparison of peaks of activity from gel slices and tacks fixed and stained demonstrated that some diffusion occurred in the first method (Fig. 3XX & XXII). Therefore development of a method whereby the visualisation of the PGAM bands after fixing would be more accurate and increase definition. Specific antibodies to PGAM may also be employed in a new method if used in conjunction with e.g. a colourimetric system. Specific antibodies could be applied to gel tracks directly after fixing and the binding be recognised by a linked assay procedure. This system may have potential as a diagnostic aid should the sensitivity of analysis be increased. Techniques have been developed to enable direct IEF of enzymes in frozen cryostat sections (20 μ) from muscle biopsy samples (Thompson et. al, 1982). Once focusing is complete zymograms (copies) may be made and several enzymes analysed from the same sample. The fact that DMD patients have an abnormal 'fetal type' banding pattern could be employed in this type of diagnosis (Edwards et. al, 1982). Research of this type into carrier samples may also reveal abnormalities of diagnostic significance.

A further use of focusing techniques may be the separation of PGAM isozymes by the use of preparative IEF techniques. This method allows the separation of isozymes by pH and also easy extraction of isotypes from the gel matrix. The separated isoenzymes could then be used to raise muscle (M) and brain (B) specific antibodies which may further increase the sensitivity of a diagnostic RIA system.

In conclusion, the results described herein indicate that future research into the relationship of the enzyme PGAM to DMD should be pursued via the development of a sensitive RIA. The use of the enzyme linked PGAM assay to analyse samples from those 'at risk' of carrying DMD did not reveal significant differences from the normal. However in light of the low sensitivity of this method re-analysis by RIA may be profitably pursued. This research demonstrated that specific anti PGAM antibodies could be raised but were not of sufficient titre to be utilised in this context. Purification procedures demonstrated the feasibility of purifying human muscle PGAM. The addition of an extra purification step could result in the production of pure PGAM with which to raise specific antibodies. An additional use would be that of standard in the RIA with which to compare unknowns. A sensitive RIA would then be used to reaffirm the results of Chown et. al (1984) and reinvestigate normal and 'at risk' enzyme levels. The increased sensitivity of this method may reveal abnormalities not detectable by other methods. Antibodies raised against specific isotypes may further increase sensitivity of analyses and preparative IEF may be employed to provide antigen. A further use of IEF techniques employed in this research may be direct focusing from biopsy samples. The sensitivity of detection in this system also may be increased by the use of antibodies.

Finally, the results reported in this section indicate that further research into PGAM abnormalities in DMD may reveal this enzyme to be a powerful diagnostic aid.

Table 3a

Plasma Phosphoglycerate mutase (PGAM) activities from normal females.

Age Range (year of birth)	Phosphoglycerate mutase (U ⁻¹)	Activity Range (U ⁻¹)
	Mean $\pm$ SEM n S.D.	
1911-1920	90.43 1 -	
1921-1930	57.42 $\pm$ 10.25 3 17.75	43.92-77.52
1931-1940	58.31 $\pm$ 5.38 17 22.2	31.01-106.79
1941-1950	69.62 $\pm$ 4.18 30 22.88	28.42-110.24
1951-1960	74.82 $\pm$ 6.47 17 26.66	28.42-117.13
1961-1970	37.91 $\pm$ 1.71 2 2.42	36.2-39.62
1911-1970	66.57 $\pm$ 2.81 71 24.22	28.42-117.13

PGAM activities (determined in triplicate) for 71 normal females are expressed in (SI) units per litre (U⁻¹) plus or minus the standard error of the mean (SEM). Values are segregated by date of birth into units of 10 years and also shown as a mean for 71 analyses.

Table 3b

Serum Phosphoglycerate mutase (PGAM) activities from normal females.

Age Range (year of birth)	Phosphoglycerate mutase (U ⁻¹)		Activity Range (U ⁻¹)
	Mean ± SEM	n . S.D.	
1911-1920		41 1 -	
1921-1930	64.02 ± 6.97	6 17.07	44.79-88.71
1931-1940	56.17 ± 4.58	18 19.45	21.53-90.43
1941-1950	54.13 ± 3.28	20 14.66	3 9.62-96.46
1951-1960	50.27 ± 5.6	13 20.2	24.12-98.96
1961-1970	66.53 ± 14.27	4 28.54	39.62-106.79
1911-1970	55.05 ± 2.31	65 18.63	21.53-106.79

PGAM activities for 65 normal women were processed as described for Table 3a.

Table 3c

Comparison of plasma and serum PGAM activities for 9 normal females.

Code Number	Date of birth	Plasma PGAM (U l^{-1})	Serum PGAM (U l^{-1})	Percentage difference between Plasma and Serum PGAM
37538	17/6/49	74.07	53.39	-28%
38023	27/10/41	89.57	39.62	-56%
38068	16/2/50	56.84	58.57	+3%
38960	4/2/41	31.01	37.89	+22%
39999	1/9/24	77.52	63.73	-17%
49867	24/4/64	68.9	106.79	+55%
52272	10/8/57	71.52	43.06	-39%
54703	17/10/45	106.8	47.37	-56%
59932	1958	68.9	42.2	-39%

Each plasma and serum PGAM value shown represents the mean of three determinations.

Table 3d

Plasma enzyme activities for 23 females 'at risk' as carriers of DMD.

For each individual, percentage carrier risk, phosphoglycerate mutase (PGAM), creatine kinase (CK), and carbonic anhydrase III values were determined. Each enzyme analysis was made in triplicate and the mean value stated. Activity levels for PGAM and CK were expressed as (S.I.) units per litre (U l^{-1}) and CaIII as nanograms per mililitre (ng ml^{-1}).

Table 3d

Name	Carrier Risk (%)	PGAM (U ⁻¹)	Ck (U ⁻¹)	CaIII (ngml ⁻¹)
S.T.	100	24.98	229	91
U.D.	100	42.83	185	66
M.J.S.	100	24.12	44	762
B.	100	38.07	125	56
M.B.B.	100	25.84	180	62
E.C.	100	50.85	132	65
J.R.	100	37.03	158	83
C.B.	78	17.23	115	36
S.C.	66	37.89	97	37
W.H.	62	14.64	113	36
P.P.	57	29.28	84	52
I.S.	53	38.76	82	47
M.T.	44	30.93	87	71
T.P.	43	80.09	99	68
F.	18	37.89	94	27
A.D.	17	49.09	61	25
M.W.	15.4	15.51	80	46
D.C.	12	19.81	49	18
M.S.	11	28.42	36	20
B.MCG.	3.7	20.69	50	48
T.S.	0.25	16.36	84	31
A.C.	0.006	41.34	38	30
P.C.	0.005	27.56	55	21

Table 3e

Plasma phosphoglycerate mutase (PGAM) activities for 6 individuals sampled at time intervals.

Name	Date of Bleed	PGAM U ⁻¹ _l
I.S.	9/2/81	38.76
	22/2/82	37.03
	26/8/82	37.90
M.S.	15/11/82	28.42
	22/11/82	12.92
T.S.	21/7/82	36.17
	18/8/82	16.36
M.B.B.	28/4/82	29.28
	5/5/82	25.84
M.T.	22/7/82	30.93
	29/7/82	40.48
D.S.	9/11/82	21.53
	15/11/82	20.67
	22/11/82	21.53

For each individual the date of sampling is shown accompanied by the corresponding PGAM activity. Each enzyme value represents the mean of three determinations.

Table 3f

Cellogel electrophoresis of iodinated phosphoglycerate mutase.

<u>Tube</u>	<u>Counts per minute</u>
1 (origin)	13,119
2	121,027
3	24,971
4	6,581
5	5,152
6	1,300
7	124
8	35
9	43
10	654
11	542

Cellogel electrophoresis of iodinated protein was carried out at pH 9.6 for 30 minutes at 100 volts in tris/EDTA/barbitone buffer. The track was cut into 1cm strips and radioactivity counted.

Table 3g

Adsorption of phosphoglycerate mutase to Cibacron-blue

<u>Time</u> <u>(min.)</u>	<u>PGAM</u> <u>(U⁻¹)</u>
15	1574
30	984
45	1008
60	910
120	990

Aliquots containing PGAM activity were mixed with cibacron-blue media. At time intervals the mixture was centrifuged and enzyme activity remaining in supernatant assayed.

Table 3h

Desorption of phosphoglycerate mutase from cibacron-blue.

<u>Time (min)</u>	<u>PGAM₁ (U l⁻¹)</u>
0	262
15	300
30	434
45	758
60	896
120	1000
180	900

Desorption of PGAM from cibacron-blue was facilitated by the addition of 2,3 diphosphoglycerate. At time intervals the mixture was centrifuged and enzyme activity eluted into supernatant measured.

Figure 3I

Creatine kinase and phosphoglycerate mutase activities for women 'at risk' carriers for DMD.

Creatine kinase (CK) and phosphoglycerate mutase (PGAM) data from Table 3d are shown plotted for each individual 'at risk' carrier for DMD. MJS, a 'known' carrier is indicated thus $\square$. Other individuals with a calculated carrier risk probability of 100% are shown thus $\circ$ and those with less than 100% are shown by $\bullet$. The upper limits of normal (95th percentile of normal) for both enzymes is indicated. The value for CK is 200 U l^{-1} (from Heath et. al 1982) and for PGAM 93 U l^{-1} .

Figure 3I

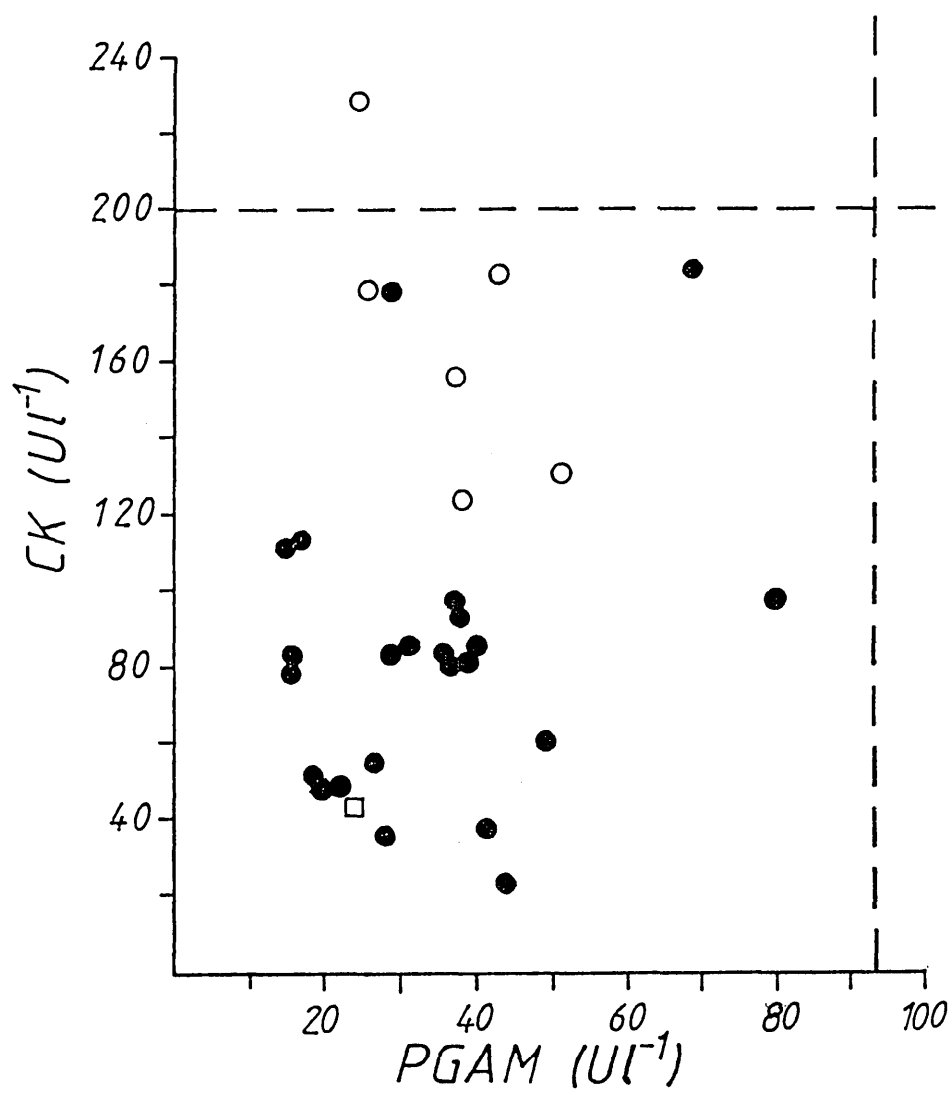


Figure 3II

Carbonic anhydrase III and Phosphoglycerate mutase activities for women 'at risk' carriers for DMD.

Carbonic anhydrase III (CaIII) and phosphoglycerate mutase (PGAM) data from Table 3d are treated as described for Figure 3I. The upper limit of normal (95th percentile of normal) for CaIII is 79 ng.ml^{-1} (from Heath et. al 1982) and for PGAM is 93 U.l^{-1} .

Figure 3II

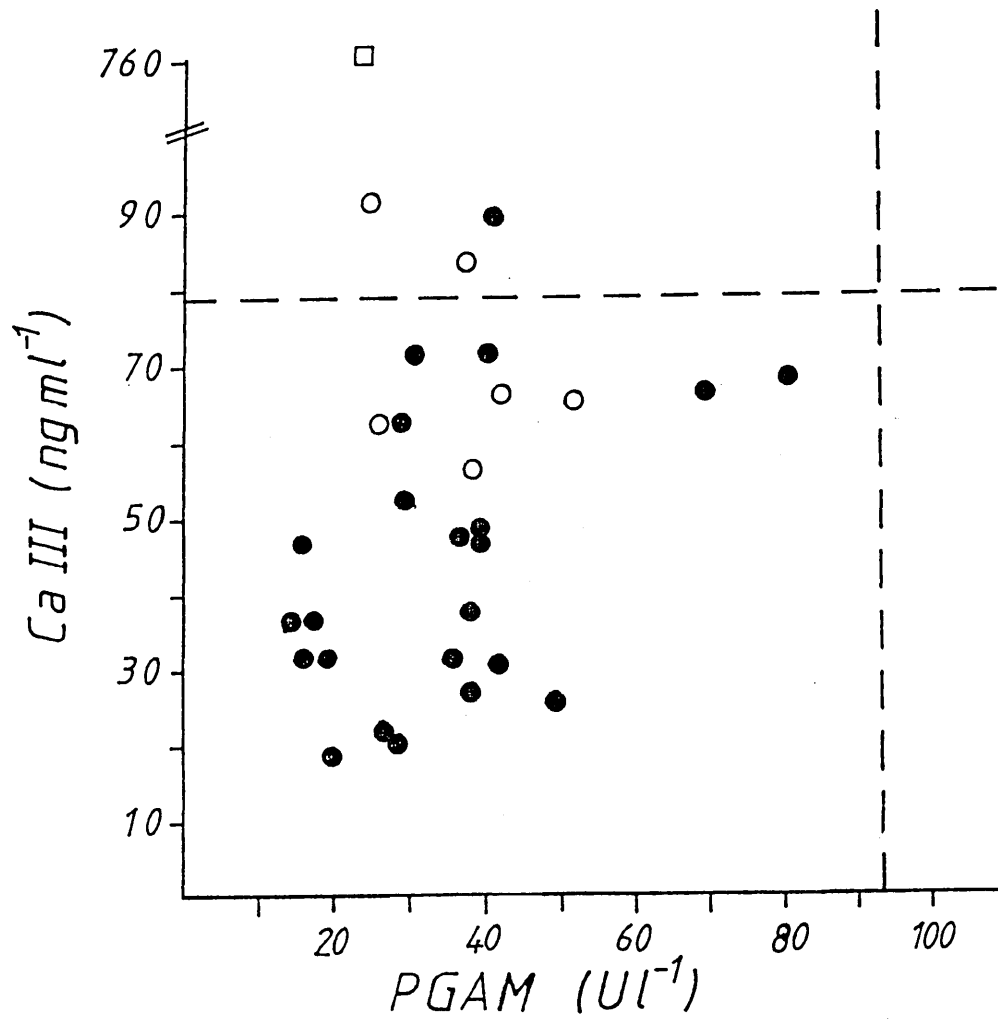


Figure 3III

Carbonic anhydrase III and Creatine kinase activities for women 'at risk' carriers for DMD.

Carbonic anhydrase III (CaIII) and creatine kinase (CK) data from Table 3d are treated as described for Figure 3I. The upper limit of normal (95th percentile of normal) for CaIII is 79 ngml^{-1} (from Heath et. al, 1982) and for PGAM is 93 U l^{-1} .

Figure 3III

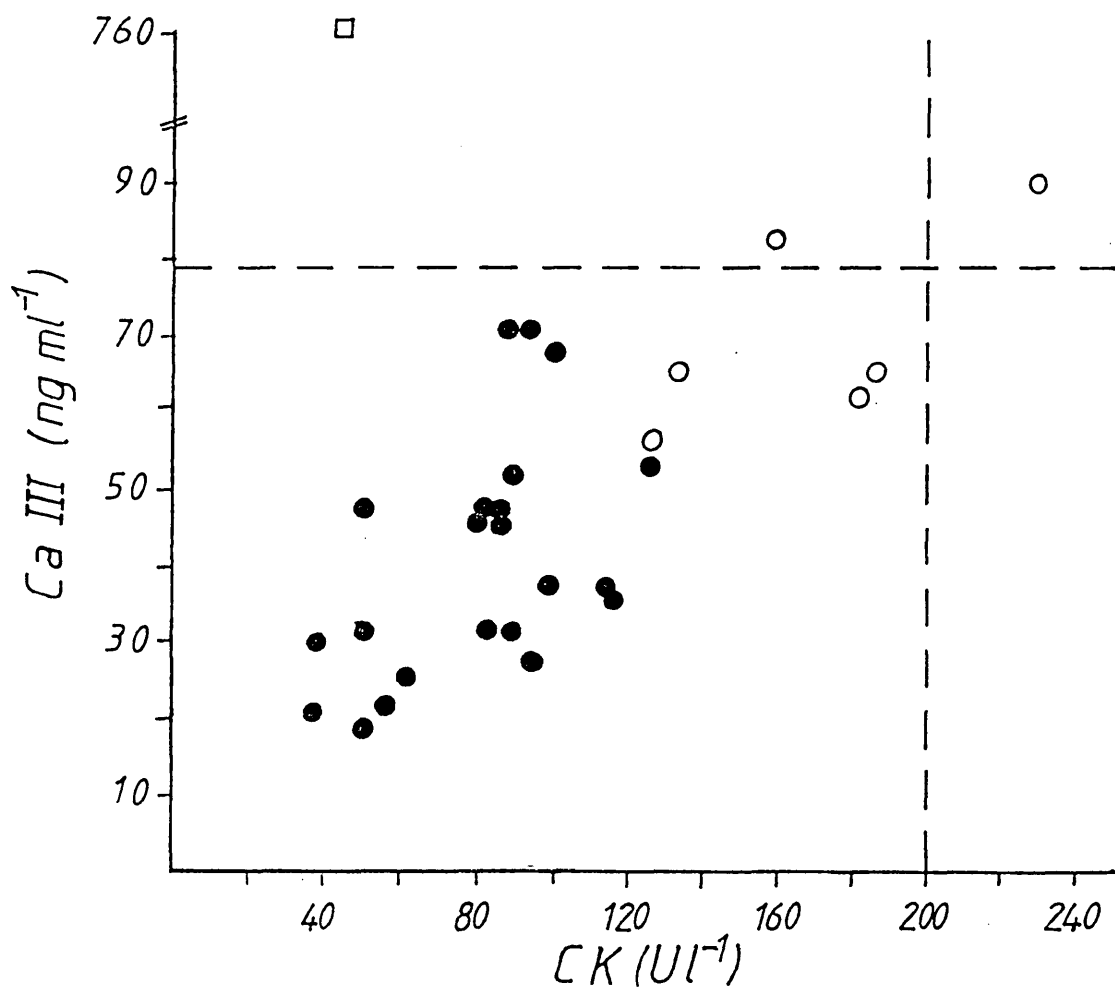


Figure 3IV

SDS-polyacrylamide gel electrophoresis of rabbit and human PGAM.

SDS-PAGE analysis was carried out on rabbit muscle PGAM and extracts of human muscle containing PGAM activity. Samples (5mg protein) in 62 mM Tris HCl, pH 6.8, containing 2% SDS, 5% mercaptoethanol, glycerol and bromophenol blue were heated to 95°C for 15 minutes prior to loading into wells. Electrophoresis was undertaken at 25 mA constant current (8-9 volts. cm⁻¹). Tracks contained:-

1. Rabbit muscle PGAM.
2. Purified human muscle PGAM.
3. Ammonium sulphate precipitate of human muscle PGAM.
4. Initial homogenate of human muscle PGAM.
5. Standards; Bovine serum albumin (67k), catalase (60k) ovalbumin (43k) and cytochrome C (12.4k).

Figure 3IV

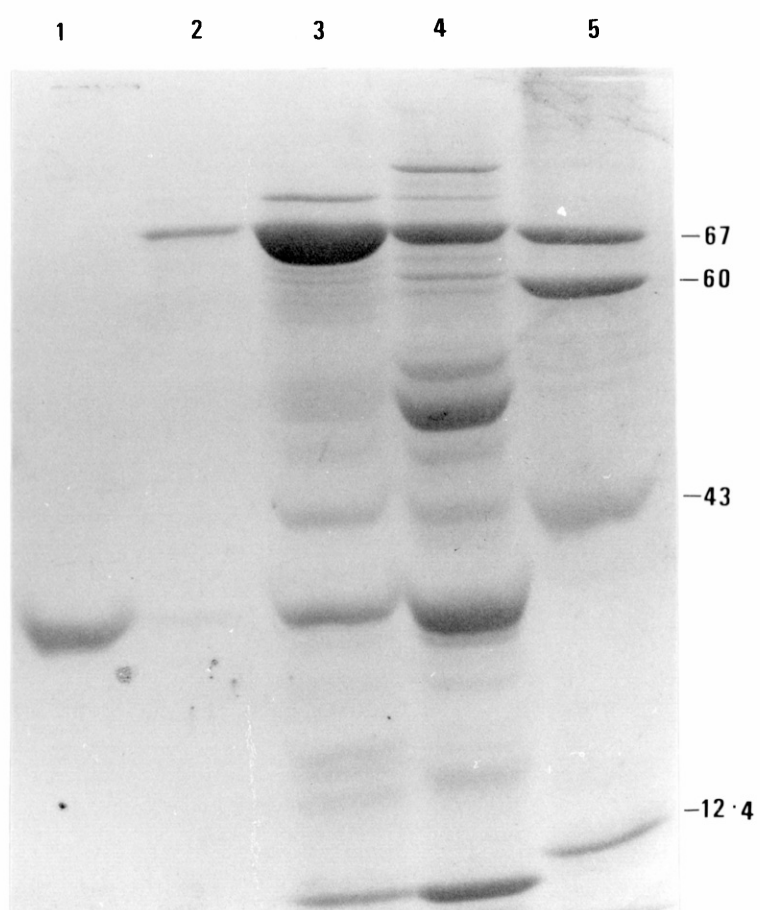


Figure 3V

Ochterlony double diffusion analysis of guinea pig anti PGAM serum.

Antiserum obtained from two guinea pigs inoculated with pure rabbit muscle PGAM. was analysed by ochterlony double diffusion techniques. The wells contained 5 μ l each of:

1. Guinea pig anti PGAM antiserum from guinea pig 1.
2. Normal serum from guinea pig 1.
3. Guinea pig anti PGAM antiserum from guinea pig 2.
4. Normal serum from guinea pig 2.
5. Rabbit muscle PGAM.

Figure 3V

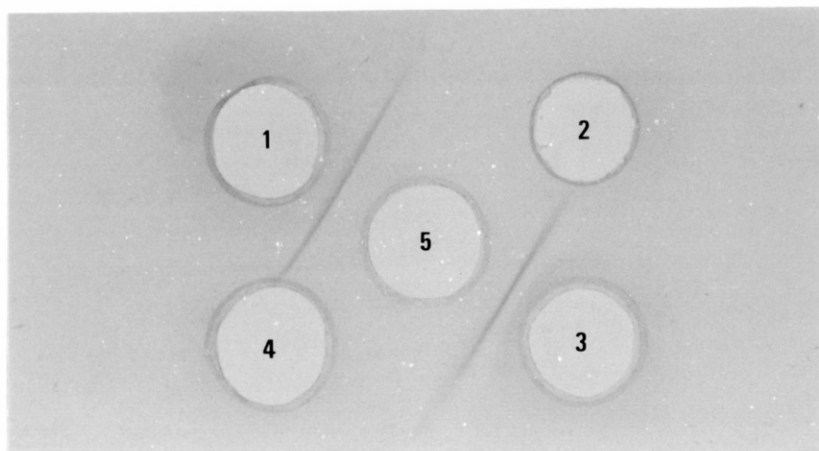


Figure 3VI

Ochterlony double diffusion analysis of antiserum from guinea pig 2.

Anti PGAM antiserum from guinea pig 2 assessed against rabbit muscle PGAM and homogenate from human feotal muscle. The wells contained 5 μ l each of:-

1. Rabbit muscle PGAM (neat).
2. Rabbit muscle PGAM (2 x dilution).
3. Human feotal muscle homogenate.
4. Anti-PGAM antiserum from guinea pig 2 (neat).

Figure 3VI

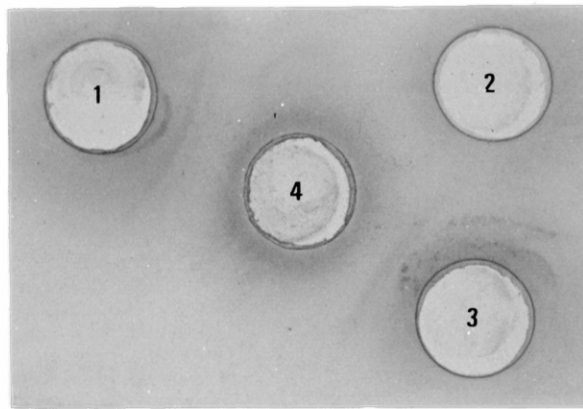


Figure 3VII

Column elution profiles for iodinated phosphoglycerate mutase.

Elution profiles for ^{125}I -PGAM from G-75 columns after iodination by Bolton-Hunter method. The differing iodination conditions are described as follows.

- A. 10 μg PGAM was incubated at 22°C with 500 μCi Bolton-Hunter reagent for 1 hour. Fractions (1ml) eluted from the column were collected and radioactivity levels counted. There was 45% incorporation of ^{125}I into PGAM.
- B. 10 μg PGAM was incubated at 4°C with 125 μCi Bolton-Hunter reagent for 1 hour. Fractions (2ml) eluted from the column were collected and radioactivity levels counted. There was 34% incorporation of ^{125}I into PGAM.

In both instances the first peak is the enzyme.

Figure 3VII

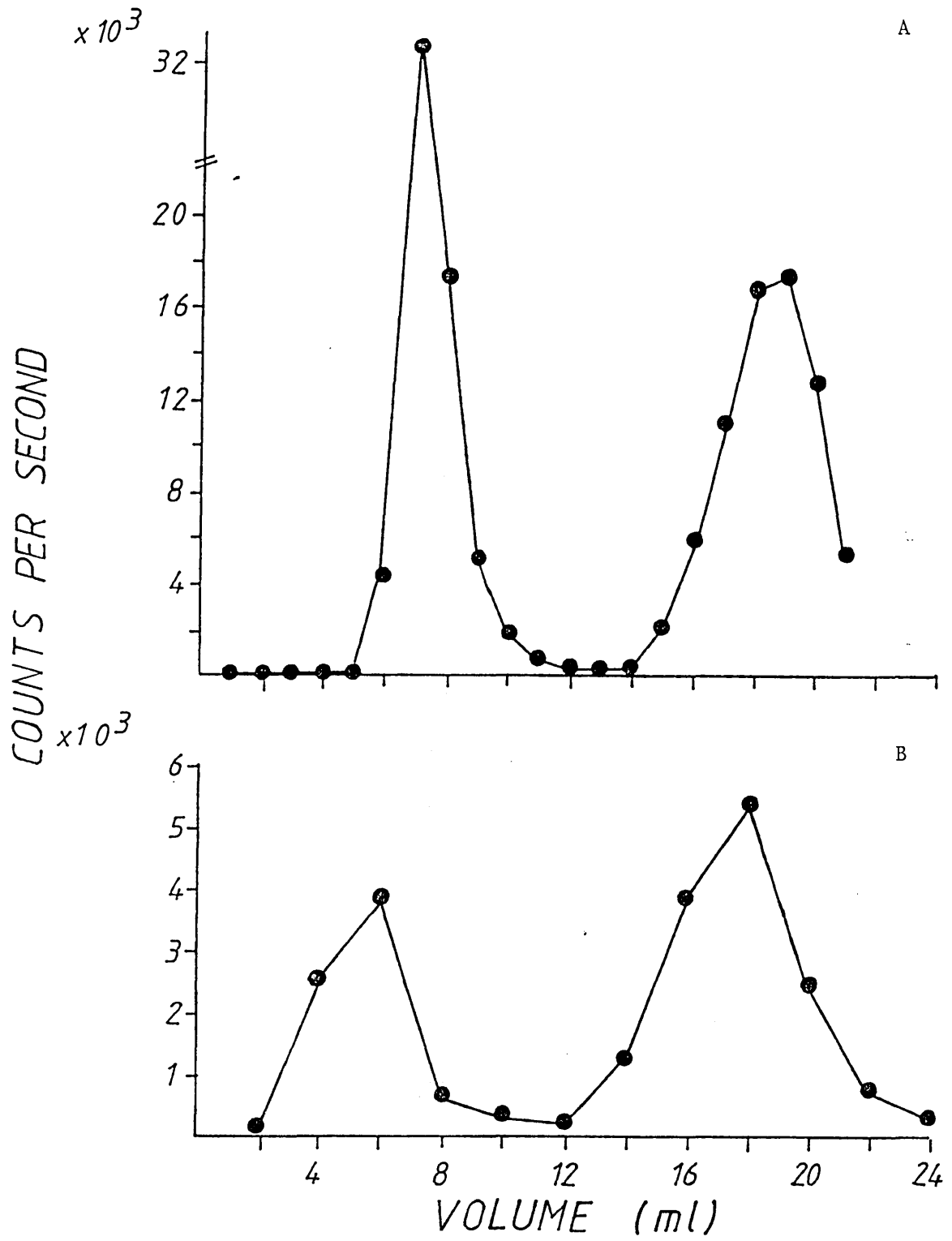


Figure 3VIII

Column elution profile for iodinated phosphoglycerate mutase.

Elution profile for ^{125}I -PGAM from G-75 column after iodination by chloramine-T method. 25 μg PGAM was incubated at 22°C with 500 μCi ^{125}I in sodium phosphate buffer (pH 7.5) containing 10 μl chloramine-T ($2\text{mg}\cdot\text{ml}^{-1}$). Fractions (2ml) eluted from the column were collected and radioactivity levels counted. There was 60% incorporation of ^{125}I into PGAM.

Figure 3VIII

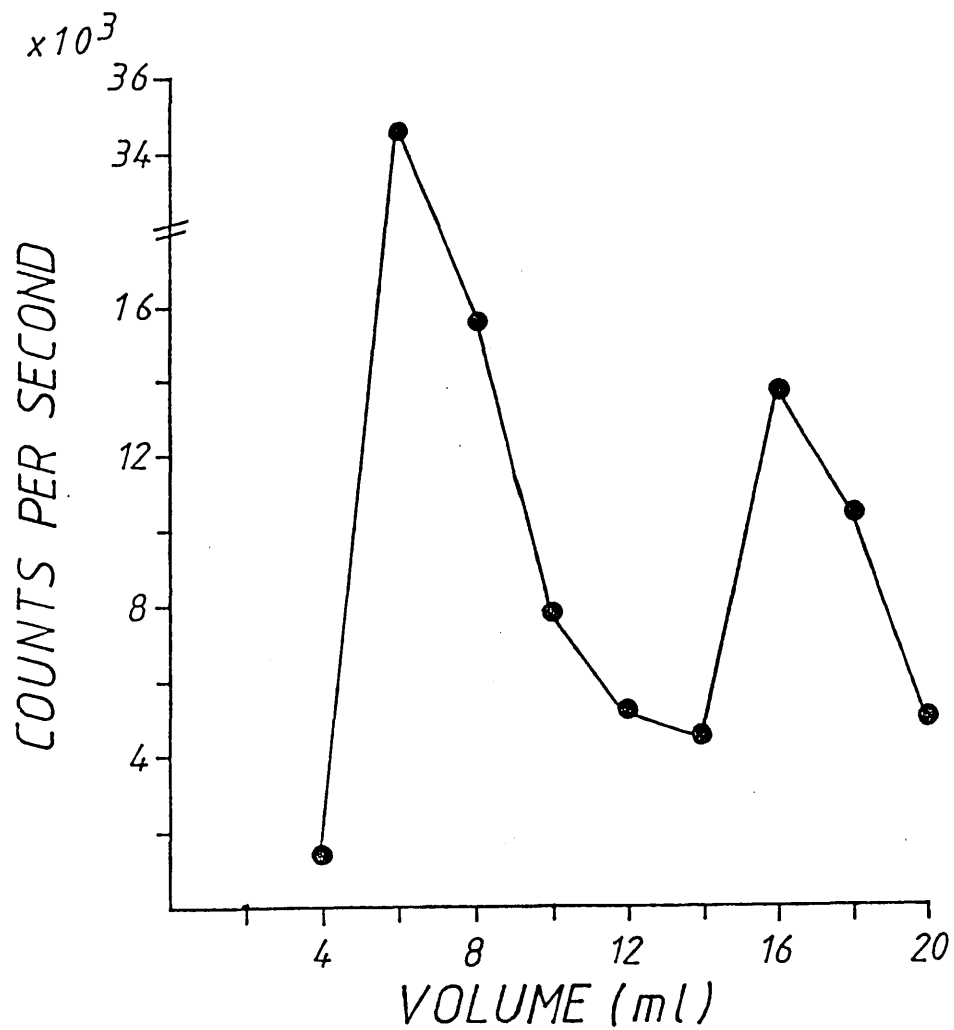


Figure 3IX

Standard curve for rabbit ^{125}I -PGAM.

A standard curve for iodinated PGAM is shown as $B/B_0\%$ against PGAM concentration in ng.ml^{-1} .
Where B_0 is the amount of ^{125}I -PGAM bound by 1/100 dilution of antibody and B is the amount of ^{125}I bound at each standard concentration.

Figure 3IX

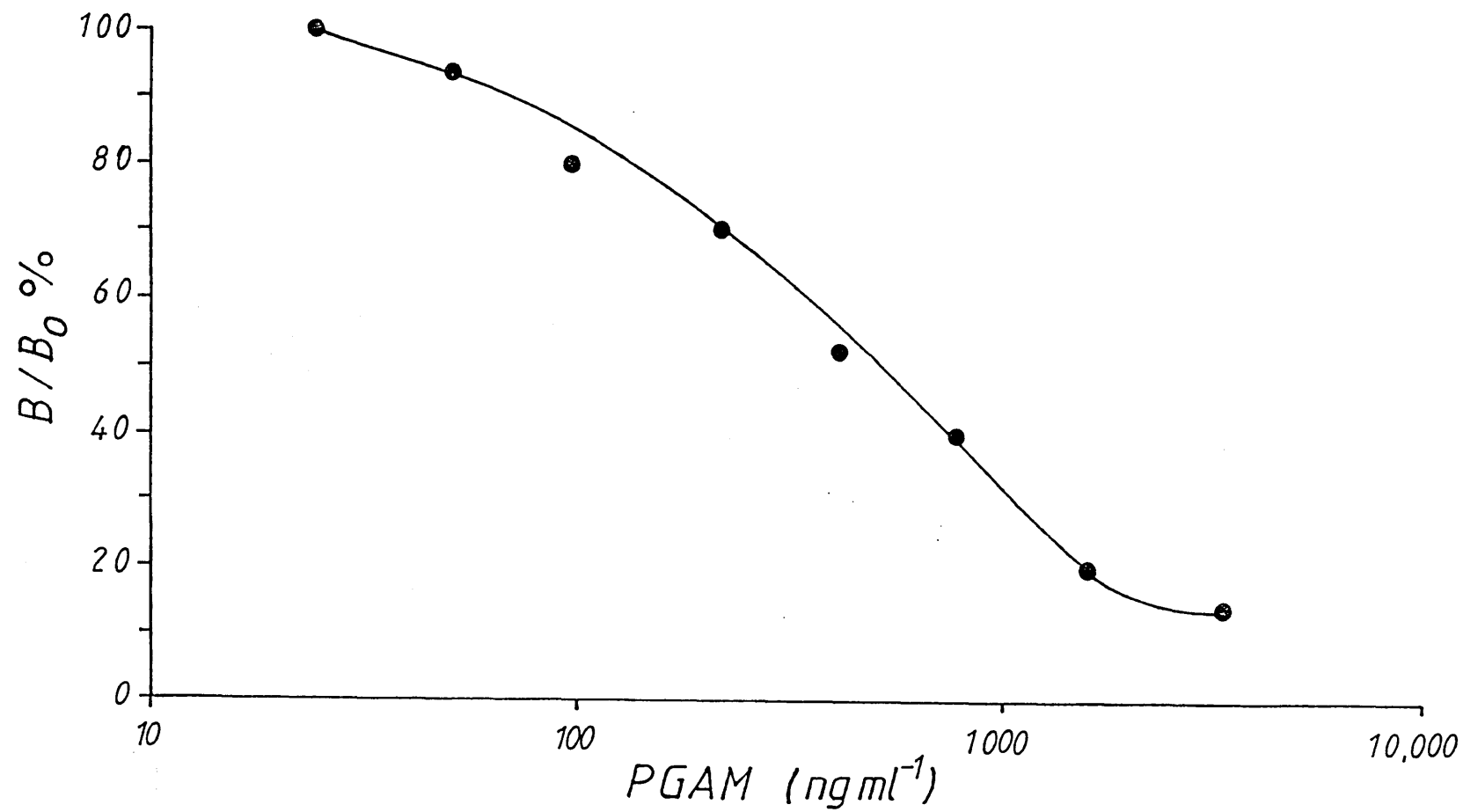


Figure 3X

The relationship of ammonium sulphate saturation to protein concentration and phosphoglycerate mutase activity.

The supernatant protein concentrations (■) and PGAM activities (●) are shown for homogenate containing a range of ammonium sulphate saturations (0-100%).

Figure 3X

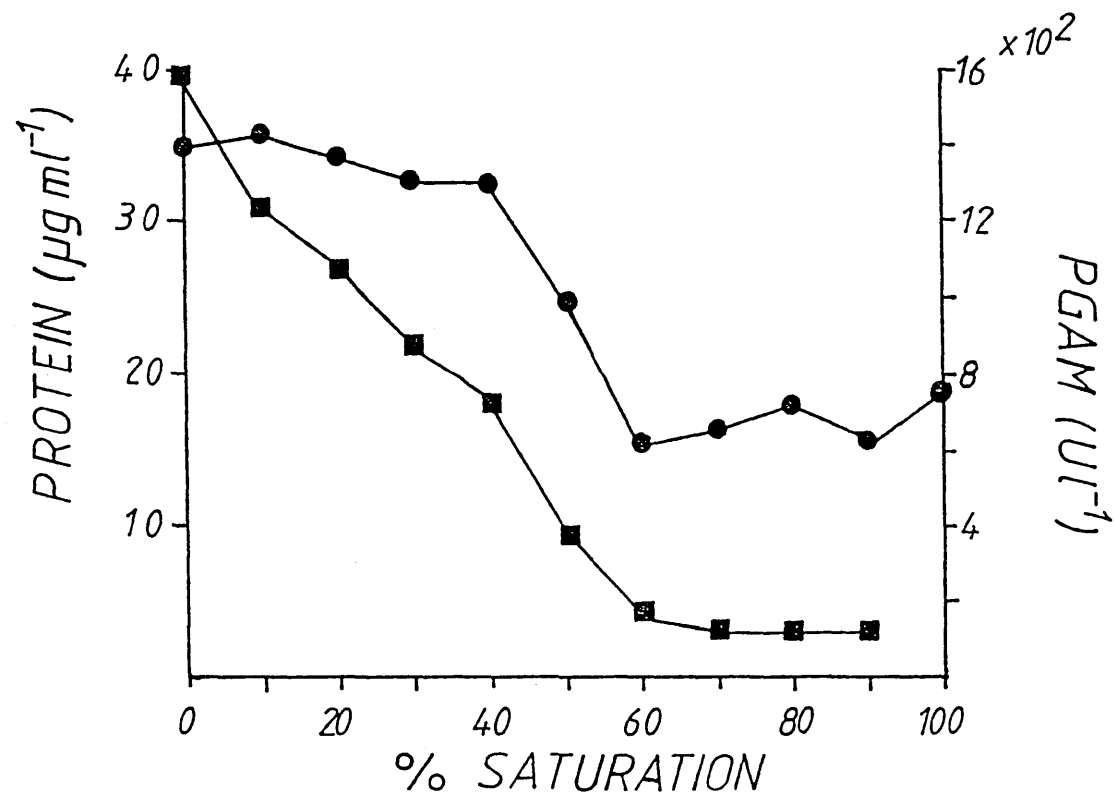


Figure 3XI

SDS-polyacrylamide gel electrophoresis of chicken pectoral muscle phosphoglycerate mutase.

SDS-PAGE analysis was carried out on chicken pectoral muscle phosphoglycerate mutase from three stages of batch purification. Details of pre electrophoresis and electrophoretic procedures are as described for Figure 3IV.

1. Standards; Bovine serum albumin (67k), catalase (60k), ovalbumin (43k), α chymotrypsin (24k) and cytochrome C (12.4k).
2. Crude homogenate of chicken pectoral muscle.
3. Final precipitate from ammonium sulphate fractionation.
4. PGAM containing fraction eluted from cibracron blue procedure.

Figure 3XI

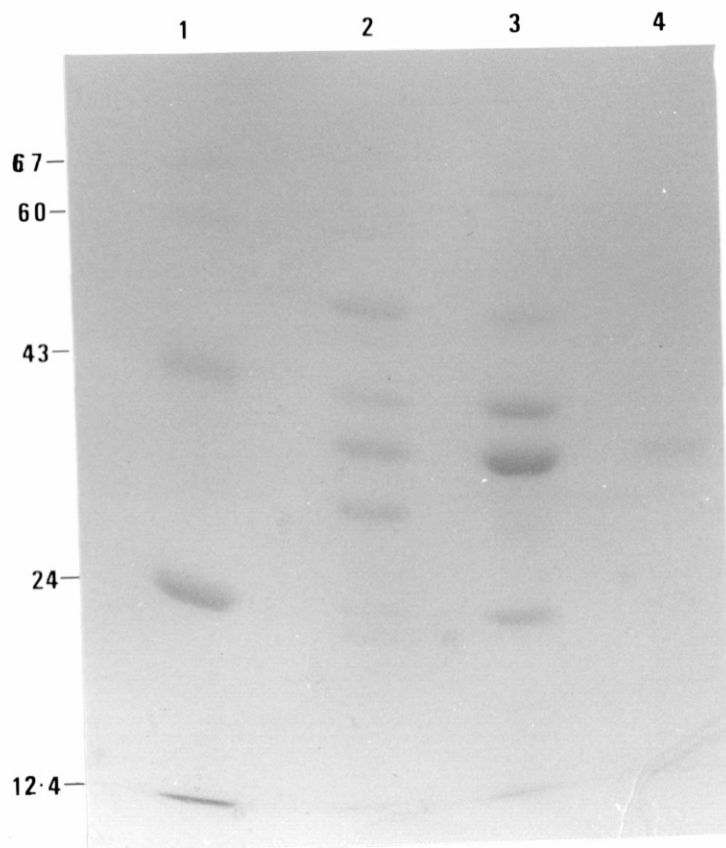


Figure 3XII

Protein elution profile of dialysed rabbit muscle PGAM from cibacron blue column.

Absorbance changes at 280 nm were monitored for fractions collected from cibacron blue column.

All fractions were 3 ml except numbers 18 to 30 which were 1 ml. Application of sample (1mg PGAM),

5mM 2,3-diphosphoglycerate (2,3 DGP), 1M sodium chloride and buffer to top of column are indicated.

Figure 3XII

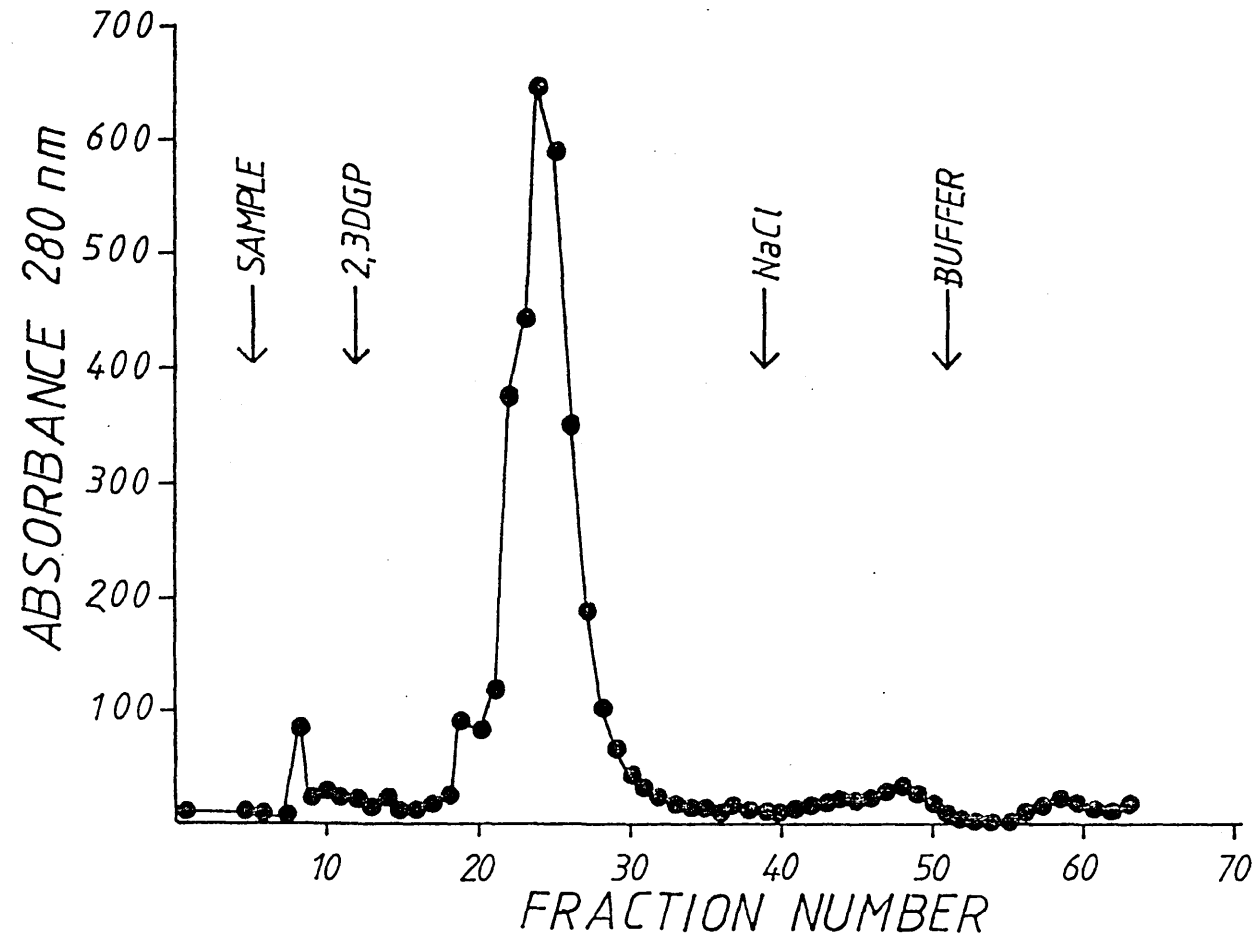


Figure 3XIII

Protein elution profile of rabbit muscle PGAM (in ammonium sulphate) from cibacron blue column.

Absorbance changes at 280 nm were monitored for fractions collected from cibacron blue column. All fractions were 3 ml volumes. Application to column of 2,3 diphosphoglycerate (2,3 DGP) is indicated.

Figure 3XIII

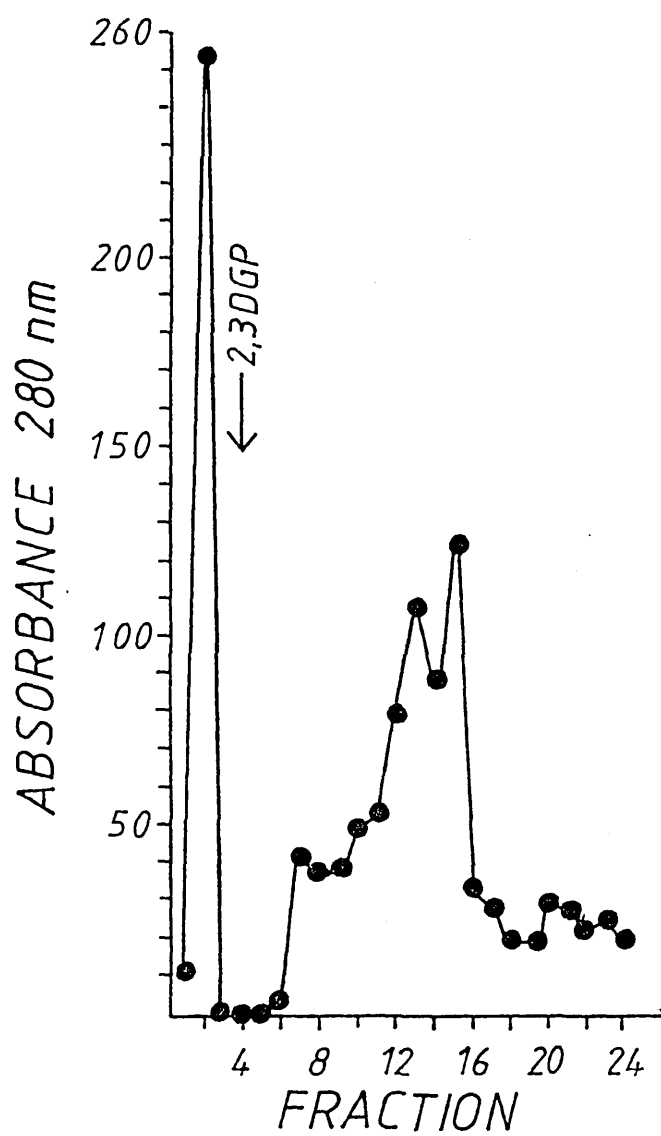


Figure 3XIV

Elution profile for chicken pectoral muscle PGAM from a cibacron blue column.

Purified chicken pectoral PGAM was applied to a cibacron blue column and eluted fractions were monitored for changes in absorbance at 280 nm (●) and after specific elution with 2,3 diphosphoglycerate (2,3DGP), PGAM activity was measured (■). All fractions collected were 1ml.

Figure 3XIV

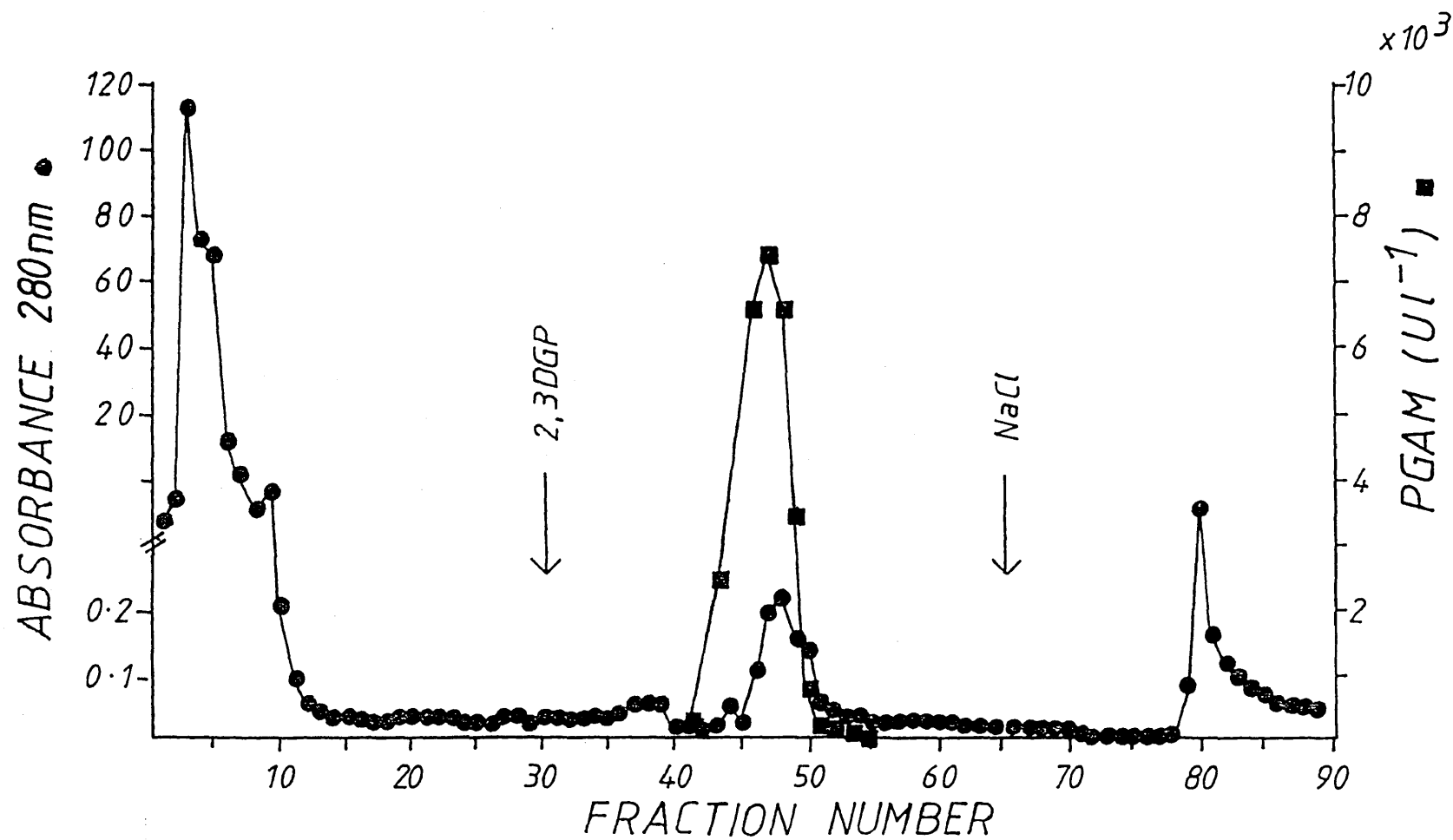


Figure 3XV

SDS-polyacrylamide gel electrophoresis of human (foetal and adult) muscle phosphoglycerate mutase.

SDS-PAGE analysis was carried out on partially purified foetal and adult muscle PGAM. Details of preparation for and electrophoretic procedures are as described for Figure 3IV.

Tracks contained:-

1. Human foetal muscle : PGAM containing fraction from cibacron blue column separation.
2. Human adult muscle : PGAM containing fraction from cibacron blue column separation.
3. Rabbit muscle PGAM (dialysed).

Figure 3XV

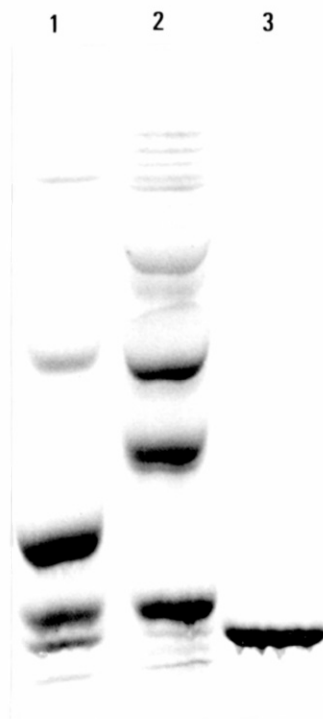


Figure 3XVI

SDS-polyacrylamide gel electrophoresis of adult muscle phosphoglycerate mutase after application to cibacron-blue columns.

SDS-PAGE was carried out on adult human muscle PGAM containing fractions after one and two passes on cibacron blue columns. Details of preparation for and electrophoretic procedures are as described for Figure 3IV. Tracks contained:-

1. Standards; Bovine serum albumin (67k), Catalase (60k), ovalbumin (43k), Lactate dehydrogenase (36k), cytochrome c (12.4k).
2. 2nd Pass; PGAM containing fraction eluted from second cibacron blue column.
3. 1st Pass; PGAM containing fraction eluted from first cibacron blue column.
4. Rabbit muscle PGAM (dialysed).

Figure 3XVI

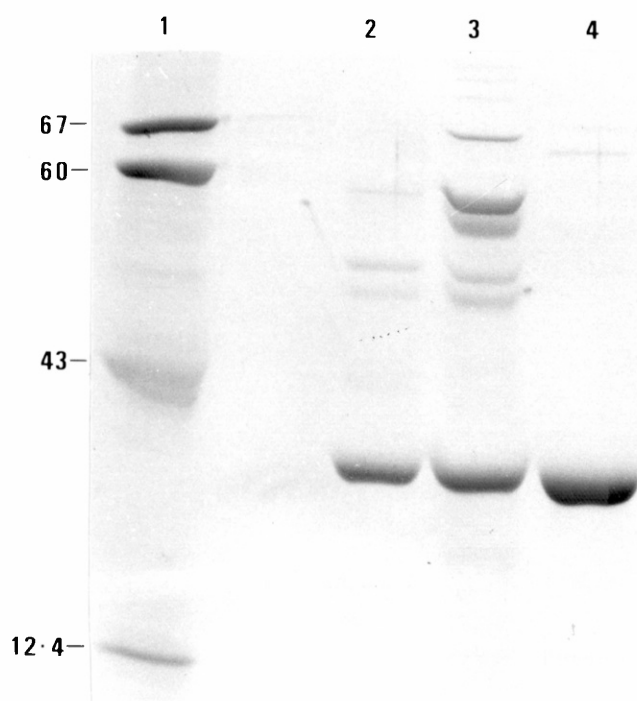


Figure 3XVII

Comparison of pH gradient measured by surface electrode and elution from gel slices.

The pH gradient generated in an isoelectric focusing gel is shown in A by surface electrode and in B after elution of gel slices in distilled deionised water.

Figure 3XVII

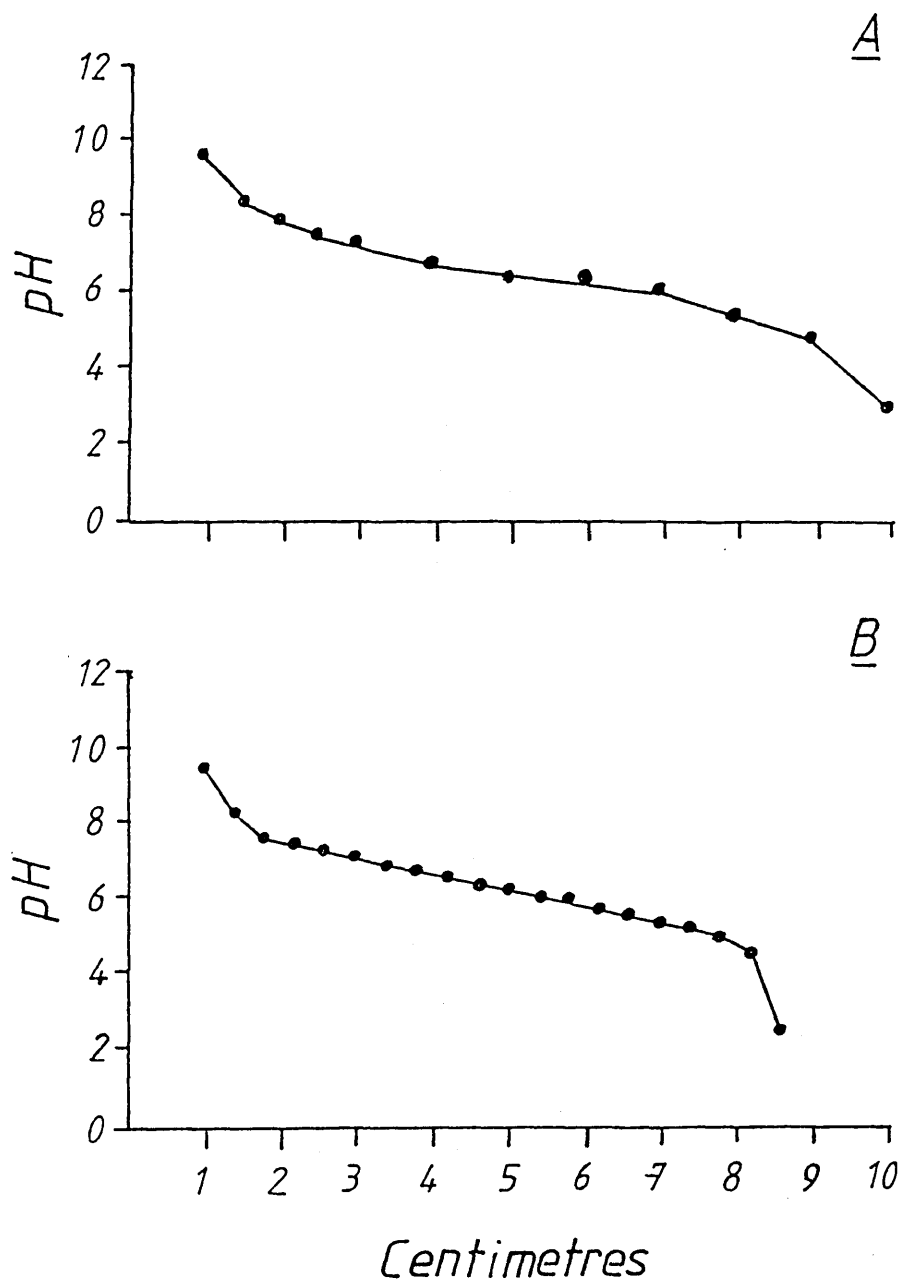


Figure 3XVIII

The isoelectric banding pattern for rabbit muscle phosphoglycerate mutase.

Dialysed rabbit muscle PGAM was applied to a pH 3-10 range isoelectric focusing gel and isoelectric focusing carried out at constant power and 1.5k volts. The focusing bed was kept at constant temperature (1°C). On completion the gel track was sliced into 0.2cm segments and placed in distilled deionised water overnight. The upper figure demonstrates the pH measured from the eluant (●). The lower figure indicates the peaks of PGAM activity measured in the gel eluant (○).

Figure 3XVIII

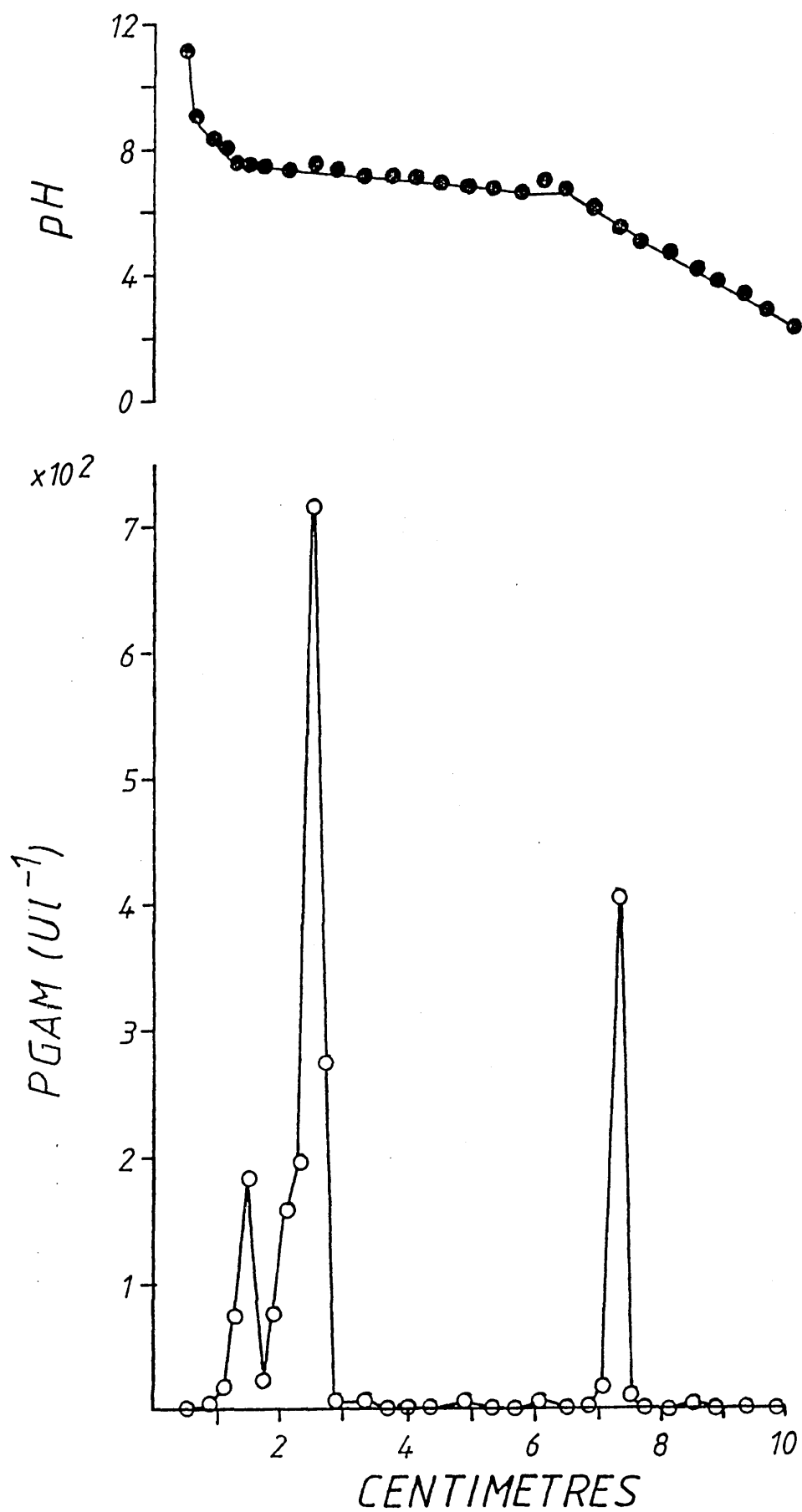


Figure 3XIX

The isoelectric banding pattern for mouse pectoral muscle and brain phosphoglycerate mutase.

A. Mouse brain homogenate was applied to a pH3-10 range isoelectric focusing (IEF) gel.

Focusing was carried out at constant power and 1.5k volts. The gel support was kept at constant temperature (1°C). On completion the gel track was sliced into 0.4 cm segments and placed in distilled deionised water overnight. The upper figure demonstrates the pH measured (●) in the gel eluant and the lower figure shows PGAM activity measured in the gel eluant (○).

B. Mouse pectoral muscle homogenate was applied to the pH3-10 range IEF gel and treated as described for Figure 3XIX A.

Figure 3XIX.

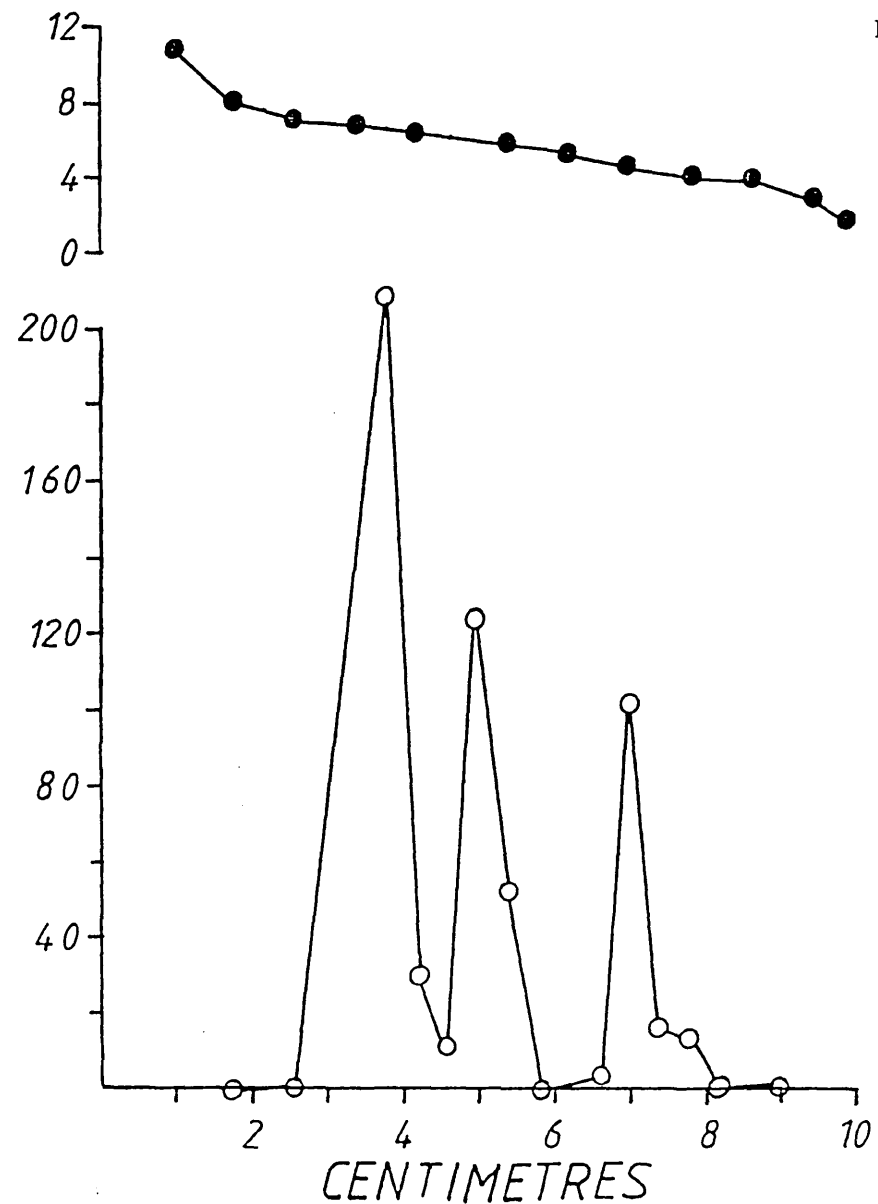
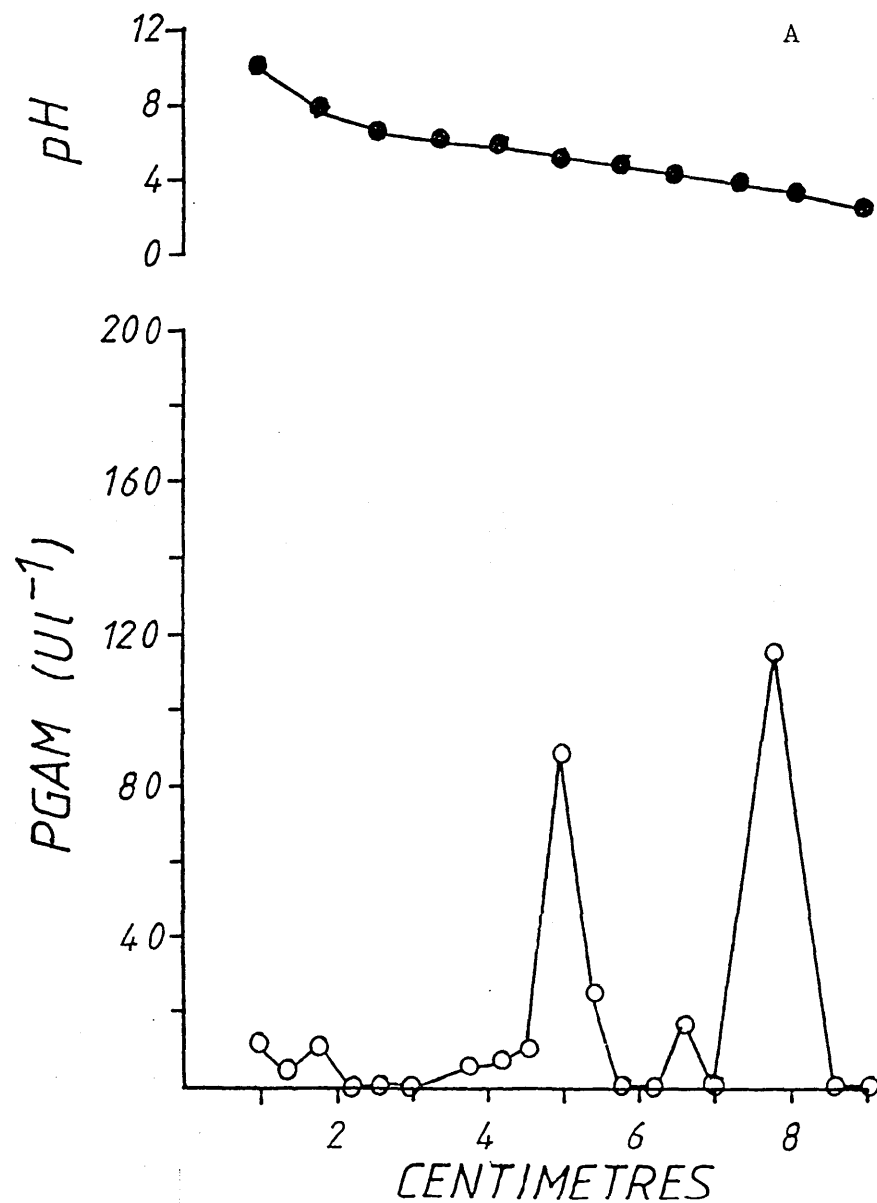


Figure 3XX

The isoelectric banding pattern for partially purified adult muscle phosphoglycerate mutase.

Partially purified adult human muscle homogenate was applied to a pH 3-10 range isoelectric focusing (IEF) gel. Focusing was carried out at constant power and 1.5k volts. The gel support was kept at constant temperature (1°C). On completion the gel track was sliced into 0.2cm segments and placed in distilled deionised water overnight. The upper figure demonstrates the pH measured (●) in the gel eluant and the lower figure shows PGAM activity measured in the gel eluant (○). The inserted photograph demonstrates a duplicate track fixed and stained for protein content.

Figure 3XX

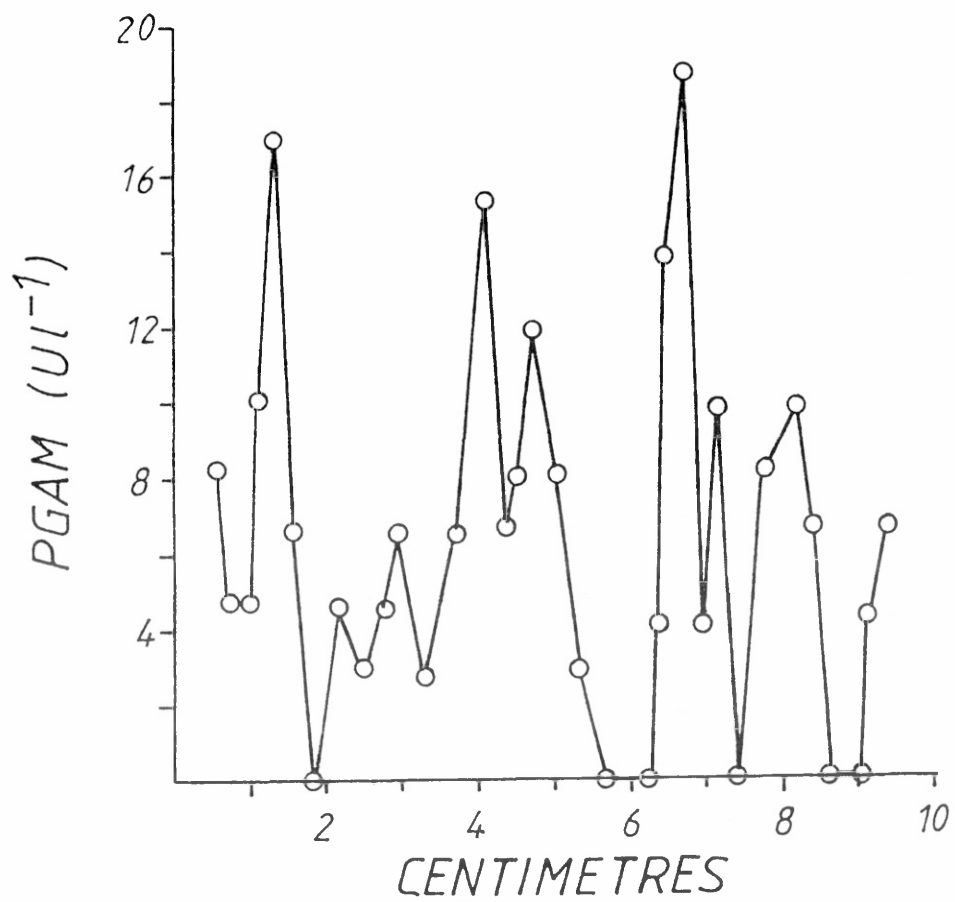


Figure 3XXI

The isoelectric banding pattern for partially purified foetal muscle phosphoglycerate mutase.

Partially purified foetal human muscle was applied to a pH 3-10 range isoelectric focusing gel. Focusing was carried out and results treated as described for Figure 3XX.

Figure 3XXI

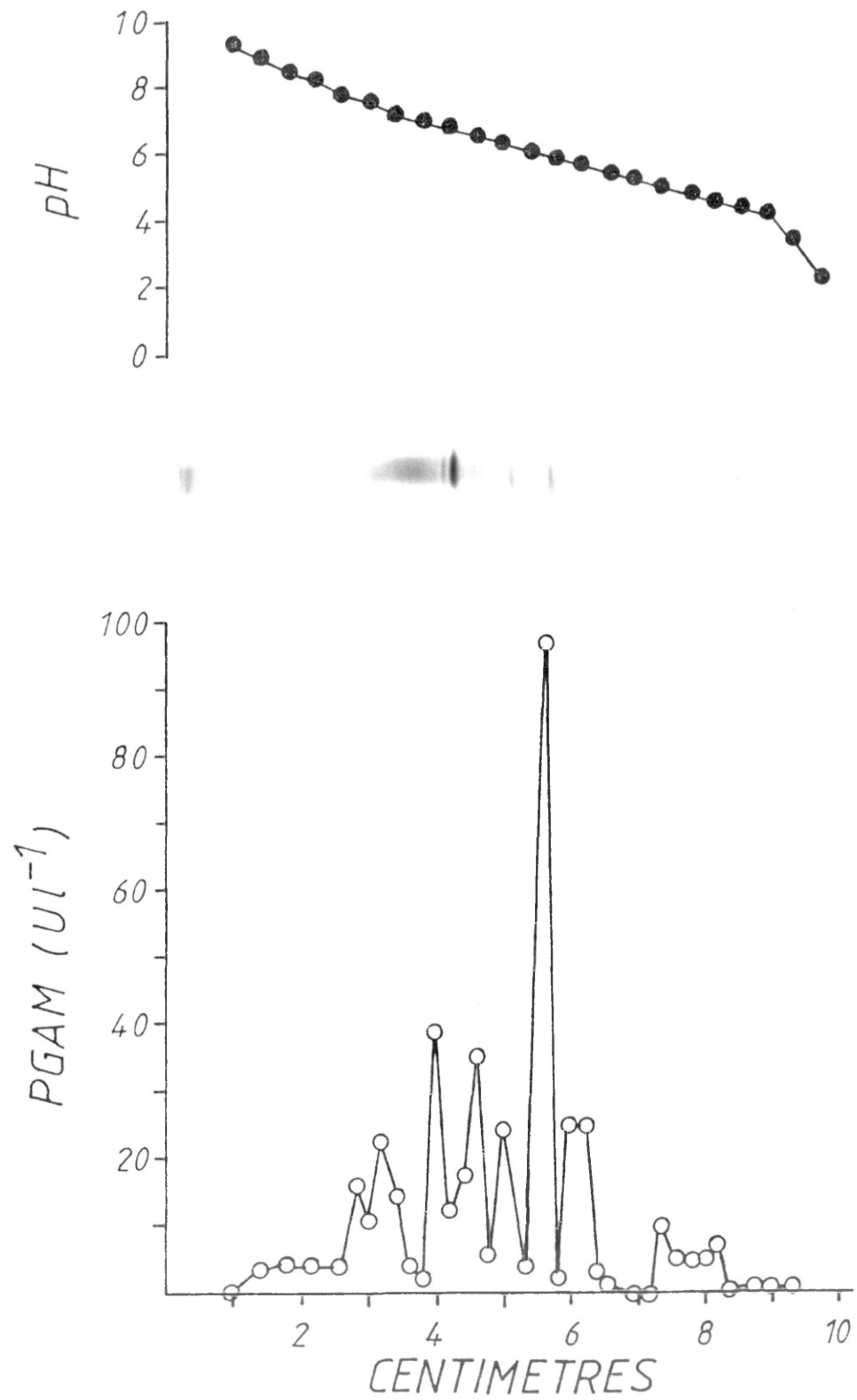


Figure 3XXII

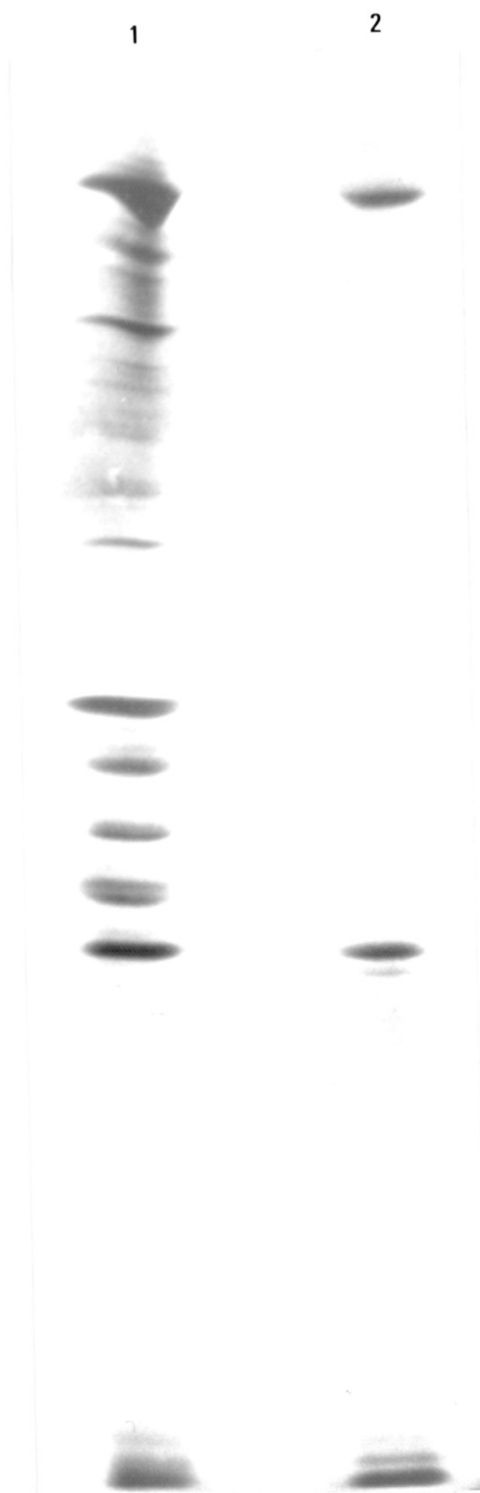
The isoelectric banding pattern for adult muscle phosphoglycerate mutase.

The PGAM containing fractions eluted after first and second 'passes' of cibacron blue columns were applied to a pH 3-10 range isoelectric focusing gel. IEF was carried out as described for Figure 3XVIII. On completion, the pH forming peptides were eluted, the gel fixed and then strained for protein contented.

Track 1 : PGAM containing fraction resulting from one pass
of a cibacron blue column.

2 : PGAM containing fraction resulting from a second
pass of a cibacron blue column.

Figure 3XXII



SECTION FOUR

MURINE (mdx) DYSTROPHY

INTRODUCTION

Animal models have played an integral role in the study and treatment of disease, especially so in the instance of Duchenne muscular dystrophy (DMD) where the primary myopathy remains unknown. Although no animal model could exactly mirror the human disease, the degree of similarity may often facilitate an understanding of causal elements and potential therapeutics.

Several animal models of DMD have been identified; mouse, chicken, hamster, mink, turkey, dog, sheep, duck and cow (Harris and Slater, 1979). Due to practicalities of husbandry, the majority of research has been conducted with the mouse, hamster and chicken models. The dy/dy and dy^{2j}/dy^{2j} strains of mice, the syrian hamster (BIO 14.6) and dystrophic chicken show many similarities with DMD (Wrogemann et. al, 1979; Michelson et. al, 1955; Wilson et. al, 1979). The two mouse strains (dy/dy and dy^{2j}) differ in the extent of expressed dystrophic symptoms. Of the two strains, the expressed dystrophic symptoms in the dy/dy line are more severe than those apparent in dy^{2j}/dy^{2j} . Features common to these mice and DMD include calcium leakage from muscle, sarcoplasmic reticulum alterations, variation in fibre size, necrosis of muscle tissue, proliferation of connective tissue and abnormalities of muscle enzymes (Mendell et. al, 1979; McCaman 1963). In contrast to the pseudohypertrophy of DMD these models demonstrate a 'true' hypertrophy and the extent of calcium leakage is less pronounced. Regeneration observed at the site of necrosis in DMD, occurs at some distance from the necrotic foci in the dystrophic mouse where other tissues are additionally affected (Bradley and Jaros, 1979; Cullen and Mastaglia, 1980; Summers and Parsons, 1978). Finally, further research has demonstrated defects in myelination and nerve transmission which exclude these strains of mice from being designated a true

myopathy (Strickland et. al, 1979).

In the instance of the hamster, which exhibits an autosomal recessive trait, the initial fibre breakdown was similar to that observed for DMD (Mendell et. al, 1979). Features in common with DMD include calcium deposition in muscle, variations in fibre size, fibre splitting, hyalinisation and necrosis. There is however, an absence of connective tissue infiltration and all the above defects are subsidiary to a cardiomyopathy (Homburger, 1979). There are several avian models of DMD and of the New Hampshire lines available, the line 413 dystrophic chicken and line 412 normal have provided an excellent system with which to investigate and treat the disease (Barnard and Barnard, 1979; Entrikin et. al, 1981; Cosmos and Butler, 1980). The similarities with DMD include early onset of symptoms, hypertrophy, fibre splitting, necrosis and fatty infiltration of muscle tissue (Wilson et. al, 1979; Barnard, 1980). The elevation of plasma enzymes, also similar to those observed in DMD, and the ease with which samples may be obtained from the chicken have made this a suitable model for use in both fundamental research and drug monitoring (Barnard and Barnard, 1978; Entrikin et. al, 1981; Cosmos and Butler, 1980). Here also the greatest difference between the model and DMD is the mode of inheritance. In the chicken it is autosomal, and both male and female birds express dystrophic symptoms (Barnard, 1980). It is clear that a mammalian model for DMD, expressing similar symptoms to avian dystrophy accompanied by X-chromosome linkage, would provide a more appropriate system with which to extend the current understanding of the primary myopathy in DMD.

In 1984, Bulfield and colleagues described of a novel mouse mutation, designated mdx, for which preliminary research data indicated a possible role as a 'true' mammalian model for DMD. The mdx mutation was isolated in C57BL/10ScSn mice during a screening program designed to select for enzyme mutants in the glycolytic pathway (Bulfield, 1981). The mutation resulted in a three fold increase in plasma PK activity over normal levels. Further research demonstrated the mutation to be X-linked and located in the Hp Bqa region of the mouse X chromosome in a similar position to equivalent genes of the human chromosome. The gene defect was accompanied by necrosis of muscle tissues (Bulfield et. al, 1984). In the light of these observations the mdx mouse strain appeared suitable for analysis and consideration as a mammalian model of DMD.

Analysis of this novel mutant was approached by measurement of plasma and muscle enzymes known to be abnormal in Duchenne and avian dystrophy. Confirmatory histological analysis was performed by J. Jarvis. The results of analysis are assessed by taking into account current knowledge of these parameters in dystrophy. The plasma enzymes measured were creatine kinase (CK), pyruvate kinase (PK), isocitrate dehydrogenase (ICD) and phosphoglycerate mutase (PGAM). In addition to these enzymes, glucose 6-phosphate dehydrogenase (G6PDH) was also measured in muscle homogenates. The analysis of PGAM and G6PDH was carried out in collaboration with P. Chown. Elevated levels of all these plasma enzymes have been noted in avian dystrophy (Barnard and Barnard, 1979; Liu et. al, 1980; Chown et. al, 1983; Chown et. al, 1984). Abnormalities in plasma of Duchenne patients for CK, PK and PGAM have also been recorded (Zatz et. al, 1980; Chown et. al, 1984). In muscle, reductions of CK, PK and PGAM and increases in ICD and G6PDH have been observed in avian dystrophy (Allan unpublished results; Chown et. al, 1983; Chown

et. al, 1984). Comparison of results with known histological data from avian and human dystrophy were also undertaken, (Dubowitz, 1973; Pizzey and Barnard, 1983).

Overall the aim of this research was to document the pathogenesis of the mdx mutation and to evaluate the novel strain with respect to other muscular dystrophies.

MATERIALS

All chemicals were supplied by Sigma, Poole, Dorset, UK, with the exception of sodium heparin (1000 Uml^{-1}) which was bought from Duncan Flockhart Limited and CK (NAC- activated) and PV (UV method) test kits from BCL Lewes, Sussex, UK.

METHODS

Animals

The mdx mice were a gift from Dr. G. Bulfield, ARC Poultry Research Centre, Roslin, Fife, U.K. They were subsequently bred and maintained in the animal facility at Imperial College, London. The mice strain C57 BL/10 were utilised as normal controls.

SAMPLE PREPARATION

Plasma

Blood was withdrawn from mice by one of two methods dependent upon any additional requirements for experimental muscle tissue. In the first method, allowing subsequent use of muscle tissue heparin (10 units) was injected into the animal and blood was taken by cardiac puncture. Where only blood was required, mice were tail bled into heparinised tubes. In both methods, the blood was centrifuged for 3 minutes in a haematocrit centrifuge and the supernatant plasma fraction retained for assay.

Muscle

The muscles taken for enzyme assay were pectoral and gastrocnemius dissected from anaesthetised animals and immediately weighed on ice. Tissue for homogenisation was chopped at 0°C and added to ice cold 0.05M tris-HCl pH 7.00 (100 mg wet weight/ml buffer) for disruption in a glass:glass hand homogeniser (maximum volume 3ml). The resulting homogenate was centrifuged at 10691xg for 30 minutes at 4°C and the supernatant retained for assay.

ENZYME ASSAYS

Creatine kinase (ec 2.7.3.2)

Plasma and muscle creatine kinase (CK) activity was determined by CK (N-acetyl cysteine activated) method described in detail in Section 1 p.42 of this thesis.

Pyruvate kinase (ec 2.7.1.40)

Plasma and muscle pyruvate kinase (PK) activity was measured using the UV method described in Section 1 pp. 42-43 of this thesis.

Isocitrate dehydrogenase (ec 1.1.1.42)

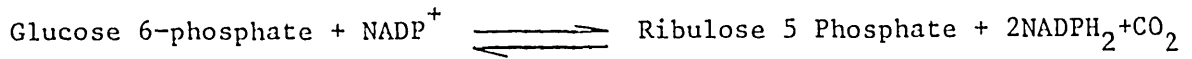
Plasma and extra-mitochondrial isocitrate dehydrogenase (ICD) activities were determined using the method described in Section 2 p. 159 of this thesis.

Phosphoglycerate mutase (ec 2.7.5.3)

The phosphoglycerate mutase (PGAM) determinations were carried out (in conjunction with P. Chown) using the method of DiMauro et. al, (1982) described in Section 3 p. 186 of this thesis.

Glucose 6-phosphate dehydrogenase (ec 1.1.1.49)

The glucose 6-phosphate dehydrogenase (G6PDH) activity of muscle samples were analysed (in collaboration with P. Chown) by a method adapted from Pennington (1963).



Samples (25-50 μl) were added to 0.1 M tris HCl pH 7.6 containing 10 mM mgCl_2 and 0.25 mM glucose 6-phosphate (25 μl) to the buffer mixture. Increase in absorbance at 340 nm resulting from NADPH_2 production was monitored (Chown et. al, 1984).

ENZYME ACTIVITY CALCULATION

Enzyme activities were calculated by methods described in Section 1 p. 43 of this thesis.

BODY AND MUSCLE WEIGHT MEASUREMENTS

Body weight measurements were taken using a Sartorius^R programmable balance. Values stated were the mean of five weighings. Individual muscle weights were determined using a Mettler^R analytical balance.

RESULTS

Plasma

Activity levels of muscle enzymes creatine kinase (CK), pyruvate kinase (PK), phosphoglycerate mutase (PGAM) and isocitrate dehydrogenase (ICD) were measured in plasma from mice over an age range from 3 to 52 weeks (Table 4a).

Creatine Kinase

At all ages measured, except week 7, levels of activity of this enzyme in mdx were significantly greater (student's t-test) than those of the corresponding normal mice. In the normal mice, plasma CK levels increased from a mean value of 3652 UL^{-1} at week 3 to reach a peak of 8107 UL^{-1} at week 24. In the instance of the mdx mice

the initial three fold increase above the corresponding normal mice at week 3 was raised to an eight fold increase at week 6 but subsequently diminished. In all instances the variation within a sampling point was greater for mdx mice than for normals. This difference was especially evident at weeks 6 and 24.

Pyruvate kinase

Significant differences between normal and mdx mouse plasma PK were observed at all ages measured. No general trend of increasing or decreasing activity over the trial period was observed for normal mice, but individual values varied between 746 UL^{-1} (week 3) and 351 UL^{-1} (week 8). Similarly for mdx mice, no general trend was observed but activity levels increased to seven times that of the corresponding normal at week 3 and to a maximum of twenty one times normal levels at week 8. Greater variation within sampling points were observed for the mdx mice than the normals.

Phosphoglycerate mutase

Plasma PGAM levels in mdx mice were significantly greater than those of normal mice at all time points measured. Normal plasma enzyme values were spread over a range from 918 UL^{-1} (week 8) to 2456 UL^{-1} (week 3) but no overall trend of increased or decreased activity over the trial period was observed. Similarly mdx PGAM activities demonstrated no regular pattern and activities ranged from 4334 UL^{-1} (week 52) to $11,729 \text{ UL}^{-1}$ (week 24). The differences between normal and mdx mice were not as great as those observed for PK, in the instance of PGAM the greatest difference observed was a seven fold increase above that of normal at weeks 6 and 8.

Isocitrate dehydrogenase

Activity levels of this enzyme in normal plasma were essentially the same at weeks 3 and 52 but at intermediate time points varied from 14.4 UL^{-1} (week 8) to 28.53 UL^{-1} (week 4). In the instance of mdx mice ICD levels at week 52 were twice that of the week 3 determination, however, between these time points values ranged from 19.96 UL^{-1} (week 7) to 51.83 UL^{-1} (week 52). At weeks 6, 8 and 32 mdx mouse ICD levels were significantly greater than corresponding normal values. At all other time points, with the exception of week 7, mdx activity levels were not significantly greater than those of the normal mice.

In summary, all the enzymes analysed demonstrated elevated plasma levels in mdx mice. In the instances of CK, PK and PGAM significantly raised activity levels were observed from week 3 onwards.

MUSCLE

The two muscles selected for enzyme analysis were the pectoral muscle and gastrocnemius muscle. The enzymes measured from muscle extracts were CK, PK, PGAM, ICD and glucose 6-phosphate dehydrogenase (G6PDH).

Pectoral muscle

Creatine kinase

A general trend of increasing CK activity with age was observed for both normal and mdx mice. In the instance of normal mice, activity values ranged from 1424 Ug^{-1} (week 3) to 4428 Ug^{-1} (week 52). The range for mdx mice was 1379 Ug^{-1} (week 4) to 4280 Ug^{-1} (week 52). At weeks 4, 24 and 32 normal levels were significantly greater than those of the corresponding mdx mice. At the remainder of the time points no significant differences were observed (Table 4b).

Pyruvate kinase

Both normal and mdx mouse PK levels followed the same general trend of increasing activity with age which reached peak values of 209 U g^{-1} and 174 U g^{-1} respectively. At all time points,

normal activity levels were greater than those of the corresponding mdx mice but significant differences were only observed at weeks 3, 4 and 6 (Table 4b).

Phosphoglycerate mutase

No general trend of increased or decreased activity was observed for either normal or mdx pectoral muscle PGAM. Throughout the age ranged analysed normal mouse enzyme levels were greater than those of the corresponding mdx mice but only at weeks 3, 4, 6 and 8 were significant differences observed (Table 4b).

Isocitrate dehydrogenase

In normal and mdx mouse plasma, the ICD activity levels demonstrated no general trend over the age range analysed. Normal levels, however, were significantly greater than those of the corresponding mdx mice at all times analysed, with the exception of the weeks 4 and 6 points (Table 4b).

Glucose 6-phosphate dehydrogenase

No prevailing trend of increasing or decreasing glucose 6-phosphate dehydrogenase (G6PDH) activity overall was observed for either normal or mdx mouse muscle extracts. The mdx mouse G6PDH levels were greater than those of the corresponding normal mice over a range from 3 to 52 weeks. Significant differences, however, were only observed from weeks 3 to 24 (Table 4b).

In summary, of the enzymes measured, only CK and PK demonstrated a general trend of increased activity with age. No

distinct trends were observed for the remainder of the enzymes analysed. Normal levels of enzyme activity were greater than those of corresponding mdx mice for all enzymes measured except G6PDH. In the latter instance, G6PDH activities in mdx pectoral muscle were greater than those of the normal mice at all analyses.

Gastrocnemius muscle

Creatine kinase

The overall trend observed for both normal and mdx mouse CK activity levels was an increase in activity with age. In the instance of normal mice the activity values ranged from 1588 Ug^{-1} (week 3) to a peak of 6560 Ug^{-1} (week 24). In the mdx mouse the enzyme activities spread from 1272 Ug^{-1} (week 3) to 3884 Ug^{-1} (week 52). With the exceptions of the week 3 and 6 values, the normal levels were greater than those of the corresponding mdx levels (Table 4c).

Pyruvate kinase

The general trend observed for normal and mdx mice was an increase in activity from week 3 to week 52. The normal and mdx mouse activity ranges extended from 124 Ug^{-1} (week 3) to 255 Ug^{-1} (week 24) for the former instance and from 78 Ug^{-1} (week 4) to 194 Ug^{-1} (week 24) for the latter. Throughout the trial period mdx mouse values were less than those of the corresponding normal mice. With the exception of weeks 6, 7 and 52 significant differences were demonstrated between normal and mdx mice (Table 4c).

Phosphoglycerate mutase

Throughout the trial period normal levels of PGAM were greater than those of the corresponding mdx mice. The normal range extended from 527 Ug^{-1} (week 3) to 725 Ug^{-1} (week 52) and that for the mdx from 342 Ug^{-1} (week 4) to 669 Ug^{-1} (week 52). Only between weeks 3 and 6 were the normal mouse activity levels significantly greater.

than those for the corresponding mdx mice (Table 4c).

Isocitrate dehydrogenase

No general trend of increased or decreased activity was observed for either normal or mdx mice where ICD levels exhibited similar patterns of activity. The only significant difference between normal and mdx mouse ICD levels was observed at week 52 where normal values were greater than those of the corresponding mdx mice (Table 4c).

Glucose 6-phosphate dehydrogenase

Although no distinct trend of increased or decreased activity was observed for this enzyme, throughout the trial period mdx mouse levels were greater than those of the corresponding normal mice. G6PDH levels in mdx mice were significantly greater than those of normal at all time points with the exception of weeks 4 and 52 (Table 4c).

In summary, of the enzymes measured in gastrocnemius muscle, only CK and PK demonstrated a general trend of increasing activity with age. No distinct trends were observed for the other enzymes analysed. Normal levels of enzyme activity were greater than those of corresponding mdx mice for the enzymes CK, PK and PGAM. No differences between normal and mdx ICD levels were observed. In the instance of G6PDH, the mdx mouse levels were greater than those of the corresponding normal mice.

WEIGHT ANALYSIS

Whole mouse

The mean body weight for normal mice increased from a value of 11.8g at week 4 to a peak weight of 34.5g at week 24. Thereafter the body weights declined to a value of 23g at week 52. In contrast, mdx mouse body weights increased from 8.6g at week 3 to a peak value of

32.7g at week 7 followed by a dramatic loss of weight by week 8 to 21.8. Subsequently, body weights increased to reach a second peak of 34g at week 52. Consequently mdx mouse weights were significantly greater than those of the corresponding normal mice at weeks 7 and 52 but at other time points were significantly less than those of the normal mice (Table 4d).

Pectoral muscle

Normal muscle weights increased from a value of 0.027g at week 3 to 0.15g at week 52. Similarly, mdx mouse muscle weights increased from 0.052g at weeks 3 to a peak value of 0.307g at week 52. The dramatic drop in weight observed for whole mouse body weight was not found in this particular muscle. The pectoral muscle weight for mdx mice, with the exception of week 4, was greater than those of the corresponding normals and significant differences were observed at weeks 3, 6 and 52 (Table 4d).

Gastrocnemius muscle

Normal gastrocnemius muscle weights increased steadily from 0.047g at week 3 to a maximum value of 0.28g at 32 weeks and subsequently declined to 0.23g by week 52. The mdx muscle weights increased from 0.03g at week 4 to a peak of 0.279g at week 52. At weeks 4, 8 and 24 the mdx mouse weights were less than those of the corresponding normals and at the remainder of the time points were greater than the normals. Significant differences were observed at weeks 4, 7 and 24 (Table 4d).

In summary normal whole mouse body and individual muscle weights increased during the trial period to reach peak values between 24 and 32 weeks, followed by a decline in old age (week 52). The mdx mouse body and individual muscle weights followed a pattern which did not decline by 52 weeks. The mdx whole mouse weights alone dropped dramatically between 7 and 8 weeks to increase thereafter. At

this period the mdx muscle weights remained static then increased to peak values at 52 weeks.

HISTOLOGY

Gastrocnemius muscle

Normal mouse muscle was demonstrated to contain only type 2A and 2B fibres, which were clearly distinguished by NADH-TR staining. In mdx mice the definition of fibre types was less pronounced and in many type 2B fibres observed diformazan granules indicated an increased oxidative capacity.

Gross pathological changes were observed in both fibre types of mdx mice. Changes in fibre area distribution reflected the enlargement of type 2B fibres. Areas of degeneration and intense nuclear propagation were observed to involve type 2A fibres. Fibre splitting was observed for both fibre types. At the 6 and 8 week samplings muscle fibre structure was completely lost in certain cases. Where fibres were absent, phagocytic cells were present in large numbers but no collagen was observed. In mature mdx mice the 'foci of degeneration' were absent but groups of very small type 2A fibres were observed. The most striking features were the presence of central nuclei and type 1 fibres.

Verification of the presence of regenerating fibres was carried out by acridine orange (AO) staining. Regenerating fibres (small, basophilic, centrally nucleated) were identified by haematoxylin/eosin or haematoxylin/sirius red staining, then the section was stained acridine orange (AO) and viewed under UV illumination. The RNA stained by this process fluoresced under UV light. Confirmation of regeneration was provided by longitudinal sections also stained by AO. In these, characteristic chains of central nuclei were observed in myotubes.

In other muscles investigated (pectoral, plantaris, tibialis anterior, soleus, extensor digitorum longus and plantaris) degeneration was also apparent indicating no clear differentiation in expression between fast and slow twitch muscles.

Comparison of mdx mouse to other muscular dystrophies

Investigation of this model was based on enzyme analysis of plasma and muscle, weight assessments and histological analyses. Comparisons of mouse plasma enzyme results with those of avian dystrophy, hamster dystrophy and, where available DMD was undertaken. The muscle values were assessed in comparison with those from normal and dystrophic chicken data. Direct age-for-age comparisons were difficult in view of the diverse life spans of the experimental models, but certain important trends were noted.

The plasma enzymes measured in this research are well documented in normal and dystrophic chickens (New Hampshire) lines 412 and 413 (Liu et. al, 1980; Chown et. al, 1984; Barnard and Barnard 1979; Allan unpublished results). Elevated levels of the plasma enzymes CK, PK, PGAM and ICD observed for mdx mice were a feature shared with the dystrophic chicken. CK activity levels in mdx mice were observed elevated above normal in a similar fashion to that observed in avian and puchenne dystrophy. The normal mouse levels, however, were greater than those observed in avian dystrophy; covering the range from 3002 UL^{-1} to 8107 UL^{-1} as compared with the range 200 UL^{-1} to 300 UL^{-1} for normal chickens. Variability within a sampling point and between samplings observed in avian and human dystrophy were also observed in both normal and mdx mice (Liu et. al 1980).

Plasma PK levels detailed in dystrophic chickens and hamsters increased to a plateau then declined as a result of losses of muscle

tissue (Liu et. al, 1980). Although elevated PK levels were observed for mdx mice no such decline in activity was discovered. The elevation of mdx mouse PK activities above normal was in the same range as that observed for both avian and hamster dystrophy ie. maximum of 21 fold increase in the instance of the mdx mouse as compared with 29 and 17 fold increases for chicken and hamster respectively. DMD sufferers and carriers of the disease also have elevated plasma levels of PK and as a consequence of this it has been utilised as a diagnostic enzyme for the disease (Zatz et. al, 1980; Griggs et. al, 1985).

In the dystrophic chicken, plasma PGAM levels followed a similar pattern of activity to that demonstrated by PK, this phenomenon was also observed in the mdx mouse plasma. The measured levels of PGAM activity for both normal and mdx mice were greater than those observed in humans and chickens. The values for normal and mdx mice were 2349 UL^{-1} and 11729 UL^{-1} respectively as compared with 70 UL^{-1} (adult human), 111 UL^{-1} (DMD patients), 257 UL^{-1} (normal chicken) and 4000 UL^{-1} (avian dystrophy).

The ICD levels noted for both normal and mdx mice were greater than those observed for normal and dystrophic chickens (Figure 2I, Table 4a). In this instance, however, the patterns of enzyme activity did not mimic those observed for normal and dystrophic chickens.

In summary, many similarities between plasma enzyme activity patterns were observed between these models of dystrophy, in particular the pathological elevation of the enzymes CK, PK, PGAM and ICD. In the latter stages of the disease in avian and human dystrophy levels of these enzymes decline as a result of the losses in muscle tissue. This was not observed in the mdx mouse.

Comparison of normal and dystrophic chicken pectoral muscle enzyme analysis with that of normal and mdx mice revealed reductions in CK, PK and PGAM levels in both the dystrophic chicken and mdx mouse when compared with their respective normals (Chown et. al, 1984; Allan unpublished research). Similarly, G6PDH levels in the dystrophic chicken and mdx mouse were elevated in comparison with their respective normals. The ICD activity patterns in mdx mouse alone did not correspond to those of the dystrophic chicken when compared to the respective normals.

The most predominant feature observed by histological analyses was central nucleation of muscle fibres. This change of nuclei distribution has been attributed to result from regeneration of fibres (Dubowitz, 1973).

From the histological analysis the mdx mice appear to go through a 'crisis' at 6 to 7 weeks followed by a period of regeneration, after which no further degeneration was observed. Research has indicated that regenerated fibres are more resistant to degeneration and this effect was observed for the mdx mouse (Carnwath, personal communication). The plasma and muscle levels observed in mdx mice were consistent with this regeneration pattern. However in the dystrophic chicken, muscle fibres decline to be replaced by collagen and fat and the elevated plasma enzymes diminished in turn. In contrast to avian and human dystrophy, the mdx mouse demonstrated no collagen replacement of muscle fibres and insignificant levels of fatty infiltration. Consequently muscle enzyme levels remained elevated throughout the trial period.

In summary, in the initial stages of the myopathy in the mdx mouse both the muscle enzyme levels and histological data agree with those recorded for avian dystrophy and DMD. In the latter stages

of pathogenesis in avian and Duchenne dystrophy plasma enzyme levels were reduced as a result of losses of muscle tissue, a feature not observed in the mdx mouse.

DISCUSSION

The objective of this research was to investigate the mdx mouse mutant by analysis of plasma and muscle enzyme levels, weight analysis and histological parameters in order to permit comparisons with DMD. Confirmation of an X-linked mammalian model of DMD would provide a superior diagnostic aid to those models currently available.

This research has revealed plasma levels of all the enzymes analysed to be elevated above normal in the mdx strain. In both muscles analysed, CK and PK activities were reduced and G6PDH levels increased in the mutant mouse. The remaining enzyme analysed, ICD, was reduced in pectoral muscle when compared to normal levels but unaffected in the gastrocnemius muscle.

The normal whole mouse body weights followed a pattern of weight increase that would be expected of a healthy animal with a corresponding decline with old age. However, the mdx mice deviated from this pattern by sudden total weight loss between weeks 7 and 8 followed by a steady increase in weight thereafter. No such dramatic deviation from the normal was observed for the individual muscle weights examined in the study and the source of weight changes.

Histological analysis revealed gross pathological changes in mdx mouse muscle tissue. These included fibre splitting, variations of fibre size, increased oxidative capacity, phagocytosis and areas of regeneration.

Comparison of the above mdx mouse results with data available for avian hamster and human dystrophy revealed many similarities at the early stages of pathogenesis. However, in the later stages many

differences were apparent, particularly the lack of collagen and fatty replacement of muscle tissues in mdx mouse. The mdx mouse apparently recovers from the 'crisis' and muscle tissue regenerates hence the plasma enzymes remain elevated. This contrasts with DMD and AMD where losses of muscle tissue result in declined plasma enzyme activity levels.

In conclusion, from the enzyme and histological analyses, the mdx mouse appeared to have a transient muscle wasting disease which was not progressive like DMD since recovery was effected. This conclusion is in agreement with the results of Dangain and Vrbova (1984), who also noted this feature of mdx mouse pathogenesis. In contrast to DMD and AMD, no collagen replacement of muscle tissue was observed. Several features consistent with a DMD model have been observed in the mdx mouse. These included elevated plasma enzyme levels and corresponding muscle enzyme content, initial changes in fibre type and composition and no demyelination of nerves (J. Cavanagh personal communication). In contrast, dissimilarities were also observed. The muscles observed in this research recover integrity with time and thus no collagen or fatty infiltration was observed. The elevated plasma enzyme levels also did not decline in geriatric mice, whereas muscle fibre loss in AMD resulted in lowered plasma enzyme levels. In DMD and AMD, fast twitch muscles are selectively attacked by the disease but this was not observed in the mdx mice where the initial degenerative process was observed in both fast and slow twitch muscles. Physically no differences between normal and mdx mice could be observed. Overall the results of this research indicate that the mdx mouse mutant is not an ideal model for DMD. However, at the initial stages of pathogenesis the similarities are such that it may provide a useful tool to

investigate the mechanisms initiating the dystrophic process. The ability of mdx mouse to regenerate muscle tissue precludes its use for drug trials but may provide insight into the mechanisms of muscle repair after injury or disease.

Table 4a

Plasma enzyme activities from normal and mdx mice.

The enzymes creatine kinase, pyruvate kinase, phosphoglycerate mutase and isocitrate dehydrogenase were measured in normal and mdx mouse plasma. At each time point the mean activity is expressed in (SI) units per litre plus or minus the standard error of the mean ($\text{U l}^{-1} \pm \text{SEM}$). The number (n) of animals per time point is also given..

- o significantly different from normal at $P < 0.05$ by student's t-test
- + significantly different from normal at $P < 0.025$ by student's t-test
- x significantly different from normal at $P < 0.01$ by student's t-test
- * significantly different from normal at $P < 0.005$ by student's t test

Table 4a

Age (weeks)	Creatine kinase		Pyruvate kinase		Phosphoglycerate mutase		Isocitrate dehydrogenase	
	Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM	
	n		n		n		n	
	mdx	normal	mdx	normal	mdx	normal	mdx	normal
3	11130 $\pm$ 2094* 5	3652 $\pm$ 678 5	5795 $\pm$ 944* 5	746 $\pm$ 139 5	7969 $\pm$ 376* 5	2456 $\pm$ 731 5	24.5 $\pm$ 3.6 4	20.1 $\pm$ 3.1 4
4	11217 $\pm$ 1705* 4	3002 $\pm$ 748 6	4305 $\pm$ 242* 4	437 $\pm$ 178 6	6329 $\pm$ 1038* 4	1257 $\pm$ 312 6	38 $\pm$ 4 3	28.5 $\pm$ 6.5 3
6	34830 $\pm$ 6579* 6	4174 $\pm$ 569 5	5985 $\pm$ 522* 6	564 $\pm$ 64 5	9171 $\pm$ 1280* 7	1626 $\pm$ 260 5	35.6 $\pm$ 3.3* 6	17.4 $\pm$ 2.8 5
7	6432 $\pm$ 1401 7	7437 $\pm$ 1171 6	10444 $\pm$ 1807* 7	689 $\pm$ 78 6	9472 $\pm$ 1087* 7	2195 $\pm$ 421 6	20 $\pm$ 2.6 ^o 7	35 $\pm$ 7.4 6
8	8862 $\pm$ 2173 [†] 5	3081 $\pm$ 961 6	7399 $\pm$ 376* 5	351 $\pm$ 75 6	6175 $\pm$ 1348* 5	918 $\pm$ 262 6	69 $\pm$ 17* 5	14 $\pm$ 1.7 4
24	29211 $\pm$ 6839* 10	8107 $\pm$ 3376 6	10776 $\pm$ 1044* 10	681 $\pm$ 169 6	11729 $\pm$ 1707* 10	2349 $\pm$ 769 6	31 $\pm$ 3.4 7	26.2 $\pm$ 2 6
32	7431 $\pm$ 1947 ^o 4	3427 $\pm$ 163 3	5139 $\pm$ 266* 4	394 $\pm$ 20 3	5855 $\pm$ 1159* 4	1257 $\pm$ 135 3	32 $\pm$ 14 [†] 2	17 $\pm$ 2.1 3
52	13092 $\pm$ 4457 ^o 3	5657 $\pm$ 1660 3	4298 $\pm$ 182* 3	417 $\pm$ 69 3	4334 $\pm$ 312* 3	1856 $\pm$ 714 3	51.8 $\pm$ 24.6 3	23.6 $\pm$ 2.6 3

Table 4b

Enzyme activities from normal and mdx mouse pectoral muscle.

The enzymes creatine kinase, pyruvate kinase, phosphoglycerate mutase, isocitrate dehydrogenase and glucose 6-phosphate dehydrogenase were measured in buffered extracts of pectoral muscle. At each time point the mean activity is expressed as units per gram of wet weight tissue (Ug^{-1}) plus or minus the standard error of the mean (SEM). The number (n) of animals per time point is also given.

- o significantly different from normal at $P < 0.05$ by student's t-test.
- † significantly different from control at $P < 0.025$ by student's t-test
- x significantly different from control at $P < 0.01$ by student's t-test
- * significantly different from control at $P < 0.005$ by student's t-test

Table 4b

Age (weeks)	Creatine kinase		Pyruvate kinase		Phosphoglycerate Mutase		Isocitrate dehydrogenase		Glucose 6: phosphate dehydrogenase		
	Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		
	n		n		n		n		n		
	mdx	normal	mdx	normal	mdx	normal	mdx	normal	mdx	normal	
3	1722 $\pm$ 352 4	1424 $\pm$ 70 5	71 $\pm$ 10 ^x 4	109 $\pm$ 6 5	355 $\pm$ 22 [†] 3	455 $\pm$ 28 5	2.9 $\pm$ 0.24 [†] 3	3.9 $\pm$ 0.25 5	0.5 $\pm$ 0.09 ^o 3	0.3 $\pm$ 0.03 5	
4	1379 $\pm$ 107* 3	3208 $\pm$ 89 4	77 $\pm$ 5* 3	135 $\pm$ 8 4	326 $\pm$ 54 ^x 3	530 $\pm$ 19 4	4.8 $\pm$ 0.75 3	4.8 $\pm$ 0.21 4	0.6 $\pm$ 0.14 [†] 3	0.2 $\pm$ 0.02 4	
6	2173 $\pm$ 271 7	1740 $\pm$ 220 5	105 $\pm$ 11* 7	174 $\pm$ 6 5	380 $\pm$ 21* 7	794 $\pm$ 17 5	2.2 $\pm$ 0.06 6	2.2 $\pm$ 0.2 5	0.45 $\pm$ 0.05* 7	0.3 $\pm$ 0.02 5	
7	2272 $\pm$ 397 7	3055 $\pm$ 618 6	134 $\pm$ 13 7	144 $\pm$ 15 6	533 $\pm$ 49 7	617 $\pm$ 63 6	1.6 $\pm$ 0.12* 7	2.4 $\pm$ 0.1 6	0.4 $\pm$ 0.05* 7	0.2 $\pm$ 0.03 6	
8	2089 $\pm$ 271 4	3419 $\pm$ 774 4	110 $\pm$ 3 4	160 $\pm$ 27 4	441 $\pm$ 22 [†] 4	704 $\pm$ 102 4	1.8 $\pm$ 0.22 [†] 4	2.6 $\pm$ 0.23 4	0.7 $\pm$ 0.13 [†] 4	0.31 $\pm$ 0.06 4	
24	3242 $\pm$ 223 [†] 10	4178 $\pm$ 369 5	174 $\pm$ 13 10	209 $\pm$ 19 5	511 $\pm$ 53 10	522 $\pm$ 50 5	2.0 $\pm$ 0.09 ^o 10	2.6 $\pm$ 0.24 5	0.45 $\pm$ 0.05* 10	0.2 $\pm$ 0.03 5	
32	2451 $\pm$ 620 ^o 5	3939 $\pm$ 287 3	126 $\pm$ 20 5	148 $\pm$ 6 3	551 $\pm$ 77 4	642 $\pm$ 50 3	1.81 $\pm$ 0.14* 3	3.8 $\pm$ 0.23 3	0.44 $\pm$ 0.1 4	0.3 $\pm$ 0.05 4	
52	4280 $\pm$ 324 3	4428 $\pm$ 257 3	169 $\pm$ 8 3	200 $\pm$ 15 3	725 $\pm$ 83 3	776 $\pm$ 41 3	2.4 $\pm$ 0.43* 3	5.2 $\pm$ 0.4 3	0.8 $\pm$ 0.4 3	0.64 $\pm$ 0.07 3	

Table 4c

Enzyme activities from normal and mdx mouse gastrocnemius muscle.

The enzymes measured and data handling are as described for Table 4b.

- o significantly different from normal at $P < 0.05$ by student's t-test;
- † significantly different from normal at $P < 0.025$ by student's t-test;
- x significantly different from normal at $P < 0.01$ by student's t-test;
- * significantly different from normal at $P < 0.005$ by student's t-test.

Table 4c

Age (weeks)	Creatine kinase		Pyruvate kinase		Phosphoglycerate Mutase		Isocitrate dehydrogenase		Glucose 6 phosphate dehydrogenase	
	Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM	
	n		n		n		n		n	
	mdx	normal	mdx	normal	mdx	normal	mdx	normal	mdx	normal
3	2254 $\pm$ 442 4	1588 $\pm$ 98 5	103 $\pm$ 10 ^o 4	124 $\pm$ 4.7 5	455 $\pm$ 24 ^o 3	527 $\pm$ 70 5	2.7 $\pm$ 0.11 3	2.5 $\pm$ 0.08 4	0.3 $\pm$ 0.02* 3	0.16 $\pm$ 0.01 5
4	1272 $\pm$ 89* 3	4270 $\pm$ 617 4	78 $\pm$ 11 [†] 3	171 $\pm$ 28 4	342 $\pm$ 51 ^x 3	719 $\pm$ 106 4	3.9 $\pm$ 0.5 3	3.7 $\pm$ 0.4 4	0.25 $\pm$ 0.1 3	0.2 $\pm$ 0.01 4
6	2603 $\pm$ 346 7	2015 $\pm$ 193 5	123 $\pm$ 14 7	156 $\pm$ 24 5	404 $\pm$ 36 ^o 7	665 $\pm$ 114 5	2.1 $\pm$ 0.2 6	1.7 $\pm$ 0.09 5	0.49 $\pm$ 0.05* 7	0.2 $\pm$ 0.03 5
7	2382 $\pm$ 450 7	2976 $\pm$ 315 6	148 $\pm$ 14 7	149 $\pm$ 13 6	641 $\pm$ 48 7	650 $\pm$ 84 6	1.7 $\pm$ 0.11 7	2.1 $\pm$ 0.12 6	0.4 $\pm$ 0.05* 7	0.15 $\pm$ 0.01 6
8	2870 $\pm$ 430 4	3854 $\pm$ 798 4	151 $\pm$ 6 [†] 4	189 $\pm$ 11 4	547 $\pm$ 14 4	649 $\pm$ 60 4	2.2 $\pm$ 0.2 4	2.1 $\pm$ 0.1 4	0.54 $\pm$ 0.1 [†] 4	0.23 $\pm$ 0.03 4
24	3641 $\pm$ 177* 10	6560 $\pm$ 704 5	194 $\pm$ 10 ^x 10	255 $\pm$ 22 5	599 $\pm$ 60 5	535 $\pm$ 49 10	1.8 $\pm$ 0.08 9	2.5 $\pm$ 0.14 5	0.39 $\pm$ 0.05* 10	0.13 $\pm$ 0.01 5
32	2683 $\pm$ 699 ^x 5	5411 $\pm$ 398 3	131 $\pm$ 24 [†] 5	205 $\pm$ 8 3	632 $\pm$ 66 4	711 $\pm$ 19 3	1.8 $\pm$ 0.12 3	2.1 $\pm$ 0.16 3	0.31 $\pm$ 0.06 [†] 4	0.15 $\pm$ 0.01 3
52	3884 $\pm$ 184 ^x 3	5396 $\pm$ 318 3	200 $\pm$ 20 3	184 $\pm$ 14 3	669 $\pm$ 19 3	725 $\pm$ 56 3	2.4 $\pm$ 0.1 [†] 3	3.2 $\pm$ 0.21 3	0.4 $\pm$ 0.1 3	0.2 $\pm$ 0.03 3

Table 4d

Whole body and individual muscle weight analysis for normal and mdx mice.

The mean weight value per time point is stated in grams plus or minus the standard error of the mean (g $\pm$ SEM). The number (n) of animals per analysis is also given.

- o significantly different from control at $P < 0.05$ by student's t-test.
- † significantly different from control at $P < 0.025$ by student's t-test.
- x significantly different from control at $P < 0.01$ by student's t-test
- * significantly different from control at $P < 0.005$ by student's t-test

Table 4d

Age (weeks)	Pectoral muscle weight(g) $\pm$ SEM		gastrocnemius muscle weight(g) $\pm$ SEM		whole mouse weight(g) $\pm$ SEM	
	<u>mdx</u>	normal	<u>mdx</u>	normal	<u>mdx</u>	normal
3	0.0518* ± 0.003 3	0.027 ± 0.0058 5	0.048 ± 0.0069 3	0.047 ± 0.009 5	8.6 ± 0.38 3	
4	0.0217 ^x ± 0.0075 3	0.041 ± 0.0005 4	0.033* ± 0.009 3	0.089 ± 0.01 4	8.55 ^o ± 0.39 3	11.83 ± 1.33 3
6	0.078 [†] ± 0.007 6	0.055 ± 0.006 5	0.1125 ± 0.011 6	0.104 ± 0.004 5	17 ± 0.96 3	
7	0.092 ± 0.026 4	0.0857 ± 0.013 5	0.1572 ^o ± 0.02 4	0.1139 ± 0.006 5	32.7* ± 1.25 2	23.3 ± 0.66 3
8	0.098 ± 0.016 4	0.076 ± 0.011 4	0.1035 ± 0.006 4	0.1176 ± 0.011 4	21.18 [†] ± 1.09 4	24.73 ± 0.58 4
24	0.1221 ± 0.008 10	0.1159 ± 0.015 5	0.1637 [†] ± 0.007 10	0.1779 ± 0.002 5	29.64* ± 0.59 7	34.5 ± 1.33 5
32	0.248 ± 0.015 3	0.2141 ± 0.02 3	0.2997 ± 0.031 3	0.277 ± 0.012 3	28.7 ± 2.09 3	30.4 ± 0.57 3
52	0.307 ^x ± 0.04 3	0.1513 ± 0.02 3	0.2789 ± 0.027 3	0.2316 ± 0.023 3	34.3* ± 1.29 3	23.3 ± 0.97 3

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

The research described within this thesis addresses particular aspects of the treatment and diagnosis of muscular dystrophy. Avian dystrophy has been utilised as a model for the critical assessment of the effective therapeutic potential of particular drugs in the treatment of dystrophic symptoms. In addition the avian system has been applied in an investigation of enzymes having value as diagnostic markers.

The assessment of the therapeutic potential of drugs in avian dystrophy has been detailed in Section one. The nineteen drugs under scrutiny may be individually classified within six distinct categories (serotonin antagonists, calcium antagonists, neurotrophic agents, anti-proteases and chelating agents), targeted at well documented features of dystrophic pathogenesis (Mendell et al., 1971; Duncan, 1978; Dubowitz and Heckmatt, 1980; Weinstock et al., 1958; Clark, 1984). The results of these trials, as characterised by reductions in activity levels of selected plasma enzymes (CK, PK, ICD, PGAM) and improvements in flip capability, have shown that treatment with individual drugs may alleviate particular dystrophic symptoms. The most pronounced beneficial effects were observed after treatment with the calcium antagonist, Nifedipine and the anti-protease, Map-1. Further trials under varied dosage regimes and investigations into alternative methods of administration are required to complete the therapeutic profiles of these drugs in avian dystrophy. The aminopyridines, although demonstrating profound effects on avian dystrophic symptoms, were accompanied by extreme toxic side effects sufficient to preclude their further research and development. The contrasting therapeutic effects, in avian dystrophy, of the calcium antagonist, cinnarizine and its difluoro derivative flunarizine may be usefully employed to elucidate

molecular mechanisms of muscle damage in dystrophy.

The investigation in avian dystrophy of the oxidative enzyme Isocitrate dehydrogenase has been described in Section two. Previous research had indicated that in avian dystrophy this enzyme may be elevated at hatch (Cardinet et al., 1972). If this phenomenon was repeated in lines 412/413 chickens and in man, this enzyme would provide a marker of diagnostic significance at early stages of the disease. The results discussed in Section two do not support this view but do show significant differences of activity levels between normal and dystrophic birds both in muscle and plasma at later stages of the pathogenesis. Such differences may be usefully employed to monitor changes during therapeutic drug trials.

Phosphoglycerate mutase has previously been evaluated in avian dystrophy and elevated activity levels demonstrated at various stages of pathogenesis (Chown, et al., 1984). Subsequent limited analyses of specimens from Duchenne sufferers confirmed this view (Chown, et al., 1984). In order to evaluate the potential of this enzyme for the diagnosis of human carriers of DMD, this study initially established the natural variation of normal PGAM in the plasma and serum from normal women. From the normal values the upper limit of normal (95th percentile) was calculated and used to assess PGAM levels determed from women 'at risk' carriers of DMD. In studies of female 'at risk' carriers paired analysis of PGAM with two other established markers (creatine kinase and carbonic anhydrase III) did not reveal any advantages of the use of PGAM in this type of analysis (Heath, et al., 1982). The enzyme-linked assay system employed above, depended upon multiple activities and proved susceptible to inaccuracies resulting from the heterogeneity of the system components. Improved and accurate methods were developed based upon radioimmuno assay

techniques. In order to establish an accurate radioimmuno assay, anti-PGAM antibodies were raised against a commercial preparation of rabbit muscle PGAM. Human muscle PGAM was also affinity purified for application as standards in such analysis. The investigation indicated that modified procedures of enzyme purification and quantitative analyses utilising radioimmuno assay techniques would promote improved carrier and foetal diagnoses.

Section four details an enzyme study of a noval mouse mutant proposed as a new mammalian model for DMD (Bulfield et al., 1984). Analysis of plasma and muscle samples by selected enzymes (CK, PK, PGAM, ICD and G6PDH) enabled evaluation of the mutant mouse with reference to avian and Duchenne muscular dystrophy. In mice upto the age of 7 weeks many enzymological similarities with other dystrophies were observed. However, by one year the dystrophic symptoms were apparently held in abeyance. This may indicate the exciting possibility that this unique mouse strain may provide a mammalian model suitable for both studies of dystrophic pathogenesis and also mechanisms of muscle damage and repair.

In conclusion, the research described and discussed herein has highlighted aspects of enzymology of dystrophy which, in conjunction with current advances in medical genetic manipulation (Monaco et al., 1985), may provide a future basis for accurate diagnoses and controlled treatment of Duchenne muscular dystrophy.

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