BIOCHEMICAL, PATHOLOGICAL AND CLINICAL ASPECTS OF

DILATED CARDIOMYOPATHY IN DOBERMAN PINSCHER DOGS

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by

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ABSTRACT

BIOCHEMICAL, PATHOLOGICAL AND CLINICAL ASPECTS OF DILATED CARDIOMYOPATHY IN DOBERMAN PINSCHER DOGS

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Nicotinamide adenine dinucleotide dehydrogenase (NADHdh) is the largest of the respiratory chain complexes and the most severely affected in Dobermans with dilated cardiomyopathy. This study tested the hypothesis that the rotenone inhibitable activity of myocardial and gastrocnemius muscle NADHdh was reduced in normal Dobermans, Dobermans with DCM, and Dobermans with occult heart disease, when compared with healthy mixed breed dogs.

Myocardial NADHdh activity was significantly ($p < 0.05$) reduced by 68.5% in Dobermans with DCM (17 ± 7 ηmol/min/mg myocardial protein, n=8) compared to normal mixed breed dogs (54 ± 17 ηmol/min/mg myocardial protein, n=10).

There was a significant ($p < 0.05$) reduction in gastrocnemius muscle NADHdh activity by 59.1% in normal Dobermans (2.87 ± 1.3 ηmol/min/mg muscle wet weight, n=5), and by 57.4% in Dobermans with DCM (2.8 ± 1.9 ηmol/min/mg muscle wet weight, n=5) compared to normal mixed breed dogs (4.86 ± 1.6 ηmol/min/mg muscle wet weight, n=8).

Raised myocardial lactic acid concentrations indicated a significant ($p < 0.05$) compensatory increase in anaerobic metabolism in response to reduced capacity for aerobic metabolism (decreased NADHdh activity) in the myocardium from Dobermans with DCM (188 ± 83 μmol/g protein) compared to normal Dobermans (109 ± 36 μmol/g protein) and normal mixed breed dogs (109 ± 26 μmol/g protein).

A significant ($p < 0.05$) increase in severity of myocyte necrosis, and myocardial interstitial fibrosis was observed in normal Dobermans compared to normal mixed breed dogs. Forty percent of normal Dobermans (4/10) had focal myocardial necrosis. Three of these 4 normal Dobermans with focal myocardial necrosis, also had either glomerulonephritis or renal tubular necrosis.

Significantly reduced myocardial and gastrocnemius muscle NADHdh activity in Dobermans with DCM, compared to normal mixed breed dogs, may be secondary to some other genetic defect, or due to a defect in the nuclear or mitochondrial genes that code for NADHdh, and predispose this breed to DCM.

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a	radius
acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
aLVFW	average left ventricular free wall dimension
aLVIDd	average left ventricular internal diameter in diastole
aLVIDs	average left ventricular internal diameter in systole
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AST	aspartate transaminase
AT_1	angiotensin II receptors
ATP	adenosine triphosphate
ATP synthetase	adenosine triphosphate synthetase, complex V
AVP	arginine vasopressin
Bax	a protein involved in apoptosis
Bcl-2	a mitochondrial protein involved in apoptosis
Bio 14.6	Syrian hamster carrying the autosomal recessive cardiomyopathic gene, *cm*
Bio 14.6 HAM	normal control Syrian hamsters
bp	base pairs
$C_{16}H_{32}O_2$	palmitic acid
$C_6H_{12}O_6$	glucose

CHF	congestive heart failure
CK	creatine kinase
CO_2	carbon dioxide
CoA	coenzyme A
complex I	mitochondrial nicotinamide adenine dinucleotide dehydrogenase; NADH ubiquinone oxidoreductase
complex II	succinate dehydrogenase
complex III	cytochrome reductase (ubiquinol ferricytochrome C oxidoreductase)
complex IV	cytochrome oxidase (ferrocytochrome C oxygen oxidoreductase)
CP	creatine phosphate
csa	cross-sectional area
DCM	dilated cardiomyopathy
dG	deoxyguanosine
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DP	Doberman Pinscher dog
DP-DCM	Doberman Pinscher dog with dilated cardiomypopathy
DP-N	normal Doberman Pinscher dog
DP-OCC	Doberman Pinscher dog with occult heart disease
e	exponential
ECG	electrocardiogram
EDRF	endothelium-derived relaxing factor

EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
EPSS	E point to septal separation
ET_A	endothelin-A receptor
ET_B	endothelin-B receptor
FAD	flavin adenine dinucleotide (oxidized form)
$FADH_2$	flavin adenine dinucleotide (reduced form)
$FADH_2$	flavin mononucleotide (reduced form)
F	F value is a measure of the relationship between the dependent variable and independent variables
F_o	protein channel subunit of ATP synthetase
G	wall stress in a spherical chamber
G_i	inhibitory guanosine protein
G_s	stimulatory guanosine diphosphate protein
h	wall thickness
H^+	proton
H_2O	water
H_2O_2	hydrogen peroxide
HADH	β-hydroxyacyl coenzyme A dehydrogenase
HeLa	human HeLa cell line
HNE	4-hydroxynonenal
human KB	human KB cell line
J-2-N	cardiomyopathic strain of Syrian hamster

L	long axis
LV	left ventricle
LVFW	left ventricular free wall dimension
LVIDd	left ventricular internal diameter in diastole
LVIDs	left ventricular internal diameter in systole
M	minor axis
MHC	major histocompatibility complex
mm Hg	millimeters of mercury
mm^2	area in millimeters squared
M-mode	motion-mode echocardiography
mouse L	mouse cell line L
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
Na^+-K^+ ATPase	adenosine triphosphate dependent, sodium-potassium transport pump
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADHdh	nicotinamide adenine dinucleotide dehydrogenase; respiratory chain complex I; NADH ubiquinone oxidoreductase
NE	norepinephrine
NO	nitric oxide
NOS	nitric oxide synthetase

NOS_c	nitric oxide synthetase, constitutive form
NYHA	New York Heart Association
O_2	oxygen
$O_2^{\bullet-}$	superoxide radical
ODH	oxoglutarate dehydrogenase
$OH^{\bullet}$	hydroxyl radical
oxo8dG	8-oxo-7,8-dihydro-2-dexoyguanosine
P	pressure
$p < 0.05$	the probability that the difference between the groups or correlations is due to chance in less than 5 percent of cases
^{31}P-MRI	nuclear magnetic resonance spectra for phosphorus-31
P/O ratio	number of ATP formed for each atom of oxygen consumed, sometimes called ATP/ O ratio
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pH	hydrogen ion concentration
P_i	inorganic phosphate
PO_2	partial pressure of oxygen
QRS	electrocardiographic wave formed by electrical cardiac impulse
RPP	heart rate pressure product (heart rate multiplied by the left ventricular developed pressure)
RQ	respiratory quotient
SDS	sodium dodecyl sulphate

SNO-Hb	s-nitrosohemoglobin
SR Ca^{2+}-ATPase	sarcoplasmic reticulum, adenosine triphosphate dependent, calcium- transport pump
TGF-β_1	transforming growth factor β_1
tRNA	transfer ribonucleic acid
ubiquinol-10	ubiquinol; coenzyme Q; vitamin Q_{10}
VPC	ventricular premature contracture
VPD	ventricular premature depolarization
vs	versus
Vitamin C	ascorbate; ascorbic acid
WHO	World Health Organization
WSd	wall stress index in diastole
WSs	wall stress index in systole

CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

Dilated cardiomyopathy is an important myocardial disease of people (Kelly and Strauss, 1994), and several animal species (Robinson and Maxie, 1993) including Doberman Pinscher dogs (Hazlett *et al*, 1983). A study of 193 Dobermans, 0.4 to 11 years of age, with no signs of heart disease revealed 46% with echocardiographic changes and 16% with electrocardiographic changes suggestive of occult heart disease (O'Grady and Horne, 1992). Biochemical studies of myocardium from Dobermans that died with dilated cardiomyopathy demonstrated reduced mitochondrial respiratory chain enzymes and myoglobin along with a marked reduction in mitochondrial ATP production when compared to healthy mixed breed dogs (McCutcheon *et al*, 1992).

1.2. DILATED CARDIOMYOPATHY IN PEOPLE AND ANIMALS

1.2.1. Definition of dilated cardiomyopathy

Cardiomyopathy has been defined as "a disease of the myocardium associated with cardiac dysfunction", in a recent report of the World Health Organization (Richardson *et al*, 1996). Previously, the World Health Organization definition of cardiomyopathy was restricted to "heart muscle diseases of unknown cause" (WHO Technical Report, 1984). Veterinary reference sources define cardiomyopathy as: idiopathic myocardial disease (Robinson and Maxie, 1993); a general diagnostic term designating primary myocardial disease of unknown cause (Blood and Studdert, 1990);

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and, primary dysfunction of the myocardium of unknown cause (Jones *et al*, 1997). "Idiopathic" is often used in the literature, in conjunction with cardiomyopathy, to describe myocardial diseases of unknown cause (Jones *et al*, 1997; Richardson *et al*, 1996; Wynne and Braunwald, 1992).

Three major categories of cardiomyopathy are recognised in both the human and veterinary literature, based on functional impairment:

• **Dilated cardiomyopathy (DCM)** is characterized by reduced myocardial contractility with increased ventricular end-diastolic volume (Robinson and Maxie, 1993). Dilated cardiomyopathy is the most common form of cardiomyopathy in people (Abelmann, 1996). In older literature, DCM is termed congestive cardiomyopathy (Wynne and Braunwald, 1992).

• In **hypertrophic cardiomyopathy**, there is left and/or right ventricular hyprertrophy, which is usually asymmetric and involves the interventricular septum (Richardson *et al*, 1996; Robinson and Maxie, 1993).

• **Restrictive cardiomyopathy** is characterized by endomyocardial fibrosis, reduced ventricular compliance, and impaired ventricular filling (Robinson and Maxie, 1993).

Other categories of cardiomyopathy that are recognised by the WHO include arrythmogenic right ventricular cardiomyopathy, unclassified cardiomyopathies (e.g., fibroelastosis) and specific cardiomyopathies (e.g., ischemic, valvular, hypertensive, and metabolic cardiomyopathies) (Richardson *et al*, 1996).

1.2.2. Clinical aspects of dilated cardiomyopathy

Dilated cardiomyopathy is characterized by ventricular dilation, impaired systolic function and is often associated with arrhythmia, thromboembolism, and sudden death or death due to heart failure (Manolio *et al*, 1992; Richardson *et al*, 1996). The recorded prevalence and incidence of dilated cardiomyopathy in people has increased since the 1970's due to improved diagnostic techniques (particularly echocardiography) and increasing awareness by clinicians (Manolio *et al*, 1992; Wynne and Braunwald, 1992). Dilated cardiomyopathy in people has an annual incidence in Europe and USA ranging from 2 to 14.5 cases per 100,000 (Acquatella *et al*, 1990; Manolio *et al*, 1992). There is a 2.5 fold increased risk of DCM in black compared to white people; black men have the highest prevalence. Case control studies have shown that the 2.7 fold increased risk of DCM in black men was not explained by differences in income, alcohol intake, body size or other confounders (Manolio *et al*, 1992).

Dilated cardiomyopathy is recognized to occur in cattle (Holstein, Japanese Black and polled Hereford, Simmentaler/Red Holstein), dogs (Doberman Pinscher, Boxer, Portugese water dog, Dalmatian, English Cocker Spaniel, German Shepherd, Great Dane, Irish Wolfhound and St. Bernard), ferrets, Syrian hamsters (Calvert, 1995; Eschenhagen *et al*, 1995; Robinson and Maxie, 1993; Damhach *et al*, 1996; Freeman *et al*, 1996), rats, mice and turkeys (Van Vleet and Ferrans, 1986). Cardiomyopathy has been reported to occur in polled Hereford calves as a lethal autosomal recessive disorder, in association with the wooly haircoat syndrome (Whittington and Cook, 1988). Multifocal ventricular premature contractions were demonstrated to be the cause of cardiac arrhythmia in 4 polled Hereford calves with cardiomyopathy and the wooly haircoat syndrome

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(Whittington and Cook, 1988). A hereditary cardiomyopathy of Simmentaler/Red Holstein cattle is endemic in Switzerland (Eschenhagen *et al*, 1995). Cardiac disease is recognised to occur consistently in dogs over 3 months of age with Duchenne's type of muscular dystrophy. Myocardial changes are milder and occur later than the skeletal muscle dystrophy in affected dogs. Ventricular wall thickness and overall dimensions of the hearts from dogs with Duchenne's type of muscular dystrophy were within the normal range, except in one 6 year old dog which had mild cardiomegaly (Valentine *et al*, 1989). Strains of normal and cardiomyopathic (J-2-N) white Syrian hamsters have been bred from the Bio 14.6 cardiomyopathic strain of golden Syrian hamsters (Nagano *et al*, 1994). Cardiomyopathy has been experimentally induced in commercial turkey poults by the addition of 0.07% furazolidone in the feed (O'Brien *et al*, 1993).

Current techniques used to diagnose and prognosticate DCM in people include physical examination, echocardiography, Holter monitoring, thoracic radiography, gated radionuclide scanning (for gallium-67 citrate and indium-111 antimyosin), left- or right-sided cardiac catheterization, and endomyocardial biopsy (Manolio *et al*. 1992; De Maria, *et al* 1992; Stevenson and Perloff, 1988). These diagnostic aids vary in accuracy and invasiveness (table 1.1.) (modified from Manolio *et al*, 1992).

In human medical practice, a presumptive diagnosis of DCM may be established by history, symptoms of dyspnea, palpitations, chest pain, syncope, fatigue and reduced exercise tolerance, after the exclusion of ischemic, hypertensive, valvular, pericardial and congenital heart and vascular disease (Acquatella, *et al*, 1990; De Maria *et al*, 1992).

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DIAGNOSTIC AID	INVASIVENESS Low ⟶ High			SPECIFITY FOR DCM Low ⟶ High		
Physical examination	✓			✓		
Thoracic radiography		✓		✓		
Echocardiography	✓					✓
Gated radionuclide scan		✓				✓
Endomyocardial biopsy			✓	✓		
Right heart catheterization			✓			✓
Left heart catheterization			✓			✓

Table1.1. Comparison of diagnostic tests used to diagnose DCM, in terms of the invasiveness versus specificity of the test for DCM (modified after Manolio *et al*, 1992).

Some physical signs in people with DCM (and other heart diseases) include an external jugular vein pulse (following peak right atrial pressure) and a murmur of mitral regurgitation (Stevenson and Perloff. 1988).

Veterinary cardiologists use clinical examination, auscultation. thoracic radiography. electrocardiography and echocardiography to diagnose cardiac dysfunction in dogs (Calvert, 1995). Both the terms occult and prodromal are commonly used to signify the early stages of cardiomyopathy (Calvert, 1995; Wynne and Braunwald, 1992). Prodromal stages of DCM may be diagnosed on clinical examination by a combination of familial history of DCM, syncope, auscultation of an abnormal heart rhythm, electrocardiography or during continuous, 24 to 48 hour ambulatory electrocardiographic (Holter) monitoring (Calvert, 1995; Calvert *et al.* 1997). Recently, stress echocardiograms performed along with the administration of a β1-agonist (dobutamine) revealed that elevated LVID-S[1] and diminished diastolic function (decreased ratio of peak early to late diastolic mitral filling velocity) were strong independent predictors of occult DCM in Doberman Pinscher dogs (Minors, 1995). As the cardiomyopathy progresses, left ventricular systolic function is reduced and the left ventricular ejection fraction decreases to 35-50% of normal in the early decompensatory stages (Calvert, 1995). In some dogs with early decompensated cardiac changes, auscultation may reveal a gallop heart rhythm (due to an abnormal third heart sound), various forms of tachyarrhythmias and/or systolic heart murmur (due to mitral regurgitation) (Calvert, 1995). With a further decline in cardiac systolic function to the stage of overt cardiac decompensation, left ventricular ejection fraction can decline to 30-40% of normal for the

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[1] The left ventricular internal dimension in systole was measured on 2-dimensionally-guided M-mode echocardiographs, from the right parasternal long axis of the left ventricle (Minors, 1995).

dog, leading to a wide range of abnormal clinical signs. Some dogs with left-sided congestive heart failure due to DCM will exhibit coughing, dyspnea and pulmonary crackles on auscultation (due to pulmonary edema). Right-sided or bilateral cardiac failure due to DCM can lead to distended jugular veins and jugular pulses, pleural effusion and muffled heart sounds, and ascites in dogs. Generalized weakness, pale mucous membranes, prolonged capillary refill time, weak femoral pulses, and hypothermia can be seen in dogs with reduced cardiac output due to DCM (Calvert, 1995).

Endomyocardial biopsy allows the study of heart tissue throughout the course of disease, however this technique does involve risk to the patient, and the use of this technique in DCM remains controversial (Manolio et al, 1992; Wynne and Braunwald, 1992). An analysis of 546 endomyocardial biopsies in 464 people reported complications in 6% of cases, with 2 people dying following cardiac perforation (Deckers et al. 1992). Endomyocardial biopsy may detect myocarditis or systemic infiltrative disease but is not specific for DCM. In the future, endomyocardial biopsy, combined with polymerase chain reaction or in situ hybridization, may be useful for the detection of genetic defects or infection (Wynne and Braunwald, 1992).

Thoracic radiography in people with DCM may reveal cardiomegaly, pulmonary venous congestion and interstitial edema (Stevenson and Perloff, 1988). The increase in cardiothoracic ratio and heart volume, which can be detected on plain thoracic radiography, provides an insensitive indicator of increased left ventricular end-diastolic volume (Steiner and Levin, 1992).

Generalized globoid cardiomegaly is commonly seen in thoracic radiographs from giant breeds of dogs with advanced DCM. Distention of the pulmonary (cranial and caudal lobar) veins is often seen in dogs with congestive cardiac failure. Pulmonary edema, pleural effusion and ascites are often present in radiographs from giant breed dogs with congestive cardiac failure due to DCM (Calvert, 1995).

Echocardiography is a technique in which an ultrasonic beam is projected from the chest surface to pass through the heart. Receiving transducers placed at the skin surface capture sonic reflections and create real-time images of the heart. Border forming echoes are interpreted to estimate the physical dimensions of the heart. These cardiac dimensions are compared to a normal range and used to evaluate the heart function. M (motion)-mode echocardiography is a plot of echogenic ultrasound representing various intensities (brightness) of reflection from tissue interfaces on the Y-axis versus time on the X-axis (Henik, 1995). Quantitative evaluation of M-mode echocardiographs allows measurement and assessment of cardiac chambers, wall and valve orifice dimensions as well as wall and valve motion (Nyland and Mattoon, 1995). The earliest M-mode changes in people developing DCM may be an increase in the end-systolic ventricular dimension. Later, both end-diastolic and end-systolic dimensions are increased. Ventricular wall thickness is frequently normal and with dilation there is a net increase in ventricular mass. Most human cardiologists measure the ejection fraction to assess the global left ventricular systolic function (Feigenbaum, 1994). The ejection fraction is the percent (or fraction) of the left ventricular diastolic volume that is displaced during systole (Feigenbaum, 1994). The left ventricular (LV) ejection fraction (EF) can be calculated using M-mode echocardiography as follows:

$$EF = \frac{LV\ diastolic\ volume - LV\ systolic\ volume}{LV\ diastolic\ volume}$$

$$EF = \frac{LV\ stroke\ volume}{LV\ diastolic\ volume}$$

Both cine magnetic resonance imaging, and contrast angiography have been used to estimate left ventricular volumes and ejection fraction (Wynne and Braunwald, 1992).

Depressed ejection fraction ($\leq$ 40 to 45%) is a commonly accepted criterion for impaired systolic function and the most powerful predictor of mortality in people with DCM (Manolio *et al*, 1992).

Two-dimensional echocardiography with Doppler interrogation and color flow imaging provides quantifiable graphical images illustrating the morphological and physiological changes of the heart that occur in chronic DCM (Stevens and Perloff, 1988). Typical changes seen on two-dimensional echocardiographic examination of people with DCM include a spherical enlarged left ventricle with global severe hypocontractility of the heart (Acquatella *et al*, 1990). Internal dimensions of the ventricles at end-diastole are increased, septal or free wall thicknesses are normal or reduced, and mitral regurgitation is frequently seen in people with chronic DCM (Acquatella *et al*, 1990; Stevenson and Perloff, 1988).

Another estimator of left ventricular systolic function is fractional shortening (FS) which can be calculated by using M-mode or two-dimensional linear dimensions. An advantage of the fractional shortening (or fractional area change) is that the measurements are not squared or cubed which may magnify errors (Feigenbaum, 1994). If the fractional shortening of the left ventricle is measured as a change in cross-sectional area or internal diameter, it may not be a true measure of global systolic function if the

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ventricle is not contracting symmetrically. Asymmetrical ventricular contraction can occur in people with ischemic heart disease (Feigenbaum, 1994).

$$\text{Fractional shortening of the left ventricle} = \frac{\text{LVIDd} - \text{LVIDs}}{\text{LVIDd}} \times 100$$

Where: LVIDd = left ventricular internal diameter in diastole; LVIDs = left ventricular internal diameter in systole.

Fundamental laws and formulae relating stress to pressure and physical dimensions of spherical and elliptical chambers have been applied to the whole heart in an effort to assess forces acting on myocardial fibres within the ventricular wall. Laplace's law defines wall stress (force per unit of cross sectional area of wall) in a spherical chamber as:

$$G = \frac{Pa}{2h}$$

Where   G = wall stress in a spherical chamber

P = pressure

a = radius

h = wall thickness

Another estimator of wall stress can be derived from the ratio of left ventricular internal diameter over the left ventricular free wall dimension (LVFW). The wall stress

$$\text{index in systole (WSs)} = \frac{\text{LVIDs}}{\text{LVFWs}} \text{ and left ventricular wall stress index in diastole (WSd)}$$

$$= \frac{\text{LVIDd}}{\text{LVFWd}} \text{ (Braunwald } et\ al, 1992).$$

Another indicator of the global systolic function comes from measures of mitral valve movement. Mitral valve motion is influenced by the blood flow through this valve (Feigenbaum, 1994). Decreased movement of the mitral valve E point indicates reduced

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stroke volume, and increased anterior displacement of the interventricular septum implies increased diastolic volume (Feigenbaum, 1994).

Typical echocardiographic findings in giant breed dogs with DCM include an increase in LVIDd and LVIDs, leading to a reduction in the left ventricular fractional shortening by 50% at the onset of congestive heart failure (Calvert, 1995). The E point to septal separation is commonly increased in these giant breed dogs with DCM (Calvert, 1995).

Scaling of echocardiographic data has been used by researchers to account for the variations in dimensions of the heart that accompany changes in body weight and age of the person or animal (Baig et al, 1998; O'Grady et al, 1986; Kerkhof et al, 1998; Sisson and Schaeffer, 1991; Smucker et al, 1990). Linear and area dimensions of the heart, obtained by echocardiography, were reported to be correlated to body size in 18 normal dogs (O'Grady et al, 1986). When compared by simple linear regression, fractional shortening and ejection fraction (indices of left ventricular function) were not correlated to body surface area or body weight (O'Grady et al, 1986). These authors did not test the relationship between indices of left ventricular dimension, or function, by nonlinear regression (O'Grady et al, 1986). Other authors have normalized echocardiographic dimensions by expressing left ventricular end-diastolic dimension per 10 kilograms of body weight (Smucker et al, 1990). Indexing of echocardiographic measurements for body weight to the power of 0.333 has been reported to be more accurate than scaling echocardiographic dimensions linearly to body weight in growing dogs (Sisson and Schaeffer, 1990). An age variance of left ventricular diameters has been reported recently in dogs with cardiac disease (Kerkhof et al, 1998). When dogs with cardiac

disease were categorized into 3 groups (with divisions at 3 and 7 years), and the left ventricular dimensions were compared, the regression coefficients for the younger group differed from the two older groups (Kerkhof *et al*, 1998). Left ventricular internal diameter in diastole in asymptomatic relatives of patients with DCM has been compared by a formula combining body surface area (BSA) and age:

$$LVIDd \text{ (predicted)} = 45.3 \times BSA^{0.3} - 0.03 \times age - 7.2$$

Twenty nine percent of aysymptomatic relatives of people with DCM were reported to have echocardiographic abnormalities. Twenty seven percent of these aysymptomatic relatives progressed to develop overt DCM (Baig *et al*, 1998).

Electrocardiograms (ECG) of people with DCM may reveal nonspecific ST-T segment abnormalities, Q wave "infarct patterns " (which may be caused by myonecrosis due to a cardiotropic virus), and left bundle branch block (Stevenson and Perloff, 1988).

QRS wave disturbances and heart rhythm abnormalities are usually detectable in ECGs of dogs with heart failure due to DCM (Calvert, 1995). Low voltage R waves are associated with global myocardial degeneration and are commonly seen in Boxers and Doberman Pinschers in the end stages of DCM. Where a prolonged QRS wave is coupled with a low voltage R wave in dogs with DCM, the R wave downstroke tends to be slurred (Calvert, 1995). Atrial fibrillation is seen in about 60 to 70% of giant breed dogs in end-stage DCM and may be important in precipitating decompensated cardiac failure (Calvert, 1995). Ventricular arrhythmias are most commonly seen in Boxers and Dobermans with DCM, but can be seen in any dog with DCM (Calvert, 1995).

Holter monitoring is a noninvasive technique for continuous electrocardiogram recording, typically recording on two channels for 24 hours (Zipes, 1992). This allows

for the quantification of the frequency and complexity of arrhythmias, and the correlation of arrhythmias with the patient's symptoms. In a study of 218 people with invasively confirmed DCM, ventricular arrhythmias were recorded in 205 patients (94%) using 24 hour Holter monitoring and exercise stress testing (De Maria *et al*, 1992). High-grade ventricular arrhythmias, including multiform ventricular premature complexes, ventricular pairs and tachycardia were recorded in 60% of the patients with DCM (De Maria *et al*, 1992). Despite the frequent detection of high-grade ventricular arrhythmias, the patients were unaware of this dysrythmia during the Holter monitoring (De Maria *et al*, 1992). The grading in severity of ventricular arrhythmias (number of ventricular premature beats and tachycardia episodes) showed no significant relation to echocardiographic or hemodynamic indices of ventricular dimension (left and right ventricular end-diastolic diameter), or cardiac function tests (left ventricular ejection fraction, cardiac index, pulmonary capillary wedge, right ventricular end-diastolic and mean right atrial pressures) (De Maria *et al*, 1992). Dilated cardiomyopathy patients with a higher ventricular arrhythmia grade had a higher 3-year mortality rate (De Maria *et al*, 1992). Ventricular arrhythmia in people with DCM has been found to be a major independent predictor of prognosis, after markers of ventricular dysfunction such as left ventricular ejection fraction and stroke work index (De Maria *et al*, 1992). Arrhythmias have been reported to be more common in dogs with cardiomyopathy than in those with less severe heart disease (Hazlett *et al*, 1983).

Cardiac catheterization refers to the combined hemodynamic and angiographic procedures that are recommended for people with suspected DCM (Grossman, 1991). Left ventricular filling pressure and cardiac output can be quantified after right-sided

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cardiac catheterization (Manolio *et al*, 1992). Left cardiac catheterization allows quantification of left ventricular end-diastolic pressure and diminished ejection fraction (Manolio *et al*, 1992). Coronary arteriography allows the examination of coronary arteries for obstruction and helps to differentiate myocardial infarction due to obstructive coronary disease from the interstitial myocardial fibrosis seen in DCM (Wynne and Braunwald, 1992). Left ventriculography in people with DCM typically reveals diffuse hypokinesis, in contrast to people with coronary artery disease, in which there is often a portion of the left ventricular wall which continues to exhibit normal motion (Wynne and Braunwald, 1992). The typical long axis (L) to minor axis (M) ratio for the left ventricle at end diastole in people is 2:1. With DCM, this L:M ratio approaches 1:1, like a sphere (Grossman, 1991).

Magnetic resonance imaging (MRI) is a technique based on the property of atomic nuclei to absorb and release energy when placed in a magnetic field and subjected to intermittent radio-frequency pulses. The spatial organization of the tissue is determined by applying an intermittent, weak magnetic gradient and measuring the slight variations in the characteristic resonant frequency of nuclei that varies with location in the field. MRI allows the visualization of pathological abnormalities such as ventricular dilation and variations in heart wall thickness. While MRI can be carried out for many atoms that have a magnetic moment, imaging is mostly carried out for hydrogen nuclei (protons) (Higgins, 1992). Studies of the nuclear magnetic resonance spectra for phosphorus-31 (^{31}P-MRI) in the hearts of patients with DCM have allowed the comparison of myocardial phosphate metabolism with clinical cardiovascular parameters (Neubauer *et al*, 1992). Data from a group of 19 patients with DCM could not be

distinguished from the group of 19 normal volunteers on the basis of ^{31}P-MRI data (Neubauer *et al*, 1992). However, when the 19 patients with DCM were grouped according to the New York Heart Association (NYHA) classification for the clinical severity of heart failure, the myocardial creatine phosphate (CP) to adenosine triphosphate (ATP) ratio (^{31}P-MRI) decreased progressively with increasing severity of heart failure (Neubauer *et al*, 1992). The CP/ATP ratio was regarded as having a low specificity for the diagnosis of DCM (Neubauer *et al*, 1992).

Gated radionuclide scanning for uptake of gallium-67 citrate or indium-111 antimyosin is an invasive technique for the detection of acute myocardial necrosis (Manolio *et al*, 1992). This technique relies on the affinity of gallium-67 citrate or indium-111 antimyosin for tumors and inflammatory reactions. After intravenous administration of gallium-67 citrate or indium-111 antimyosin, patients can be imaged with a gamma camera for myocardial radionuclide uptake and the images photographed or captured in a computer (O'Connell *et al*, 1981).

1.2.3. Clinical chemistry

Clinical pathological changes that have been reported in dogs with congestive heart failure include hyponatremia, hypoproteinemia, hyperkalemia, increased blood volume, increased serum hepatic enzyme levels; mildly increased serum creatinine, urea, and lactate; increased serum norepinephrine, atrial natriuretic factor, renin, and aldosterone concentrations; and decreased sodium excretion (O'Brien *et al*, 1993).

Biochemical markers for the serological detection of cardiomyocyte damage include myoglobin, isoforms of creatine kinase, cardiac troponins, and lactate dehydrogenase (Maxwell *et al*, 1995; Guest and Jaffe, 1995; Fitzgerald *et al*, 1996).

Troponin T is a tropomyosin-binding protein that forms part of the troponin regulatory complex in the thin filaments of the contractile apparatus of striated muscle (Guest and Jaffe, 1995). Myocardial damage, due to infarction in people and hypoxia in chickens, releases cardiac troponin T into the blood where it can be measured by an enzyme-linked immunosorbent assay (ELISA). Serum troponin T levels were at the minimal detection limits for the assay in normal mixed-breed dogs, Sprague Dawley rats, and healthy ferrets (O'Brien et al, 1997). The technique of collecting blood by cardiac puncture in ferrets has been reported to result in high variations in serum troponin T concentrations, ranging from 0.1 to 133 $\eta g/ml$ (O'Brien et al, 1997). After damage to cardiomyocytes, cardiac troponin T remains elevated for 4 to 7 days (Guest and Jaffe, 1995). Cardiac troponin T is a cardiospecific molecule that has a low (0.5%) cross-reactivity with skeletal troponin T (Müller-Bardorff et al, 1995). Serum concentration of cardiac troponin T had 98% sensitivity and 78% specificity for the detection of myocardial infarction in a group of 370 people who were evaluated for myocardial infarction (Fitzgerald et al, 1996). Minor myocardial damage that did not meet the WHO criteria for myocardial infarction was thought be the cause for the lower cardiac specificity of cardiac troponin T (Fitzgerald et al, 1996). Serum cardiac troponin T was increased 1,000 to 10,000-fold in canine and rat models of myocardial infarction, and was highly correlated with infarct size within 3 hours of injury (O'Brien et al, 1997). Myocardial levels of troponin T level have been reported to be significantly decreased in Dobermans with DCM compared to normal mixed breed dogs, when expressed per wet weight of myocardium (O'Brien, 1997).

1.2.4. Pathological changes in people with DCM

Gross pathological changes in 152 people (16 to 78 years of age) who died with DCM included dilation of all chambers and increased weight of the heart in all individuals. Despite the increased weight of the hearts, the maximum thickness of the left ventricular free wall and ventricular septum were close to normal (Roberts *et al*, 1987). Mural thrombi were frequently found in the left ventricle and right atrial appendage. Intracardiac thrombi were found in 42 (28%) of the 148 people who had DCM confirmed at autopsy. Focal endocardial thickenings were present near the apices of the ventricles in 32 (22%) of the 148 people who died with DCM. Scar tissue was grossly visible in the left and/or right ventricular myocardium of 22 (14%) people who died with DCM (Roberts *et al*, 1987). Pulmonary and/or systemic emboli were demonstrated in 60% (79 of 131) of the people with DCM who had been studied both clinically and at autopsy. Of these 79 people, 51% (40) had pulmonary emboli, 16% (13) had systemic emboli and 33% (26) had both (Roberts *et al*, 1987). In another study of 10 DCM hearts from people who had cardiac transplants, the mean left ventricular weight was increased 2.2 fold, mean left ventricular volume was increased 4.2 fold resulting in a 48% reduction in mass-to-volume ratio compared to normal hearts (Beltrami *et al*, 1995).

Histopathological changes in the myocardium from 96 people who died with dilated cardiomyopathy included multiple patchy areas of replacement fibrosis in the left and right ventricles (Roberts *et al*, 1983). Myocardial replacement fibrosis was often accompanied by increased perivascular and perimyocyte fibrosis in the left ventricle. Interstitial inflammatory cell infiltrates were present in the ventricular myocardium of only 5% (5 in 96) of the patients who died with DCM. Only one person had multiple foci

of necrotising inflammation in the myocardium. Another person had lymphocytic infiltrates and replacement fibrosis in the myocardium, a progressive 5-week-course of congestive cardiac failure and positive serology for Coxsackie B_3 virus. Small foci of coagulative necrosis were in the left ventricle of 11% (11 out of 96), and in the right ventricle of 10% (8 out of 83) of people who died with DCM. Small perivascular aggregates of mononuclear cells in the myocardial intestitium were regarded as normal "sentinel" cells (Roberts et al, 1987). Despite the meticulous nature of this morphologically based study of DCM in 152 people, this research did not provide any clues to the cause of DCM (Roberts et al, 1987).

In a histopathological study of the myocardium from 9 people who died with DCM, a pattern of increasing fibrosis in the left ventricular free wall from the epicardium to the endocardium was reported (Unverferth et al, 1986). Fibrosis was greater in the left compared to the right side of the interventricular septum. Myocardial cell diameter was greater (22 ± 5 μm) in the DCM people compared to a control group (17 ± 2 μm) (Unverferth et al, 1986).

Morphometric studies of enzymatically isolated cardiomyocytes in normal adult human hearts have established the mean myocyte dimensions to be approximately 130 to 140 μm long and approximately 18 μm wide (Gerdes and Capasso, 1995). The ratio of cardiomyocyte length to width in normal adult humans, cats, rats, hamsters and guinea pigs is approximately 7:1 (Gerdes and Capasso, 1995). Cardiomyocytes are branching, join to adjacent fibers by intercalated discs, are usually binucleate in the adult and have nuclei located centrally (Braunwald et al, 1992). Each cardiac muscle fiber contains numerous myofibrils that extend the entire length of the fiber. Individual myofibrils are

composed of serially arranged sarcomeres that are aligned with the sarcomeres in adjacent myofibrils to give the characteristic striated pattern to cardiac muscle fibers (Braunwald et al, 1992). There is general agreement from morphometric studies of human hearts with DCM that the left ventricular cardiomyocyte length is increased (Beltrami et al, 1995; Gerdes and Capasso, 1995; and Schaper et al, 1995). In 10 humans with DCM the mean lengthening of left ventricular myocytes was 59% and mean myocyte volume was increased 2-fold in both ventricles (Beltrami et al, 1995).

Despite recent intensive morphometric studies in animal models and people with DCM, the mechanism of ventricular dilation remains controversial (Gerdes and Capasso, 1995; Poole-Wilson, 1995; Schaper et al, 1995). Three structural changes that have been reported to occur in DCM include myocyte lengthening by in-series addition of new sarcomeres (Beltrami et al, 1995; Gerdes and Capasso, 1995), side-to-side slippage of cells within the ventricular wall, and increase in volume of remaining viable myocytes (Kajstura et al, 1995).

Cardiomyocytes are normally arranged in a spiral around the ventricular free wall (Poole-Wilson, 1995). A connective tissue "skeleton", consisting of an extensive network of collagen fibers, fine microfibrils and microthreads, normally invests cardiomyocytes. The connective tissue forms fine struts that extend between and tether adjacent myocytes. Myocardial connective tissue plays an important role in myocyte orientation, intracellular force transmission in systole, and tissue compliance in diastole (Braunwald et al, 1992). In a study of myocardium from people with DCM, about 40% of left ventricular cardiomyocytes had a reduction or absence of myofilaments, contractile proteins (myosin), tropomyosin, troponin T, elastic filament (titin) and α-

actinin (Hein *et al*, 1994). The cytoskeleton component titin is a large protein that contributes to the compliance and elastic recoil of cardiac and skeletal muscle. Titin is also important in the formation of new sarcomeres, since it serves as the template for the laying down of myosin filament and the actin-tropomyosin-troponin complex. While there are reduced amounts of the titin mRNA in hearts of people with end-stage DCM, increased breakdown of titin filaments by proteolysis may also explain its absence from some myofilaments in people with DCM (Hein *et al*, 1994). Desmin and tubulin are cytoskeleton elements that provide a structural framework within cardiomyocytes to maintain the spatial arrangements of subcellular organelles. In people with end-stage DCM, about one third of left ventricular free wall cardiomyocytes have increased levels of tubulin and desmin in a disorderly array (Schaper *et al*. 1991).

Histopathological descriptions used in this thesis have been based on histomorphological changes reported in human coronary heart disease (Baroldi, 1975; Pasternak *et al*, 1992) and in veterinary reference literature (Robinson and Maxie, 1993). Three histopathological forms of myocardial necrosis have been recognised in human coronary heart disease. **Coagulative necrosis** is characterized, in the early stages, by hypereosinophilic sarcoplasm, persistence of cross-striation (even in 25-day-old infarcts), nuclear changes (hyperchromasia), and neutrophil infiltration (Baroldi, 1975). Healing involves macrophage infiltration and phagocytosis, but no granulation tissue is formed (Baroldi, 1975). **Coagulative myocytolysis,** or contraction band necrosis, is seen as a series of irregular, transverse, hypereosinophilic bands separated by lighter, slightly granular areas in the myocytes (Baroldi, 1975). These contraction bands have been interpreted as coagulation of hypercontracted sarcomeres. In the early stages, the nucleus

appears normal at the light microscope level. There is progressive lysis of the sarcoplasm and nucleus, leaving an intact, empty sarcolemmal tube. Lymphocytes may or may not be present at foci of coagulative myocytolysis (Baroldi, 1975). Occasionally macrophages, laden with granular yellow-brown material, can be seen in sarcolemmal tubes. Experimental models of heart failure have associated this change with excessive release of endogenous catecholamines (Baroldi, 1975). Prominent contraction bands can also be seen in normal myocardium that is fixed immediately after death. **Colliquative myocytolysis** is seen as swelling of myocytes, loss of striations and a homogeneous weakly eosinophilic sarcoplasm. There is early lysis of myofibrils, late nuclear lysis, and phagocytosis of necrotic myocytes (Baroldi, 1975; Pasternak *et al*, 1992). Necrosis can be induced by energy deprivation (e.g., defects in oxidative phosphorylation, mitochondrial toxins, anoxia, ischemia), gene-directed mechanisms (apoptosis, cytokines), membrane lysis (complement, lysins, porins), free radicals, blockage of protein synthesis (poisons operating at the nucleolar, ribosomal or postranscriptional level) or cytoskeleton disruption (Cheville, 1994).

While the precise mechanism of injury to myocardial cells in Dobermans with DCM is unknown, there is evidence for a 30% reduction in the level of myocardial ATP (in Dobermans with DCM compared to healthy mixed breed dogs) associated with decreased activity of mitochondrial respiratory chain enzymes and myoglobin (McCutcheon *et al*, 1992). In experimental models of myocardial ischemia, produced by coronary artery occlusion, myocyte ATP levels were 35% of normal in 15 minutes and down to only 9% of normal in 30 minutes. Mitochondrial swelling and lysis occur

coincidental to dilation and collapse of the sarcoplasmic reticulum, vesicle formation, margination of chromatin and loss of glycogen (Cheville, 1994).

Cardiac myocytes have been regarded as terminally differentiated cells which lack the ability to divide to regenerate damaged myocardium. Recently, mitotic figures have been demonstrated in myocytes from the ventricular myocardium of people with congestive heart failure due to DCM, ischemic cardiomyopathy and valvular heart disease (Quaini *et al*, 1994). In order to identify a single mitotic figure, an average of 425 mm^2 of myocardium was examined from each patient. Two to 3 mitotic figures were observed per 1,000,000 nuclei in the myocardium from people with heart failure. Proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase delta, has been demonstrated in 55% of ventricular myocardial cells in people with end-stage DCM. The presence of PCNA in myocytes is considered an indicator that these cells may enter the cell cycle, undergo nuclear deoxyribonucleic acid (DNA) synthesis and nuclear mitotic division (Quaini *et al*, 1994). While autoradiographic detection of thymidine-labeled myocytes has been used to demonstrate DNA synthesis, this technique does not differentiate whether nuclear DNA synthesis is due to nuclear hyperplasia, ploidy formation or DNA repair (Quaini *et al*, 1994).

Replacement fibrosis refers to a discrete area of myocardial scarring resulting from focal myocyte loss (Beltrami *et al*, 1995). Cardiomyocyte necrosis in cardiomyopathy may result from angiotensin II stimulating the release of catecholamines from the adrenal medulla (Campbell *et al*, 1995). Experimental infusion of angiotensin II in rats produced fibroblast proliferation both at sites of cardiomyocyte necrosis, and in myocardial perivascular locations at 2 and 4 days, but not after that time (Campbell *et al*,

1995). Macrophages and lymphocytes were present at sites of angiotensin II-induced cardiomyocyte necrosis, and persisted for 6 weeks (Campbell *et al*, 1995). With aldosterone infusion in mice, fibroblast proliferation, reactive and reparative fibrosis did not appear until week 3. Macrophage and lymphocyte infiltration occurred between weeks 3 to 8, following aldosterone infusion in rats (Campbell *et al*, 1995). **Segmental fibrosis** comprises an area of myocardial fibrosis greater than 1 cm^2 representing a healed myocardial infarct. **Interstitial fibrosis** corresponds to an increase in the thickness of connective tissue separating cardiomyocytes, in the absence of myocyte necrosis (Beltrami *et al*, 1995).

Myocardial ultrastructural changes observed in people and animals dying with DCM are the same as those found in people and dogs with longstanding ventricular hypertrophy with subsequent congestive cardiac failure and are not specific for cardiomyopathy (Ferrans *et al*, 1972; Bishop, 1986).

1.2.5. Pathological changes in dogs with DCM

Pathological findings in 8 large breed dogs (two Afghans, two Dobermans, three Great Danes and one St. Bernard), which died with DCM, included increased total heart weight (mean 440.1 g, compared to control group mean 257.4 g), and increased heart to body weight percentage (mean affected 0.79% compared to control group 0.63%) (Sandusky *et al*, 1984). All four chambers of the heart were dilated and enlarged, giving the hearts a globoid appearance. In six of the eight dogs, the left ventricular free wall was thinner than normal and the papillary muscles appeared shrunken. The hearts were pale in all dogs. White areas of myocardial scarring were present in the left ventricular

myocardium of five out of eight dogs. No thrombi were detected in the hearts. Multifocal infarcts were present in the kidneys and spleens of 2 dogs (Sandusky *et al*, 1984).

In a retrospective study of 26 dogs necropsied with heart lesions, 62% (16) were Dobermans (Hazlett *et al*, 1983). Ventricular dilation and atrio-ventricular valvular endocardiosis were prominent gross heart lesions in the 16 Dobermans. Dilation of the left ventricle was seen in 7 Dobermans and right ventricular dilation was seen in 10 Dobermans. Left atrial dilation often accompanied left ventricular dilation. Irregular areas of scarring were occasionally observed in the myocardium of Dobermans with severe heart disease. Endocardiosis of the atrio-ventricular valves appeared to be more advanced in the Dobermans with cardiac disease than is commonly seen as an aging change in dogs. Atrial thrombosis was only seen in Dobermans with cardiomyopathy. Extra-cardiac lesions associated with heart failure included pulmonary congestion and edema (with heart failure cells in alveoli), hepatic congestion, hydrothorax, ascites and subcutaneous edema (Hazlett *et al*, 1983). Dobermans with DCM were found to have both interstitial and replacement myocardial fibrosis, fatty replacement, cardiac myofiber degeneration and/or vacuolation, and arterial intimal cushion formation in the myocardium (Hazlett *et al*, 1983). However these authors did not report focal myocardial necrosis or myocarditis in Dobermans with DCM.

Histopathological changes in 8 large breed dogs that died with DCM included multifocal myocardial degeneration, necrosis and fibrosis (Sandusky *et al*, 1984). These changes were most severe around the papillary muscles of the left ventricle. Myocardial fibrosis was transmural in the left ventricle of 2 dogs. Degenerating myofibers contained

sarcoplasmic vacuoles of varying size. One dog had multifocal lymphocytic infiltration in the myocardium of the left atrium (Sandusky et al, 1984).

Ultrastructural changes in eight giant breeds of dogs that died with DCM included disruption of myofibrils, thickening of myofiber Z bands, increased intermyofibrillar spaces; increased lipofuscin granules, fat droplets and myelin figures; and mitochondrial hyperplasia (Bishop, 1986). In dogs that died with DCM, mitochondria were enlarged, irregularly shaped, occasionally with swollen, disrupted cristae. Occasionally, enlarged mitochondria were clustered together pushing myofibrils apart (Sandusky et al, 1984). Enlarged and hyperplastic mitochondria were considered to reflect deranged mitochondrial metabolism and to be a secondary change resulting from a wide range of disease processes (Bishop, 1986). The degree of ultrastructural degeneration was more severe in dogs with cardiomyopathy than in dogs with other causes of heart failure. These ultrastructural changes may account for the poor contractility in DCM, but there was no morphological evidence for the primary cause of DCM (Bishop, 1986).

1.2.6. Myocardial biochemical changes in DCM

Biochemical profile testing, aimed at studying the major metabolic pathways in the myocardium of Dobermans with DCM, revealed a marked reduction in respiratory chain enzymes and myoglobin with ATP reduction and elevated lactic acid levels (McCutcheon et al, 1992). Myocardial mitochondrial activities of the following were markedly reduced in 10 Dobermans with DCM: nicotinamide adenine dinucleotide dehydrogenase (NADHdh, respiratory chain complex I, NADH ubiquinone oxidoreductase, EC 1.6.5.3) was decreased by 60%; adenosine triphosphate (ATP)

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synthetase was reduced by 45%; myoglobin was decreased by 88%; sarcoplasmic reticulum Ca^{2+}- transport ATPase (SR Ca^{2+}-ATPase) was reduced by 42% and ATP levels were reduced by 31% compared to the levels in healthy adult mixed breed dogs. In contrast, other mitochondrial matrix enzymes, aspartate transaminase (AST), β-hydroxyacyl coenzyme A dehydrogenase (HADH) and oxoglutarate dehydrogenase (ODH) in the Dobermans with DCM were increased in activity by 9%, 14% and 22% respectively compared with the levels in healthy dogs. This study revealed a pronounced reduction in the mitochondrial oxidative production of ATP by Dobermans with DCM (McCutcheon et al, 1992). Further studies comparing myocardial enzyme activities in normal dogs, dogs subjected to rapid ventricular pacing and Dobermans with DCM demonstrated that the decreased glycolysis (indicated by the level of myocardial lactate dehydrogenase) and oxidative phosphorylation (F_1-ATP synthetase and myoglobin) in Dobermans with DCM was associated with decreased levels of the specific mRNA translated for these enzymes and myoglobin (O'Brien et al, 1995). A small (10%) increase in the mRNA content for myocardial citrate synthetase (an indicator of tricarboxylic acid cycle activity) was recorded in Dobermans with DCM (O'Brien et al, 1995). Earlier studies found no significant difference in myocardial citrate synthetase activity (per wet weight of myocardium) in Dobermans with DCM compared to normal mixed breed dogs (McCutcheon et al, 1992). A measure of the total mitochondrial protein per unit of myocardial cell protein and the expression of all values per gram of non-collagen protein for normal dogs, paced dogs and Dobermans with DCM would have assisted in the interpretation of changes in enzyme levels in both the former studies. These papers provide evidence for increased mitochondrial fat oxidation and up-

regulation of the mRNA for citrate synthetase (a mitochondrial tricarboxylic acid enzyme) and decreased gene expression for ATP synthetase and myoglobin (reflecting decreased myocardial oxidative phosphorylation) (McCutcheon *et al*, 1992; O'Brien *et al*, 1995).

Myoglobin is a small water-soluble protein that resides in the sarcoplasm and facilitates the transport of oxygen from the capillaries, through the cell wall, and down a concentration gradient to the mitochondria. Studies in various models of heart failure have demonstrated that the normal myocardial myoglobin concentrations of healthy mixed breed dogs (2.15 ± 0.52 mg/g wet weight) were reduced in dogs paced to heart failure (1.09 ± 0.34 mg/g wet weight) (O'Brien *et al*, 1992). Myocardial myoglobin was also found to be reduced in apparently healthy Dobermans (1.05 ± 0.22 mg/g wet weight) and Dobermans with DCM (0.47 ± 0.25 mg/g wet weight) (O'Brien *et al*. 1992). Thus, there is a correlation between myocardial myoglobin depletion and congestive heart failure in paced dogs and Dobermans with DCM (O'Brien *et al*, 1992). Later studies demonstrated that the mRNA content for myocardial myoglobin was reduced both in dogs subject to rapid ventricular pacing for 3 weeks and Dobermans with DCM when compared to that of adult healthy dogs (O'Brien *et al*, 1995). Experimental inactivation of myoglobin with sodium nitrite reduced the maximal rate of relaxation in feline right ventricular papillary muscle by 10% (Braunlin *et al*, 1986). This reduced relaxation of the papillary muscle was attributed to insufficient oxygen for oxidative phosphorylation, leading to reduced ATP (Braunlin *et al*, 1986).

Cardiac myocyte contraction and relaxation are regulated by precisely timed modulations of free calcium (Ca^{++}) in the sarcoplasm. This signaling function of Ca^{++}

necessitates a very low ionic concentration of the cation in the sarcoplasm. Two mechanisms function to maintain a low calcium ion concentration ($[Ca^{++}]$). These include the transmembrane transport of Ca^{++}, and the formation of reversible complexes between Ca^{++} and non-membranous (protein) ligands. Seven Ca^{++} transmembrane transport systems have been recognised in cardiomyocyte membranes. Three are located in the sarcolemma, two in the sarcoplasmic reticulum and two in the mitochondrial membranes. It is the sarcoplasmic reticulum that regulates the rapid and fine changes in $[Ca^{++}]$ linked to the contraction and relaxation cycle (Carafoli, 1985). Excitation is initiated by the cardiac action potential producing an inflow of Ca^{++} through a voltage dependent Ca^{++} channel in the sarcolemma. This inflow of Ca^{++} into the sarcolemma does not activate the contractile system directly, but activates a large release of Ca^{++} from the terminal cisternae of the sarcoplasmic reticulum (SR), the so called "calcium-induced calcium release" (Braunwald *et al*, 1992). This large release of Ca^{++} from the SR is modulated via the calcium release channel of the SR (the ryanodine receptor) and the slow calcium channel (the dihydropyridine receptor) of the transverse tubular membrane (Lytton and MacLennan, 1993). Free Ca^{++} in the sarcoplasm binds to troponin, forming activated troponin-C, which in turn binds tightly to the inhibitory molecule, troponin-I (Opie, 1997). This binding repositions troponin-M (tropomyosin) on the actin, which removes the inhibition exerted by tropomyosin on the actin-myosin interaction, thus initiating the cross-bridge cycle (Opie, 1997). While this initiation of contraction proceeds with the passive flow of calcium down its concentration gradient, relaxation necessitates the expenditure of ATP to pump Ca^{++} out of the sarcoplasm. Three enzymatic Ca^{++} pumps move Ca^{++} out of the sarcoplasm. One ATPase pump resides in

the SR while another is located in the sarcolemma (plasma membrane). A third pump, the Na-K ATPase, can participate by actively pumping Na^+ out of the cell, allowing Ca^{++} to move out of the cell via the Na^+- Ca^{++} exchange channel (Lytton and MacLennan, 1993).

In addition to the entry of Ca^{++} through the voltage-dependent gated Ca^{--} "slow channel", Ca^{++} can also enter cardiomyocytes by receptor-operated (agonist/antagonist) Ca^{++} channels in the sarcolemma. $Beta_1$ receptors in cardiomyocyte sarcolemma respond primarily to neuronally released norepinephrine producing an increased Ca^{++} influx and enhanced contractility (Braunwald *et al*, 1992). The tension developed in cardiomyocytes, the rate at which tension develops and the rate of decline in tension during relaxation are related, respectively, to the concentration of Ca^{++} available for binding to troponin, the rate with which Ca^{++} is delivered to troponin, and the rate at which Ca^{++} is removed from troponin (Braunwald *et al*, 1992). The long-term alterations in Ca^{++} pumps are controlled by the number of active Ca^{++} pump enzymes. The number of Ca^{++} pump enzymes is determined by the rates of biosynthesis versus degradation for these proteins. Biosynthesis of the Ca^{++} pump proteins commences with transcription of messenger RNA (mRNA) encoding for that particular protein. In hypothyroidism there is a decrease in both the enzyme activity and in the mRNA encoding for it (Lytton and MacLennan, 1993).

Studies of the ^{3}H-labelled ryanodine binding to the myocardial SR calcium release channel demonstrated a 50% reduction in the number of sites in Dobermans with DCM and dogs paced to heart failure, compared to that in healthy dogs (Cory *et al*,

1993). The myocardial SR Ca^{++}-ATPase pump was reduced by 42% in Dobermans with DCM compared with healthy dogs (McCutcheon *et al*, 1992).

Studies of gene expression and activity of sarcoplasmic reticulum Ca-cycling indicated that the sarcoplasmic reticulum Ca-release channel (mRNA) was more down-regulated than the SR Ca^{++}-ATPase pump in Dobermans with DCM compared to healthy mixed breed dogs. It was hypothesized that reduced movement of Ca^{--} out of the SR Ca-release channel in systole would reduce the energy (ATP) required to sequester Ca^{--} in the SR during diastole. However this reduced Ca^{++} cycling in cardiomyocytes would reduce the inotropic potential and leave the myocardium susceptible to fatigue and failure (O'Brien *et al*, 1995)

Carnitine is a zwitterionic[2] compound formed from lysine that has an important role in the transport of activated long chain fatty acids from the sarcoplasm, across the inner mitochondrial membrane into the mitochondrial matrix (Stryer, 1995). Under resting conditions, approximately 70% of myocardial metabolic energy is derived from fatty acids. More than 95% of metabolic energy derived from foods is used to form ATP in mitochondria (Guyton, 1991). Long chain fatty acids cannot penetrate the inner mitochondrial membrane before being activated in the presence of coenzyme A, ATP and acyl CoA synthetase. The formation of one molecule of fatty acid CoA requires the consumption of two high-energy phosphate bonds from ATP (Stryer, 1995). Carnitine deficiency is recognized in the myocardium of people with DCM, however the significance has not been established (Wynne and Braunwald, 1992). Myocardial

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[2] Zwitterions are biochemical forms that possess both a positive and a negative charge, but no net charge (Houston, 1995).

carnitine deficiency has been demonstrated in both boxer dogs and Dobermans with DCM (Keene *et al*, 1986).

Taurine (2-aminoethane sulphonic acid) is the most abundant free amino acid in the mammalian myocardium (Kramer *et al*, 1995). While taurine deficiency in cats is recognized to cause reversible heart failure, deficiency of taurine in people with DCM has not been established (Wynne and Braunwald, 1992). No significant differences occurred in plasma taurine concentrations in 76 dogs with DCM and normal dogs (Kramer *et al*, 1995).

1.2.7. Lactate and creatinine metabolism in healthy individuals and those with cardiac disease

Lactate is produced in the myocardium, skeletal muscle and other tissues from pyruvate by glycolysis when there is an insufficient supply of oxygen at the level of the mitochondria (Weltman, 1995; Stryer, 1995). Glycolysis is the cytosolic reaction sequence whereby glucose is converted to pyruvate with a net gain of 2 molecules of ATP per molecule of glucose. This low energy yield for the conversion of glucose to pyruvate under anaerobic conditions contrasts to the total yield of 36 to 38 ATP generated from the complete oxidation of one molecule of glucose by mitochondrial oxidative phosphorylation (Lehninger *et al*, 1993) (Fig. 1.2.). Under normal physiological conditions, the myocardium derives most of its energy from mitochondrial oxidative phosphorylation. Beta-oxidation of fatty acids provides the major source of reducing equivalents (NADH, $FADH_2$) to the respiratory chain. The complete oxidation of one molecule of palmitoyl coenzyme A to carbon dioxide and water, yields 129

molecules of ATP (Houston, 1995). When oxygen supply to the myocardial mitochondria becomes limiting, the rate of ATP synthesis declines and creatine phosphate (phosphocreatine) provides a short-term source of ATP (Lehninger *et al*, 1993).

Glycogenolysis is the reaction whereby glycogen undergoes phosphorolytic cleavage, catalysed by glycogen phosphorylase to yield glucose-1-phosphate. Glucose formed in the liver and kidney by glycogenolysis is available to the entire body via the bloodstream. However, glucose-1-phosphate, catalytically cleaved from glycogen in skeletal muscle (particularly type IIB) fibers, cannot be dephosphorylated and is not available to the bloodstream. Muscle glycogen stores provide sufficient energy for about 1.5 minutes of maximal muscle contraction in people. ATP and phosphocreatine (creatine phosphate) constitute the phosphagen energy system and can provide sufficient energy for about 10 seconds of vigorous muscle contraction in people. Creatine (methyl guanidoacetic acid) is synthesised from glycine, arginine and s-adenosyl methionine. Creatine kinase (CK) catalyzes the reversible transfer of creatine to creatine phosphate. In normal dogs, creatinine is the catabolite that is excreted from muscle into circulation at the same rate as the kidneys excrete it in the urine.

$$\text{Creatine} + \text{ATP} \underset{\longrightarrow}{\overset{CK}{\longleftarrow}} \text{ADP} + \text{H}^+ + \text{Phosphocreatine} \xrightarrow{P_i} \text{creatinine}$$
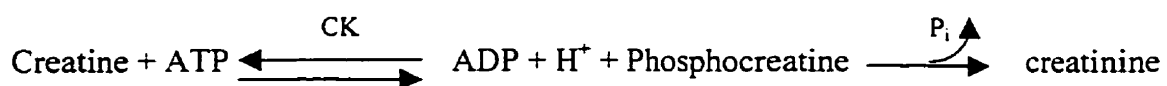
Creatinine is freely filtered through the glomerulus in the kidney at a fairly constant rate (e.g. human plasma creatinine clearance ranges from 95 to 105 ml/min) (Clarenburg, 1992).

Lactate is freely filtered through the glomerular basement membrane and is reabsorbed in the proximal convoluted tubule against a concentration gradient in people

and dogs (Dies *et al*, 1969; Poortmans and Vanderstraeten, 1994). Proximal tubular reabsorption of lactate is rate limited. Some lactate is excreted in the urine even at low glomerular filtrate loads. A direct correlation exists between lactate excretion and urine flow. When dogs were given an intravenous priming dose of 10 mg furosemide per kg body weight and then continuously infused with furosemide at 0.15 mg/kg, the expected lactic acid excretion (1.88 μmol/min) closely matched the measured urinary excretion rate (2.01 μmol/min) (Dies *et al*, 1969). In people, urinary excretion of lactate plays only a minor role in lactate removal after exercise. Only 2% of total lactate produced during moderate exercise could be recovered in the urine (Poortmands and Vanderstraeten, 1994).

Lactate metabolism, particularly elevated blood lactate, and histopathological studies of affected tissue are recognised as supporting evidence for mitochondrial disease (Scholte *et al*, 1987). Where mitochondrial diseases are suspected in people, skeletal muscle is the tissue of choice for biochemical assessment. Even when the clinical involvement of organs such as the brain or heart is more prominent, skeletal muscle is recommended for investigation of mitochondrial defects in oxidatve phosphorylation (Scholte *et al*, 1987).

1.2.8. Mitochondrial structure and function and changes with heart failure

Mitochondria are subcellular organelles in which energy from the terminal oxidation of foods is transferred to the high energy pyrophosphate bond of adenosine triphosphate (ATP). This ATP then provides the energy for the cellular functions of muscle contraction, ionic movements across plasma membranes (Ca^{++}-ATPase, Na$^+$-K$^+$ ATPase, SR Ca^{++}-ATPase), biosynthesis and signal transduction (Stryer, 1995).

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The shape of mitochondria, and number per cell, varies among tissues. In muscle, mitochondria are ellipsoid and approximately 2 to 3 μm long, and about 1 μm wide. Mitochondria constitute as much as 20% of the total cell volume in myocardial tissue. The mitochondrion has a smooth outer membrane and a highly folded inner membrane. The space between the inner and outer membranes is termed the intermembrane space. The highly invaginated inner membrane encloses a central space that contains the mitochondrial matrix (Erecinska and Wilson, 1981).

The outer mitochondrial membrane has transmembrane channels composed of the protein porin that are permeable to molecules less than 5,000 molecular weight (Lehninger et al, 1993). The inner membrane is impermeable to the passive flow of most molecules as well as protons (H^+). Specific transporter proteins, in the inner mitochondrial membrane, transport pyruvate, fatty acids, amino acids (or their α-keto derivatives), adenosine diphosphate (ADP) and inorganic phosphate (P_i) and protons (H^-) into the matrix (Lehninger et al, 1993).

In 12 dogs in which chronic heart failure was induced by intracoronary microembolization, the number of mitochondria per 100 mm^2 of myocardium was higher compared to that in controls (92 $\pm$ 5 vs 64 $\pm$ 2) (Sabbah et al, 1992). Average mitochondrial size was small in heart failure dogs compared to normal dogs (0.53 $\pm$ 0.3 vs 0.78 $\pm$ 0.04 μm^2 respectively (Sabbah et al, 1992).

Catabolism of fats, carbohydrates and some amino acids for energy occurs in 3 stages of cellular respiration. In stage 1, fatty acids, glucose and some amino acids are oxidized to yield the 2 carbon, acyl group of acetyl-coenzyme A (acetyl-CoA) (Stryer, 1995). Stage 2 involves the oxidation of acetyl groups via the citric acid (Krebs or

tricarboxylic acid) cycle to yield electrons and carbon dioxide (CO_2). The free energy released by oxidation in stage 2 is transferred as electrons to nicotinamide adenine dinucleotide (NAD^+ is the oxidized form) and flavin adenine dinucleotide (FAD, the oxidized form), and stored in the reduced electron carriers NADH and $FADH_2$. During stage 3, the reduced cofactors NADH and $FADH_2$ are oxidized by NADH dehydrogenase and succinate dehydrogenase, releasing protons (H^+) and electrons. These electrons are transferred along a chain of electron-carrying molecules (the respiratory chain) to oxygen (O_2), which is reduced to water (H_2O). The passage of electrons along the respiratory chain, to reduce molecular oxygen, is coupled to the condensation of ADP and P_i to form ATP. This process is termed oxidative phosphorylation. When electrons are transferred from NADH to O_2, they pass through 3 of the respiratory chain complexes. NADH dehydrogenase (NADHdh; NADH ubiquinone oxidoreductase: complex I), cytochrome reductase (ubiquinol ferricytochrome C oxidoreductase, complex III) and cytochrome oxidase (ferrocytochrome C oxygen oxidoreductase, complex IV). Electrons transferred from $FADH_2$ enter the respiratory chain via succinate dehydrogenase (complex II, the only citric acid cycle embedded in the inner mitochondrial membrane) and then to ubiquinone, cytochrome reductase, and then cytochrome oxidase. Cytochrome oxidase completes the sequence by transferring electrons to O_2 (Stryer, 1995). The mechanism whereby electrons flow along the respiratory chain to synthesise ATP from ADP and P_i, and reduce oxygen to water, was originally proposed in the chemiosmotic hypothesis (Mitchell, 1961). Mitchell proposed that as electrons flow along the respiratory chain, protons are actively pumped across the inner mitochondrial membrane (Mitchell, 1961). The free energy generated by the electrical potential and pH gradient across the inner

mitochondrial membrane constitutes a proton motive force (Mitchell, 1961). This proton motive force is utilised by the final respiratory complex, ATP synthetase, for the synthesis of ATP from ADP and P_i (Mitchell, 1961). Currently it is believed that the number of protons pumped from the matrix to the intermembrane space per pair of electrons transferred along the respiratory chain is 4, 2 and 4, respectively, for nicotinamide adenine dinucleotide-cytochrome Q reductase (NADH-Q reductase), cytochrome reductase and cytochrome oxidase (Stryer, 1995). The pH gradient and membrane potential created by this movement of H^- from the intermembrane space into the F_o (protein channel) component of ATP synthetase leads to the release of ATP (Stryer, 1995). Until recently, it was thought that the complete oxidation of a glucose molecule led to the formation of 36 or 38 molecules of ATP, depending on which shuttle system was used to transfer reducing equivalents (NADH formed glycolysis in the cytosol) into the mitochondrial matrix. In the myocardium, electrons from cytosolic NADH are brought into the mitochondrial matrix by the malate-aspartate shuttle (Lehninger et al, 1993). In the matrix, reducing equivalents are passed by the action of malate dehydrogenase to NAD^+, forming NADH; which in turn passes electrons to complex I. Three molecules of ATP are generated as the pair of electrons pass along the respiratory chain to O_2. In skeletal muscle, the glycerol-3-phosphate shuttle delivers reducing equivalents from NADH into complex III (not complex I), leading to the production of 2 molecules of ATP per pair of electrons (Lehninger et al, 1993). Recently the stoichiometry of mitochondrial oxidative phosphorylation has been re-examined and there is disagreement regarding the exact number of ATP generated per molecules of oxygen consumed (P/O ratio). Maximal P/O ratios of 3 for the oxidation of NADH-

linked substrates and 2 for the oxidation of succinate have become widely accepted for mitochondrial oxidative phosphorylation (Lehninger et al, 1993). Different research groups studying mitochondrial respiratory chain activity in isolated rat liver mitochondria have reported P/O levels of 2.5 or greater than 2.5 for NADH-linked substrates and 1.5 or greater than 1.5 for succinate (Hinkle et al, 1991; Lee et al, 1996). There is clearly a need for further research into the molecular mechanisms of proton translocation through respiratory complexes I, III and IV, and the energy requiring reactions that compete with ATP systems under experimental, physiological and pathological conditions (Lee et al, 1996).

Approximately 95% of the metabolic energy liberated from food is directed towards ATP production by oxidative phosphorylation in mitochondria. During resting (aerobic) conditions, cardiac muscle derives approximately 70% of its energy requirements from fatty acids, predominantly oleic and palmitic acids (Fig. 1.1.). Under anaerobic or ischemic conditions, blood glucose is utilized to generate energy via glycolysis (Fig. 1.2.). This leads to the formation of lactic acid in the myocardium (Guyton, 1996). Myocardial glycogen provides a significant contribution to overall myocardial glucose utilization in isolated working rat hearts (Henning et al, 1996). Pathways for glycogen synthesis and glycogenolysis are separate. Enzymes for glycogen synthesis and catabolism are regulated in a reciprocal fashion, such that when glycogen synthesis is stimulated, glycogenolysis is inhibited, and vice versa. Net glycogenolysis was recorded in isolated rat hearts, working aerobically, while perfused with insulin and physiological levels of fatty acids (Henning et al, 1996). Glucose was metabolized from myocardial glycogen stores by glycolysis and glucose was oxidized despite physiological

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$$C_{16}H_{32}O_2 + 23\ O_2 + 129\ (ADP + P_i) \rightarrow 129\ ATP + 16\ CO_2 + 42\ H_2O$$
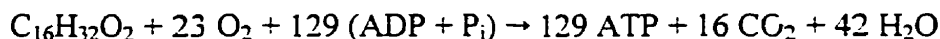
Beta oxidation of palmitic acid

$$C_6H_{12}O_6 + 6\ O_2 + 36\ (ADP + P_i) \rightarrow 36\ ATP + 6\ CO_2 + 42\ H_2O$$
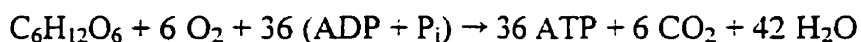
Complete oxidation of glucose

The respiratory quotient is the molar ratio of CO_2 produced divided by the O_2 consumed during oxidation of the metabolic fuels. The respiratory quotient (RQ) can be calculated as follows:

$$RQ \text{ for palmitic acid } = \frac{16}{23} = 0.7$$

$$RQ \text{ for glucose } = \frac{6}{6} = 1.0$$

The P/O (or ATP/O) ratio compares the number of ATP generated for each atom of oxygen consumed in mitochondrial oxidative phosphorylation.

$$P / O \text{ for palmitic acid is } \frac{129}{(23x2)} = 2.8$$

$$P / O \text{ for glucose is } \frac{36}{(6x2)} = 3.0$$

For the same amount of oxygen consumed, more energy can be generated from glucose than fatty acid (Houston, 1995).

Fig. 1.1. Comparison of ATP generation and O_2 utilization during the complete oxidation of a fatty acid (palmitic acid) and glucose.

$$\text{Glucose } (C_6 H_{12} O_6) + 2P_i + 2ADP + 2NAD^+ \longrightarrow$$

$$\longrightarrow 2 \text{Pyruvate } (CH_3 - \underset{\underset{O}{\|}}{C} - COO^-) + 2ATP + 2NADH + 2H^+ + 2H_2O$$

$$\downarrow$$

Lactate

$$CH_3 - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - COO^-$$
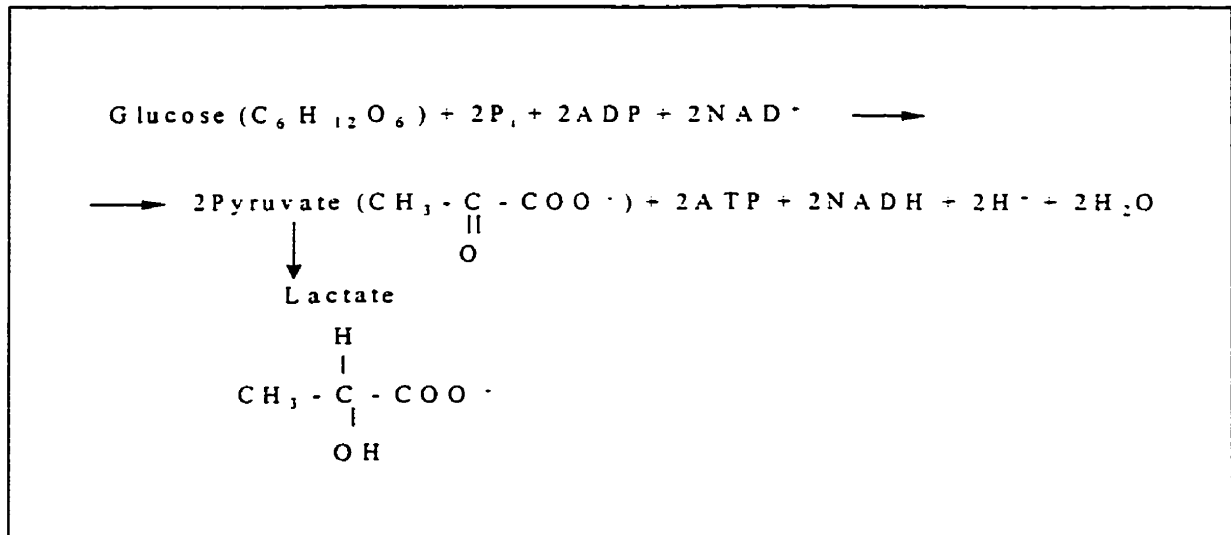
Fig. 1.2. Glycolysis (Embden-Myerhof pathway) (Stryer, 1995).

levels of fatty acids and insulin in the perfused fluids. Glucose from myocardial glycogen stores was oxidized in preference to exogenous glucose. These findings confirmed that carbohydrate metabolism in cardiomyocytes is compartmentalized. It was estimated that in the aerobic perfused working rat heart. exogenous palmitate was the preferred energy source contributing to over 50% of ATP produced. Oxidation of glucose provided 14%, and lactate oxidation contributed about 5% of ATP produced. Endogenous fatty acid (triacylglycerol) and total glucose (including that from endogenous glycogen) provided about 25% of total ATP. Glycolysis produces 7% of total ATP, with 1% to 2% of the total ATP coming from glycolysis of glucose from glycogen (Henning et al, 1996). Creatine phosphate can provide high-energy phosphate in the short term for both skeletal and cardiac muscle. In myocardial ischemia. creatine phosphate is rapidly utilized over a few minutes to supply high energy phosphate (Wynne and Braunwald, 1992). Physiological studies of saline-perfused rat hearts. based on ^{1}H nuclear magnetic resonance signal of tissue myoglobin, indicated no biochemical or physiological signs of myocardial hypoxia when the myocyte intracellular PO_2 level was above 4 mm Hg (Kreutzer and Jue, 1995). These studies indicated a sequential change in cellular metabolism, in response to limiting intracellular O_2 (Kreutzer and Jue, 1995). At a myocyte intracellular PO_2 level of 4mm Hg, there was an increase in the myocardial lactate formation rate and a decrease in the heart rate pressure product (RPP) (heart rate multiplied by the left ventricular developed pressure) (Kreutzer and Jue, 1995). When myocyte intracellular PO_2 decreased by a factor of 2, from 4 to 2 mm Hg, the myocardial oxygen consumption decreased by 13%, creatine phosphate level fell by 10% and there were negligible changes in myocyte intracellular ATP levels (Kreutzer and Jue, 1995). It

was estimated that at the critical point for intracellular PO_2 level of 2 mm Hg, the PO_2 gradient from capillary to cell was 15-30 mm Hg compared to an intracellular PO_2 gradient from cytosol to mitochondria of 1 mm Hg (Kreutzer and Jue, 1995).

Mitochondria are thought to have arisen when purple photosynthetic bacteria were assimilated into eukaryotic cells in a process termed endosymbiosis (Watson *et al*, 1992). The presence of a separate genome within mitochondria (mitochondrial deoxyribonucleic acid, mtDNA) gives rise to many unique and fascinating attributes for these organelles (Tzagoloff and Myers, 1986). Since the ovum carries several hundred thousand mtDNA while a sperm only has a few hundred mtDNA, it is predominantly the mother who transmits her mtDNA to the offspring. The relatively few mtDNA in the sperm that fertilizes the ovum, have little effect on the genotype of the embryo (Wallace, 1992). Some authors report that the mitochondria in sperm are detached at fertilization, so it is exclusively the maternal mtDNA in the ovary that are transmitted to the progeny (Ozawa, 1995). Other studies report that after 26 generations of back-crossing in mice, paternal mitochondrial DNA can be detected by the polymerase chain reaction (Watson *et al*, 1992). Each human cell contains hundreds of mitochondria and thousands of mtDNA. These mitochondria and mtDNA undergo replicative segregation at each mitosis of somatic cells and meiosis in gonadal germ cells (Brown and Wallace, 1994; Wallace, 1992). Mitochondrial DNA has been completely sequenced in people, cattle, mouse and frog. These mtDNAs from different species are almost identical in size and have the same organization and complement of genes that code for very homologous products (Tzagoloff and Myers, 1986). The human mtDNA is small, only 16,596 base pairs (bp) long, compared to nuclear DNA, which is about 10^9 bp. Mitochondrial DNA is a closed

circular molecule with two strands that differ markedly in base composition. However mtDNA is one of the most economically packaged genomes studied. Nearly every nucleotide in mtDNA appears to be part of a coding sequence, for a protein, rRNA or tRNA. During growth and division of eucaryotic cells, mitochondrial mass is increased by the addition of newly synthesized lipids and proteins to existing organelles and not by *de novo* formation of mitochondria (Tzagoloff and Myers, 1986).

Both nuclear and mitochondrial DNA contribute to mitochondrial oxidative phosphorylation complexes and metabolism. The nucleus contains genes for 80% of the oxidative phosphorylation pathway including all of the genes for mitochondrial intermediary metabolism and all the protein genes for mitochondrial biogenesis (replication, transcription, translation and ribosomal proteins). Mitochondrial DNA provides 13 protein-coded genes towards the oxidative phosphorylation apparatus, including seven subunits of NADH-ubiquinone oxidoreductase (respiratory chain complex I), one apocytochrome b of the ubiquinone cytochrome C reductase (complex III), three subunits of cytochrome C oxidase (complex IV) and two subunits of the H^--translocating ATP synthetase (complex V) (Brown and Wallace, 1994).

The amino acid sequence for the nuclear and mitochondrial encoded sub-units of mitochondrial NADHdh has not been determined in the dog. No reports are available, to date, comparing the NADHdh sub-units in different tissues of the dog. Studies of cytochrome C oxidase in the rat have demonstrated differences in nuclear-encoded sub-units between heart and skeletal muscle (Kuhn-Nentwig and Kadenbach, 1985). However, the mitochondrial-encoded sub-units for cytochrome C oxidase are believed to be identical in all tissues of the rat (Kuhn-Nentwig and Kadenbach, 1985).

Aging in people is associated with a reduction in the activity of oxidative phosphorylation enzymes, reduced ATP production and the accumulation of mtDNA deletions (Brown and Wallace, 1994; Corral-Debrinski et al. 1991; Linnane et al. 1989; Wallace, 1992; Wallace, 1994). The reduced rate of oxygen (O_2) utilization and ATP synthesis is associated with an age-associated increase in somatic mtDNA damage in post-mitotic tissue (Brown and Wallace, 1994; Corral-Debrinski et al, 1991; Wallace, 1992). Sources of defects in the oxidative phosphorylation apparatus include inherited nuclear and mtDNA defects, acquired mutations and oxidative damage to the respiratory chain by free radicals. Mitochondrial DNA has a very high mutation rate, 10 to 20 times higher than nuclear DNA.

Spontaneous damage of DNA by thermal fluctuations, reactive metabolites (free radicals) and radiation (ultraviolet light) alters thousands of DNA nucleotides in a typical mammalian cell every day. Nuclear DNA is protected from oxidants by compartmentalization away from mitochondria and peroxisomes, where most oxidants are produced. Non-replicating nuclear DNA is surrounded by histones and polyamines which may protect against oxidants. Damaged, lost or altered nuclear DNA can be repaired by a series of DNA repair nucleases. Most of these nuclear DNA repair mechanisms depend on the presence of two copies of genetic information, one in each strand of the DNA double helix (Ames, 1989; Lehninger et al, 1993). Until recently, it was thought that mammalian adult ventricular cardiomyocytes were postmitotic cells with no capacity for nuclear DNA synthesis or repair. Studies on cardiomyocytes isolated from adult cat ventricle have been shown to support active nuclear DNA repair

and synthesis of both endogenous nuclear and exogenous plasmid DNA (Kozlovskis *et al*, 1996). In contrast, the mitochondrial DNA is composed of 2 dissimilar strands, formed into a closed circle without DNA-coating proteins such as histones. Mammalian cells (mouse L, human KB and HeLa cells) were unable to remove pyrimidine dimers that had been induced in mitochondrial DNA by ultraviolet irradiation (Clayton *et al*. 1974). The level of 8-hydroxydeoxyguanosine (a base product of radiation damage) in rat livers was found to be extremely high in mitochondrial DNA (1 per 8,000 bases) compared to nuclear DNA (1 per 130,000 bases). This high level of oxidized bases in mtDNA may be due to the reactive oxygen species, inadequate mtDNA repair and the absence of histones in mitochondria (Ames, 1989). Mitochondrial DNA is located inside the inner mitochondrial membrane where reactive oxygen species are continually generated by normal electron transport, mtDNA is rapidly replicated by DNA polymerase gamma without proofreading and there is no evidence for an efficient mtDNA repair system (Linnane *et al*. 1989; Ozawa, 1995; Wallace, 1992; Yen *et al*, 1993). Mitochondrial DNA in the myocardium of mature Sprange-Dawley rats has been shown to have a half-life of 6.7 days through studying the incorporation of thymine deoxyribonucleoside-methyl-^{3}H (Gross *et al*, 1969). About 90% of inhaled oxygen is consumed by mitochondria and about 1 to 5 % of this oxygen is converted to reactive oxygen species in the mitochondria such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). Mitochondria metabolize superoxide by the manganese-containing superoxide dismutase, and hydrogen peroxide is metabolized by the selenium-containing glutathione peroxidase. Reactive oxygen species can also be scavenged by vitamin C, vitamin E, glutathione, and ubiquinol-10 (ubiquinol, coenzyme

Q, vitamin Q_{10}) (Richter *et al*, 1995; Wallace *et al*, 1995). The role of free radical scavengers in the early stages of cardiomyopathy has been investigated by comparing the free radical scavenger enzyme activity in the myocardium of an inbred strain of hamsters carrying the autosomal recessive cardiomyopathic gene, *cm* (the Bio 14.6 Syrian hamster) to that of a normal control strain (Bio 14.6 HAM) (Kobayashi *et al*, 1994). The myocardial activity of glutathione peroxidase in 30-day-old Bio 14.6 cardiomyopathic Syrian hamsters was approximately twice that of age-matched normal control (Bio 14.6 HAM) Syrian hamsters. However there was no significant difference in the myocardial glutathione peroxidase activity in 90-day-old Bio 14.6 cardiomyopathic Syrian hamsters compared to age-matched normal controls. The intraperitoneal administration of α-tocopherol (70 mg/kg per day from 21 days of age, for 70 days) reduced the total cross-sectional area of myocardial damage (fibrosis plus calcification) in Bio 14.6 hamsters compared to placebo-treated control Bio 14.6 Syrian hamsters. While the area of myocardial fibrosis was smaller in the α-tocopherol-treated Bio 14.6 hamsters, there was no significant difference from the control Bio 14.6 hamsters, which received the intraperitoneal vehicle alone. No significant difference in the myocardial superoxide dismutase activity could be demonstrated in the hearts of Bio 14.6 cardiomyopathic and normal control Bio 14.6 hamster hearts at 30 and 90 days of age (Kobayashi *et al*, 1994).

The ratio of oxidized to reduced glutathione in the liver, kidney and brain of rats and mice increases (up to 600%) with aging. Oxidative damage to mtDNA, measured by the level of 8-oxo-7,8-dihydro-2-dexoyguanosine (oxo8dG), increased with age in rats and mice. Treatment with antioxidants prevented the increased oxo8dG and oxidative damage to mtDNA with aging in mice and rats (García de la Asunción *et al*, 1996).

Superoxide, hydrogen peroxide and hydroxyl radical are mutagens produced by radiation and byproducts of oxidative phosphorylation. Lipid peroxidation produces mutagenic lipid epoxides, lipid hydroperoxides, lipid alkoxyl and peroxyl radical, and enals (α, β- unsaturated aldehydes) (Ames et al, 1993). A two aldehydic lipid peroxidation product, 4-hydroxynonenal (HNE), has been shown to interact with phospholipids in the mitochondrial membrane, enhancing cross-linking among lipid moieties and decreasing the membrane fluidity in hepatic mitochondria of rats. It was proposed that reactive products of lipid peroxidation, such as HNE, can produce age-related damage to mitochondrial membranes that decrease membrane fluidity (Chen and Yu, 1994).

Evidence is accumulating that links apoptosis to mitochondria, reactive oxygen species, intracellular Ca^{++} and the balance between Bcl-2 and Bax genes (Korsmeyer et al, 1995; Ozawa, 1995; Ritcher et al, 1995; Slater et al, 1995). Apoptosis (programmed cell death) can be induced by hypoxia, low levels of reactive oxygen species (H_2O_2) and elevation of intracellular Ca^{++} (Slater et al, 1995; Yao et al, 1996). The protooncogene product of Bcl-2 is localized to mitochondrial membranes (as well as endoplasmic reticular and nuclear membranes) where it dimerises with a structurally related protein product, from the Bax gene. The mitochondria are the target of the protooncogene products of the Bcl-2 and Bax genes, that manipulate oxygen free radical damage and determine whether a cell will undergo apoptosis (Ozawa, 1995). Apoptosis is thought to be regulated by oxygen free radical damage generated in mitochondria. This free radical damage can be down-regulated by the protooncogene product of Bcl-2 and up-regulated by the Bax gene. Mutations of mtDNA are thought to cause an imbalance of the

mitochondrial antioxidant pathways and control mechanisms for the protooncogenes Bcl-2 and Bax leading to apoptosis (Ozawa, 1995). Apoptosis has been identified ultrastructurally in sinus node tissue surgically excised from people with the long QT syndrome and in neonatal rat cardiomyocytes cultured under hypoxic conditions. Elevated levels of deoxyribonuclease I (DNAase I) in the left ventricle of 13 people with end-stage heart failure due to DCM (compared to non-diseased heart samples) implicates apoptosis in the pathophysiology of this disorder (Yao *et al*, 1996).

Mitochondrial DNA mutations have been classified into 4 groups, including missense, protein synthesis, insertion-deletion and copy number mutations (Wallace, 1992). Germ-line mutations in mtDNA can also be categorized depending on whether those cause substitutions in protein-coding genes (mit⁻) or disrupt basepairing in tRNA. Genotypes of people with mtDNA mutations can then be classified into 2 major groups, (mit⁻) and (mit⁻ + syn⁻) (Ozawa, 1995).

Deleterious mtDNA mutations in germ cells can lead to maternally transmitted disease. The accumulation of mtDNA mutations in post-mitotic somatic tissue leads to progressive decline in mitochondrial bioenergetics with aging (Wallace, 1992).

Defects in oxidative phosphorylation affect tissues that are most reliant on aerobic mitochondrial energy, such as the central nervous system, heart, skeletal muscle, kidney, liver and pancreatic islets (Wallace, 1992).

Mammalian mitochondria can contain mixtures of normal and mutant mtDNA (heteroplasmy) (Wallace, 1992). During meiosis and mitosis the proportion of normal and mutant mtDNA distributed to daughter cell varies. Over repeated cell divisions, the proportions of mutant mtDNA can drift continuously from normal to increasing

percentages of mutant mtDNA. The threshold for expression of a mtDNA mutation is dependent upon the nature of the mutation, the percentage of mutant mtDNA (per mitochondrion, cell, tissue or organ) and the reliance of each organ system on aerobic energy production from mitochondria (Wallace, 1992). The percentage of heteroplasmy for the 8993 mtDNA mutation in people determines the severity of the disease phenotype. People with greater than 90% heteroplasmy for the 8993 mutation have Leigh's disease and lactic acidemia; people with between 80 and 89% heteroplasmy have a later onset of disease with mental impairment, ataxia, and retinitis pigmentosa; with 60 to 80% heteroplasmy, people only develop a late onset retinitis pigmentosa; people with less than 60% heteroplasmy for the 8993 mtDNA mutation are asymptomatic (Robinson, 1994).

The hearts of normal people accumulate very little mtDNA mutations up to 30 years of age after which there is a gradual increase in the number of deletions (Wallace *et al*, 1995). Chronic cardiac ischemia in people produces an 8 to 2,000 fold increase in mtDNA deletions compared to age-matched controls. People who die of acute cardiac disease have fewer mtDNA deletions than age-matched people who die with chronic heart disease (Corral-Debrinski *et al*. 1991; Wallace *et al*, 1995). Mitochondrial DNA mutations lead to a vicious cycle wherein the defective mitochondrial respiratory chain enhances free radical formation resulting in increased accumulation of hydroxyl radical damage (Ozawa, 1995). An increase in the percentage of mtDNA deletions was correlated with an increase in hydroxyl radical adducts in mtDNA hydrolysate, such as 8-hydroxydeoxyguanosine (8-OH-dG) in place of deoxyguanosine (dG), in the myocardium of people with DCM and hypertrophic cardiomyopathy (Ozawa, 1995).

There is increasing interest in dietary antioxidants and other modulators of free radical metabolism in view of the evidence about free radical damage to mtDNA (Ames et al, 1993). Vitamin C (ascorbate, ascorbic acid), vitamin E (α-tocopherol) and carotenoids are antioxidants that can be acquired in the diet from fruits and vegetables (Ames et al, 1993). Ubiquinone (coenzyme Q, vitamin Q_{10}) is a component of the respiratory chain that accepts electrons from NADH dehydrogenase (NADHdh: complex I) and succinate dehydrogenase (complex II). The reduced form, ubiquinol, is an effective antioxidant in membranes. Optimal dietary levels of ubiquinone could be important in degenerative diseases (Ames et al, 1993). Dietary restriction (60% total intake of ad lib fed control group) in rats decreased free radical damage to lipids in cardiac mitochondria (decreased cardiac mitochondrial malondialdehyde level) and enhanced the antioxidant defense system (Kim et al, 1996).

1.2.9. Diagnosis and categorization of inherited cardiomyopathies

Dilated cardiomyopathy is an end-stage of disease resulting from a wide range of insults to the myocardium. It is recommended that investigations of DCM in people should be approached from the basic sciences, emphasizing biochemical, immunological and genetic studies (Manolio et al, 1992). Inherited cardiomyopathies in people can be divided into 2 broad categories: A. Disorders of cardiac energy metabolism include defects of mitochondrial oxidative phosphorylation or fatty acid oxidation, and B. abnormalities of myocardial contractile and structural proteins which include familial hypertrophic cardiomyopathy and X-linked muscular dystrophies (Table 1.2.) (Kelly and Strauss, 1994).

A) Disorders of cardiac energy metabolism

1) <u>Defects in mitochondrial oxidative phosphorylation causing cardiomyopathy.</u>
Cardiomyopathy due to defects of mitochondrial oxidative phosphorylation are usually
due to mutations in the nuclear or mitochondrial DNA. These mitochondrial diseases can
be categorized into 3 groups. The first group involves defects restricted to the
mitochondrial genome which are inherited through the maternal line. The second group
includes diseases that result from defects in both nuclear and mitochondrial genes. A
third group involves mitochondrial disease due to a defect in the nuclear genes, in which
the nuclear genome produces defective subunits for the respiratory chain complexes, or
normal proteins that regulate mitochondrial biogenesis or subunit transport and assembly.
Respiratory complex I (NADHdh) deficiency has been reported in people, and a wide
range of clinical presentations has been observed, including cardiomyopathy (Pitkanen *et
al*, 1996). The reduction in respiratory complex I and III activity in affected individuals
ranged from 35 to 80% (Pitkanen *et al*, 1996). This third group does not exhibit maternal
transmission (Bu and Rotter, 1993). Proof that a mitochondrial disease has a genetic
basis requires the specific identification of gene mutation(s) (Wallace, 1992).
Conventional investigations of gene defects have been based on polymerase chain
reaction (PCR) or Southern blot analysis.

A) Disorders of cardiac energy metabolism

1) Defects in mitochondrial oxidative phosphorylation causing cardiomyopathy

        a) Defects involving both cardiac and skeletal myopathy

i) Lethal infantile cardiomyopathy
ii) Benign infantile mitochondrial myopathy and cardiomyopathy
iii) Maternally inherited myopathy and cardiomyopathy
iv) Inherited cardiomyopathy with multiple deletions in mitochondrial DNA

        b) Defects of multiple organ-systems including cardiomyopathy

i) Myoclonic epilepsy and ragged-red fiber disease
ii) Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
iii) Kearns-Sayre syndrome

2) Cardiomyopathy due to inborn errors of fatty acid oxidation

        a) Carnitine transport defect
        b) Carnitine-acylcarnitine shuttle defects

i) Carnitine-acylcarnitine translocase defect
ii) Carnitine palmitoyltransfease II defects

        c) Defective mitochondrial fatty-acid β oxidation

i) Long-chain acylcoenzyme A dehydrogenase defects
ii) Long-chain 3-hydroxyacyl-CoA dehydrogenase defects

B) Abnormalities of myocardial contractile and structural proteins

1) Familial hypertrophic cardiomyopathy
2) X-linked muscular dystrophies
        a) Becker's muscular dystrophy
        b) Duchenne's muscular dystrophy
        c) X-linked dilated cardiomyopathy

Table 1.2. Categorization of inherited cardiomyopathy in people (Kelly and Strauss, 1994).

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A more recent approach for the detection of somatic mutations in human mtDNA includes:

a) a total detection system for deletion using 180 kinds of primer pairs;

b) a primer-shift PCR method for localizing and confirming mtDNA mutations;

c) a kinetic PCR to quantify the amount of deleted mtDNA;

d) micro high-performance liquid chromatography / mass spectrometry for the quantification of oxygen free radical damage in mtDNA (Ozawa, 1995).

Most cases of genetically defective oxidative phosphorylation in people causing cardiomyopathy are due to mutations in mitochondrial DNA (Kelly and Strauss, 1994). Deletions of mtDNA and point mutations in mitochondrial tRNA genes have been identified as mitochondrial genome mutations causing cardiomyopathy (Table 1.3.) (Kelly and Stauss, 1994).

2) Cardiomyopathy due to defective fatty acid oxidation

Inborn errors of transport proteins or enzymes for β oxidation of fatty acids are among the most common inherited metabolic diseases in people, ranging from 1 in 10,000 to 1 in 15,000 live births (Kelly and Strauss, 1994). Typically the cardiomyopathy is hypertrophic with decreased systolic function. The recorded cases of defective fatty acid oxidation in people are inherited in an autosomal recessive fashion (Kelly and Strauss, 1994).

A) Mitochondrial genome mutations

1) **Point mutations**
   - **a)** Myoclonic epilepsy and ragged-red fiber diseases ($tRNA^{Lys}$)
   - **b)** Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes ($tRNA^{Leu}$)
   - **c)** Maternally inherited myopathy and cardiomyopathy ($tRNA^{Leu}$)
   - **d)** Fatal infantile cardiomyopathy ($tRNA^{Ile}$)

2) **Deletions**
   - **a)** Inherited cardiomyopathy with multiple deletions of mtDNA
   - **b)** Kearns-Sayre syndrome

B) Nuclear genome defects (known and suspected cases)

1) Benign infantile mitochondrial myopathy and cardiomyopathy (suspected nuclear genome defect)
2) Barth's syndrome (X-linked nuclear genome defect)

Table 1.3. Classification of mutations in people causing cardiomyopathy due to defective oxidative phosphorylation (Kelly and Stauss, 1994).

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B) Abnormalities of myocardial contractile and structural proteins

1) Familial hypertrophic cardiomyopathy

Familial hypertrophic cardiomyopathy is due to mutations of the β cardiac myosin heavy-chain gene and is inherited as an autosomal dominant disease in over 50% of cases (Kelly and Strauss, 1994). The genetic basis of familial hypertrophic cardiomyopathy is heterogeneous. In many cases, point mutations have been demonstrated in the cardiac β myosin heavy-chain (chromosome 14 region q11 to q13). In other cases, mutations have been mapped to chromosomes 1q3, 15q2 and 11p13 to q13 (Kelly and Straus, 1994).

2) X-linked muscular dystrophies

Both Becker's and Duchenne's muscular dystrophies are caused by abnormalities of the dystrophin gene located at chromosome Xp21. Dystrophin is a structural protein that is expressed in brain, skeletal and cardiac muscle. While a skeletal myopathy is the main clinical manifestation of both Becker's and Duchenne's muscular dystrophies, this can be accompanied by cardiomyopathy. In Becker's muscular dystrophy, cardiomyopathy is rare and progressive cardiac failure is not a feature of this disease. Cardiac involvement is seen in over 80% of people with Duchenne's muscular dystrophy, however cardiac failure only occurs in about 10% of affected boys (Muntoni *et al*, 1993).

X-linked dilated cardiomyopathy is a primary myocardial disease causing congestive cardiac failure in teenage boys. Female carriers of this disease have a later onset (fifth decade) and slower progression of heart failure. The genetic defect was a deletion of the first muscle exon containing the muscle promoter of the dystrophin gene in all members of a family with X-linked DCM (Muntoni *et al*, 1993).

1.2.10. Pathogenesis of dilated cardiomyopathy

Dilated cardiomyopathy is a primary myocardial disease characterized by impaired myocardial contractility and the development of left ventricular or biventricular dilation. Sudden death is recognized to occur in both people and Doberman Pinscher dogs with DCM (Calvert, 1995; Tamburro and Wilber, 1992). About 30% of deaths in people with DCM are sudden. Both ventricular tachycardia and bradyarrhythmias are high risk factors for sudden death in people with DCM (Tamburro and Wilber, 1992). The pathogenesis of ventricular arrhythmias in DCM has not been clearly established. Factors which are believed to contribute to ventricular arrhythmias (VA) and sudden death include patchy myocardial fibrosis (causes heterogeneous repolarization), intramyocardial emboli (produces ischemia and scarring), depletion of serum potassium and magnesium (diuretic therapy), sympathetic activation (catecholamines may stimulate VA directly or potentiate extracellular hypokalemia), digitalis and antiarrhythmic drugs (may increase frequency, complexity and duration of VA) (Tamburro and Wilber, 1992).

Sudden death and heart disease in 10 out of 11 Dobermans was attributed to luminal narrowing of small coronary arteries and degenerations of the adjacent bundle of His (James and Drake, 1968). Five of the Dobermans that died suddenly were related to a common male ancestor (James and Drake, 1968). A subsequent survey of atrioventricular conducting system in 40 large breed dogs (including 10 Dobermans), revealed no evidence of cardiac disease, but increased fibrous connective tissue, fat and loss of conducting fibers in the interventricular septum (Sandusky *et al*, 1979). This change was interpreted as a normal aging change in dogs over 5 years of age (Sandusky *et al*, 1979).

Despite recent intensive morphometric studies in animal models and people with DCM, the mechanism of ventricular dilation remains controversial. Three structural changes that have been reported to occur in DCM include myocyte lengthening (Beltrami *et al*, 1995; Gerdes and Capasso, 1995), side-to-side slippage of cells within the ventricular wall (Beltrami *et al*, 1995) and in-series addition of new myocytes (Kajstura *et al*, 1994).

Four basic mechanisms have been investigated in studies on the pathogenesis of DCM: 1) familial and genetic factors, 2) viral myocarditis and other cytotoxic insults, 3) immune abnormalities, and 4) metabolic, energetic and contractile abnormalities. The familial and genetic causes of DCM in people have been reviewed earlier in this thesis. The modes of inheritance for familial cases of DCM in people vary widely from the maternal inheritance of point mutations in mtDNA (tRNA^{Leu}) for maternally inherited myopathy and cardiomyopathy to X-linked inheritance of Duchenne's muscular dystrophy (Kelly and Strauss, 1994).

Enteroviruses, particularly group B Coxsackie viruses, have been considered important in the pathogenesis of myocarditis and DCM (Dec and Fuster, 1994; Fujioka *et al*, 1996). Molecular hybridization studies have demonstrated enteroviral nucleic acids in the myocardium of people with DCM, as well as control subjects. In a critical study of the specific types of enterovirus in the myocardium, positive PCR results were observed in 6 (19%) of 31 people with DCM, 5 (18%) of 28 with myocarditis, 5 (22%) of 23 with other cardiac diseases, and from the pericardial effusions in 4 (57%) of 7 people with pericarditis. Investigation of the PCR-single strand conformation polymorphism in most of the clinical samples were different and not identical to the forms of group B Coxsackie

viruses recognized in human disease. It was concluded that enterovirus genomes are present in the myocardium of people with heart disease, but there are no particular forms of enterovirus in the myocardium of people with DCM (Fujioka et al, 1996).

Natural infection with canine distemper (morbillivirus), canine herpesvirus, canine parvovirus-2, and experimental infection with the herpesvirus of pseudorabies have been recorded to cause myocarditis in dogs (Van Vleet and Ferrans, 1986). Inflammatory cell infiltrates, usually involving small scattered foci of lymphocytes in the myocardial interstitium, were absent in some surveys (Hazlett et al, 1983) and only occasionally seen in other studies (Sandusky et al, 1984) of dogs that died with DCM.

Both cellular and humoral immune abnormalities have been recognized in people with myocarditis and DCM. However, whether these changes are cause or consequence, and their role in pathogenesis remains uncertain (Dec and Fuster, 1994; Lange and Schreiner, 1994). Reduced activity of natural killer cells (involved in viral defense) and suppressor lymphocytes have been identified in people with DCM (Dec and Fuster, 1994). Genes of the major histocompatibility complex (MHC, also known as human leukocyte antigen) located on chromosome 6, regulate the immune response at the cellular and molecular level. In a study of the distribution of the HLA-DQBI gene in 44 normal people and 34 people with DCM, histidine at position 30 of the HLA-DQBI gene was found more frequently in DCM patients (62%) compared to controls (36%) (Limas et al, 1995). It has been suggested that since this gene is associated with immunoregulation; the presence of histidine at position 30 of the HLA-DQBI gene may predispose to DCM via an immune mechanism (Dec and Fuster, 1994).

Circulating autoantibodies to cardiac antigens that have been identified in people with DCM include those to B_1-adrenergic receptors, mitochondrial antigens (adenine nucleotide translocator, branched-chain ketoacid dehydrogenase, M7 and adenosine diphosphate-adenosine triphosphate carrier proteins) and cardiac myosin heavy chain. It is not known whether these autoantibodies are pathogenic or reflect long-term myocardial injury (Dec and Fuster, 1994).

Abnormalities in cardiac high-energy phosphate metabolism, myocardial calcium cycling and β adrenergic receptor density and activation have been demonstrated in people and animals with DCM, other naturally occurring cases of cardiac failure and in experimentally induced heart failure (Gwathemy and Davdoff, 1993; Dec and Fuster, 1994). Changes in these metabolic, energetic and contractile processes probably represent markers in the progression of heart failure rather than initiators of DCM (Dec and Fuster, 1994).

Decreases in major markers of myocardial mitochondrial energy synthesis (ATP synthetase and adenine nucleotide translocase), myocardial calcium cycling (Ca-release channel and Ca pump activities), myocardial myoglobin and the mRNA's that regulate the synthesis of these proteins have been reported in dogs subjected to rapid ventricular pacing and Dobermans with DCM. These results provide evidence for a similar profile of metabolic and energetic changes in both dogs experimentally paced to heart failure (and DCM) and Doberman Pinschers with DCM. It was proposed that reduced levels of the mRNA's translated for key enzymes and proteins in the energy metabolism and calcium cycling of dogs paced to heart failure represent altered gene expression. Reduced levels of Ca-release channel and the SR-ATPase were interpreted as a

mechanism to conserve ATP. Decreased SR calcium release channel activity would reduce the Ca^{++} released into the sarcoplasm and also reduce the ATP required by SR-ATPase to move Ca^{++} out of the sarcoplasm during diastole (O'Brien *et al*, 1995). Reduction in myocardial ATP production and calcium cycling would reduce the rate and force of myocardial contractility (Braunwald *et al*, 1992).

Some patterns of change in the cardiovascular system are regarded as common to the dilated cardiomyopathies regardless of etiology. While reduced myocardial contractility causes decreased systolic function and ejection fraction, stroke volume is maintained by increased end-systolic and end-diastolic volumes. As ejection fraction is reduced further, ventricular volume and wall stress increase in an effort to maintain stroke volume. Heart rate may be increased to maintain normal cardiac output when stroke volume is reduced. An important factor in the further deterioration of the ejection fraction is the progressive elevation in ventricular wall stress due to internal after-load, which develops before external after-load (increased peripheral vascular impedence). With progressive ventricular dilation, atrioventricular valvular regurgitation leads to reduced stroke volume (due to loss of a regurgitant fraction), increased ventricular volume and increased ventricular filling pressure. Ventricular dysfunction in DCM leads to inadequate cardiac output ("forward failure") and elevated ventricular filling pressure is transmitted from the left ventricle to pulmonary circulation and from the right ventricle to systemic venous circulation ("backwards failure") (Stevenson and Perloff, 1988).

In response to reduced cardiac output and atrial hypertension, a complex series of neurohumoral mechanisms is initiated in the early stages of acute systolic heart failure. The neuroendocrine mechanisms of vasoconstriction include the sympathetic nervous

system, renin-angiotensin system, endothelin and arginine vasopressin. Comparisons of people with cardiac systolic and diastolic dysfunction have indicated that impaired systolic contractility is the parameter associated with activation of the sympathetic nervous system, renin-angiotensin-aldosterone axis and release of arginine vasopressin. Early activation of the sympathetic nervous system is a major noncardiac compensatory response to decreased cardiac output. There is evidence for early sympathetic activation (increased plasma norepinephrine levels) in people with asymptomatic left ventricular dysfunction (ejection fraction of $\leq$ 35%). Decreased arterial blood pressure causes a decrease in the baroreceptor inhibitory impulses which increases sympathetic activation (Benedict and Phil, 1994). Increased plasma norepinephrine (NE) results from increased release of NE from adrenergic nerve endings and its spillover into plasma. The level of plasma NE elevation is directly correlated with the severity of left ventricular dysfunction in people (Braunwald et al, 1992). Increased sympathetic activity (adrenergic drive), in the early stages of heart failure, increases myocardial contractility, raises peripheral arterial pressure and redistributes blood from nonvital vascular beds. In the late stages of severe cardiac failure, these compensatory mechanisms of sympathetic activation may be detrimental by increasing after-load, initiating cardiac arrhythmias and causing myocardial necrosis. Myocardium from people with DCM has reduced density of beta$_1$-adrenoreceptors, reduced NE in cardiac adrenergic stores and an increased ratio of G_i to G_s proteins. Reduced responsiveness to sympathetic activity in the failing heart may lead to further deterioration in congestive cardiac failure (Braunwald et al, 1997).

The pattern of marked activation of the sympatho-adrenergic system, downregulation of beta adrenoreceptors, upregulation of G_i proteins, global

desensitization of adenyl cyclase and selective subsensitisation to beta adrenergic inotropic stimulation, that has been observed in people with DCM, has been confirmed in Swiss Simmentaler/Red Holstein cattle (Eschenhagen *et al*, 1995).

Reduced cardiac output stimulates the renin-angiotensin-aldosterone axis resulting in elevation of plasma renin, angiotensin II and aldosterone leading to vasoconstriction and sodium retention. Two distinct parts of the renin-angiotensin system include plasma and locally synthesized tissue components. Little is known about the locally synthesized cardiac renin-angiotensin system. Plasma renin activity has been recorded within the normal range in people with asymptomatic left ventricular dysfunction (ejection fraction of $\leq$ 35%) (Benedict and Phil, 1994). Infusion of angiotensin II in male Sprague-Dawley rats resulted in myocyte necrosis and widespread perivascular fibrosis in both the left and right ventricular myocardium on days 2 and 4 post-infusion, but not thereafter. Neutrophil infiltration was present at foci of myocardial necrosis at days 2 to 4, whereas multifocal infiltrates of lymphocytes and macrophages were present from day 2 through the week 6. Myofibroblasts appeared in the ventricular myocardium at day 2 and persisted through to week 2 after angiotensin II infusion. Early myocyte necrosis has been attributed to angiotensin II-induced release of catecholamines from the adrenal medulla. Angiotensin II may stimulate collagen synthesis via AT_1 receptors that are known to be present on neonatal and adult cardiac fibroblasts. Alternatively, angiotensin II may stimulate myocardial fibrosis by increasing $TGF-\beta_1$ mRNA expression in cardiomyocytes or fibroblasts. Infusion of aldosterone in male Sprague-Dawley rats induced myocardial necrosis at week 3 continuing to week 6. Aldosterone was not directly cytotoxic for cardiomyocytes, but it increased urinary

excretion of potassium, producing decreased intracellular potassium levels in myocytes leading to myocardial necrosis and fibrosis. Aldosterone can stimulate myocardial fibrosis by binding to mineralocorticoid receptors on myocytes, endothelial cells and fibroblasts in the heart, without cell damage (Campbell *et al*, 1995). In Dobermans with DCM, myocardial potassium ion levels were reduced (78.9 ± 4.4 mM) compared to healthy mixed dogs (86.9 ± 4.4 mM) (McCutcheon *et al*, 1992).

Endothelin is a small peptide that is synthesized and released by endothelial cells. Over 80% of the endothelin produced is secreted locally towards the underlying vascular smooth muscle, where it binds to the endothelin-A receptor (ET_A) producing vasoconstriction (Remuzzi and Benigni, 1993). Endothelin is the most potent vasoconstricting substance known for vascular smooth muscle. Only a small amount of endothelin is secreted into the plasma, where it can bind to endothelin-B receptors (ET_B), on endothelial cells. Binding of endothelins-1 and 3 to the ET_B receptor on endothelial cells leads to release of nitric oxide (endothelium-derived relaxing factor, EDRF) which relaxes vascular smooth muscle, producing vasodilation (Benedict and Phil, 1994; Remuzzi and Benigni, 1993). Stimuli for endothelin release include norepinephrine, vasopressin and interleukin-1. Elevated plasma endothelin levels have been found in people with cardiac failure and in the canine pacing model of heart failure (Benedict and Phil, 1994).

Increased levels of arginine vasopressin (AVP) have been recorded in people with asymptomatic left ventricular dysfunction and congestive cardiac failure (Benedict and Phil, 1994). Arginine vasopressin is a potent vasoconstrictor. The administration of

arginine vasopressin antagonists to people with heart failure and elevated AVP reduces systemic vascular resistance and raises cardiac output (Colucci and Braunwald, 1997).

Vascular tone in patients with cardiac failure is determined by the complex interaction among neuroendocrine vasoconstrictor and vasodilator systems and vasoactive therapeutic agents. The humoral agents that contribute to vasodilation include atrial natriuretic peptide, dopamine, endothelium-derived relaxing factor (EDRF, nitric oxide) and vasodilator prostaglandins (Colucci and Braunwald, 1997).

Atrial natriuretic peptide (ANP) is a hormone released from the atria in response to stretching. Release of ANP from secretory granules in the atrium induces vasodilation and sodium and water excretion. Plasma levels of ANP are increased 2 to 25 fold in people with asymptomatic left ventricular dysfunction. In experimental models of heart failure there is maximal expression of ANP in both the atrial and ventricular myocardium (Benedict and Phil, 1994).

Dopamine is one of the family of endogenous catecholamines derived from tyrosine, and the immediate biosynthetic precursor of norepinephrine. When used therapeutically in people with heart failure, dopamine produces vasodilation by stimulating dopaminergic receptors in blood vessels along with the central and peripheral nervous systems. Binding of dopamine to dopamine$_1$ receptors produces vasodilation in coronary, renal, mesenteric and cerebral blood vessels by stimulation of adenyl cyclase which raises the level of intracellular cyclic adenosine monophosphate. When dopamine binds to dopamine$_2$ receptors, vasodilation occurs due to inhibition of sympathetic nerve endings. Dopamine induces diuresis by stimulating myocardial contractility through its

binding to beta$_1$-adrenoreceptors in the heart, and by preferential dilation of renal cortical blood vessels (Smith *et al*, 1992).

Endothelium-derived relaxing factor (EDRF) has been chemically indistinguishable, to date, from the powerful vasodilator, nitric oxide (NO) (Katz, 1995). EDRF/NO or a related nitroso compound is synthesized in endothelial cells from L-arginine by nitric oxide synthetase (NOS). Three isozymes of NOS include the constitutive form (NOS$_c$), the inducible form (NOS$_i$) and a third form that is found predominantly in the central nervous system (Katz, 1995). The constitutive form is the predominant NOS isozyme in endothelial cells, and its activity is dependent on the presence of calcium, calmodulin, NADPH and other electron donors as cofactors. The second NOS isozyme is mainly found in activated macrophages, and to a lesser extent in endothelial cells and vascular smooth muscle exposed to cytokines. A third NOS isozyme is predominantly found in the central nervous system and has not yet been demonstrated in endothelial cells. Nitric oxide synthesis in endothelial cells is induced by acetylcholine, bradykinin, serotonin, histamine, adenosine triphosphate and norepinephrine. Induction of NOS$_c$ by agonists is modulated by receptor-bound G proteins. Pulsatile blood flow stimulates NO synthesis in endothelial cells by a different mechanism to that of receptor mediated agonists. Continuous pulsatile blood flow causes shear stress at the endothelial surface and induces a basal level of NO release that is important in the regulation of blood pressure. NO synthesized in the endothelium moves by passive diffusion to the adjacent smooth muscle cells. Induction of NO synthesis in endothelial cells leads to vasodilation in conduit arteries, resistance arterioles and veins through activation of guanylate cyclase in vascular smooth muscle. Conflicting results

from studies of endothelium-dependent NO-mediated vasodilation in dogs with cardiac failure induced by rapid ventricular pacing have probably been due to differences in the severity of heart failure induced and differences in the experimental conditions. However, most studies have demonstrated that basal, agonist-mediated, and blood flow induced endothelial production of NO is impaired in both coronary and peripheral circulation in dogs with congestive cardiac failure induced by rapid ventricular pacing (Katz, 1995).

An important recent finding is that nitric oxide is taken up when hemoglobin is oxygenated in the lungs to form s-nitrosohemoglobin (SNO-Hb) within erythrocytes (Jia et al, 1996). The NO group is then released from SNO-Hb during the arterial-venous transit. Arterial blood was shown to have significant levels of SNO-Hb, however SNO-Hb was virtually undetectable in venous blood. The vasoactive properties of SNO-Hb were confirmed by injecting SNO-Hb-containing erythrocytes into the femoral veins of rats and demonstrating a hypotensive response (a 10% reduction in mean venous blood pressure to 8.1 mm Hg) (Jia et al, 1996). Nitric oxide is known to inhibit mitochondrial respiratory chain activity by forming nitrosyl complexes with iron-sulphur centers of aconitase, complexes I and II, and with a heme at the oxygen-binding site of cytochrome oxidase (Shen et al, 1995). It is now thought that NO produced by macrophages and during inflammation inhibits mitochondrial respiration in tissues. Nitric oxide is known to suppress contraction of both cardiac and skeletal myocytes (possibly by signal transduction that antagonizes force generation and/or possibly by direct suppression of mitochondrial energy metabolism). Nitric oxide release from vascular endothelial cells

can cause vasodilation and inhibit mitochondrial respiration in skeletal muscle (Shen *et al*, 1995).

1.2.11. Skeletal muscle changes in chronic congestive cardiac failure

Exercise intolerance is a recognised problem in people with chronic congestive heart failure (Sullivan and Hawthorne, 1995) and Dobermans in advanced stages of congestive heart failure (CHF) due to DCM (Calvert, 1995). Fatigue in skeletal muscle is defined as an inability to maintain a given force. In normal people, fatigue may occur due to metabolic, neural and neuromuscular factors. Twitch potential does not increase force of isometric skeletal muscle contraction in CHF, suggesting that central fatigue or decreased neural activation are not the causes for early fatigue in this condition (Sullivan and Hawthorne, 1995).

In people with CHF, exercise intolerance is multifactorial in origin, involving central hemodynamic abnormalities, respiratory muscle fatigue, air flow limitations, hyperpnea, reduced peripheral blood flow and altered skeletal muscle biochemistry and fiber types. Habitual exercise is recognised to play an important role in determining skeletal muscle fiber composition in normal people. Altered exercise habits in people with CHF are thought to be an important factor in leading to changes in skeletal muscle fiber composition in CHF. However deconditioning in people has not been shown to cause a decrease in type I and increase in type IIB muscle fibers (Sullivan and Hawthorne, 1995). It has been reported that skeletal muscle changes in people with CHF are similar to those due to deconditioning. Also, chronic exercise training in people with CHF has led to improved submaximal exercise performance and increased oxidative

capacity of skeletal muscle, as measured with ^{31}P-NMR spectroscopy (Delehanty and Liang, 1995).

Exercise intolerance can be detected in previously active Dobermans 6 to 9 months prior to the onset of overt congestive cardiac failure due to DCM (Calvert, 1995). In canine models of chronic heart failure, there is a relative decrease in slow twitch type I fibers and an increase in fast twitch type II fibers in the triceps brachii (lateral head) muscle (Sabbah et al, 1993). Chronic congestive heart failure was experimentally induced by multiple, sequential intracoronary embolization in adult dogs. This resulted in a decrease in the percentage of type I (from 32 ± 5 to 19 ± 2%) and an increase in type II (from 68 ± 5 to 81 ± 2%) muscle fibres in the left lateral head of the triceps muscle. Progressive atrophy was evident in both the type I and II fiber types at 1 month and 3 after the commencement of intracoronary embolization (Sabbah et al, 1993). Altered muscle fiber composition was not associated with fiber β adrenergic receptor density or structural abnormalities in the triceps brachii muscle (Sabbah et al, 1993). It was suggested that muscle fibre atrophy may have been due to reduced physical activity (Sabbah et al, 1993). Studies on the effects of short-term ventricular pacing at 260 beats per minute for 3 weeks in dogs have reported reduced cardiac output, decreased femoral blood flow and increased arterial blood lactate levels, consistent with skeletal muscle under-perfusion. In this model, skeletal muscle under-perfusion was associated with a reduced arteriovenous pressure gradient in the muscle (Wilson et al, 1986). There was no evidence of impaired skeletal muscle arteriolar vasodilation (Wilson et al, 1986).

Biochemical studies on skeletal muscle in New Zealand Large White rabbits demonstrated a higher mitochondrial content in soleus (98% type I) compared to gracilis

(99% type IIB) muscle fibers. Mitochondria from soleus muscle had twice the capacity for NADH and succinate oxidation as did those from gracilis muscle. Maximal activities of NADHdh and succinate dehydrogenase were about two fold higher in soleus compared to gracilis-derived mitochondria. Citric acid cycle enzyme activities (except for isocitrate dehydrogenase) were higher in soleus compared to gracilis-derived mitochondria (Jackman and Willis, 1996).

In human medicine, muscle is the tissue of choice for the biochemical investigation of mitochondrial defects, even when the clinical involvement of other organs such as heart and brain is more prominent. While mitochondrial deficiencies of all biotin enzymes and most coenzyme-A-linked enzymes are expressed in fibroblasts, mitochondrial respiratory chain defects have been difficult to demonstrate in cultured fibroblasts by some researchers (Scholte et al, 1987). Both skeletal muscle homogenate and isolated mitochondria have been used to identify defects in oxidative phosphorylation. Previous research workers have reported difficulties with the measurement of rotenone-inhibitable NADHdh activity in skeletal muscle (Scholte et al. 1987). Some researchers have found difficulty in measuring the rotenone-inhibitable activity of NADHdh using a cultured fibroblast method (Scholte et al, 1987). In the fibroblast assay, the rotenone-sensitive NADHdh level was very high, 78 to 86% of total NADHdh activity, compared to 19 to 50% in human muscle preparations, which created inaccuracy in the assay (Scholte et al, 1987). Other researchers have successfully developed fibroblast assay for NADHdh activity (Pitkanen et al, 1996). In human studies, the quadriceps, gastrocnemius, biceps and forearm extensor muscles have been used for the biochemical investigations (Scholte et al, 1987).

1.2.12. Dilated cardiomyopathy in Doberman Pinscher dogs

Death of Doberman Pinscher dogs (DPs) due to heart disease has been recognised since 1949 in North America (Calvert, 1986). Seven closely related male Dobermans were imported into the USA during the 1940's. These dogs were bred extensively and have ancestral links to most Dobermans in the USA today. Three of the 7 imported male dogs died with heart disease between the ages of 7 and 10 years (Calvert, 1986). Sudden death of Dobermans due to heart disease was first reported in the veterinary literature in 1968 (James and Drake, 1968). Cardiomyopathy in Doberman Pinschers was described in 1970 (Ettinger and Lord, 1970). While the etiology of this condition is unknown, the high prevalence of DCM in Dobermans, the long history of inbreeding within the breed and the tendency of the disease to occur in family lines with multiple littermates affected, suggests that this disease is inherited (Calvert, 1995). Since dilated cardiomyopathy occurs in other large breeds of dogs, there may be an association between dog size and cardiomyopathy (Smucker et al, 1990). Recent molecular biological studies have demonstrated that canine parvovirus is not associated with DCM in Dobermans (Braz-Ruivo, 1996).

Left ventricular ejection fraction in 46 apparently normal Dobermans was found to be significantly lower than that in 41 mongrel dogs, indicating occult left ventricular dysfunction in the Dobermans (Smucker et al, 1990). Half of the privately owned, apparently healthy Dobermans in this study had echocardiographic evidence of systolic left ventricular dysfunction (Smucker et al, 1990). In an echocardiographic and electrocardiographic study of 193 asymptomatic DPs, 27 dogs (14%) had an enlarged left ventricular internal dimension in diastole (LVID-d) of greater than 45mm, the end point

to septal separation (EPSS) was greater than 6mm in 25 (14%) of the dogs and minor morphologic aberrations in QRS wave were seen in 42 (23%) of the dogs. The enlargement of LVID-d and EPSS in 14% of 293 DPs indicated that occult myocardial disease is very common in Dobermans in Canada (O'Grady and Home, 1992).

Resting and dobutamine stress echocardiography were found to be safe screening tests for the early detection of systolic and diastolic cardiac dysfunction in Doberman Pinschers (Minors, 1995).

After the onset of echocardiographic and electrocardiographic abnormalities, there is a rapid progression over about a year to predominantly left-sided heart failure (Calvert, 1986). The mean age of DPs with DCM is 6.5 years (with a range of 2.5 to 14.5 years) (Calvert, 1986). Early reports suggested a preponderance of males affected (Calvert, 1986), however more recent studies indicate no sex predilection (O'Grady and Home, 1992). Typical clinical signs in DPs with advanced DCM include dyspnea, pale or cyanotic mucous membranes, increased bronchovesicular sounds (crackles), coughing, exercise intolerance, ascites and weight loss (Calvert, 1986). Pulmonary edema, irregular heart rhythm, gallop rhythm, weak pulse, pulse deficit, increased capillary refill time and systolic heart murmur may be detected (Calvert, 1986). Sudden death due to ventricular arrhythmia and fibrillation in otherwise normal DPs is becoming more commonly recognised (Calvert, 1986). In a study of 54 Dobermans with no clinical signs of disease, 14 (26%) died suddenly (Calvert et al, 1997). More than 100 VPD/24 hours was considered indicative of cardiomyopathy, particularly if depolarizations occurred as couplets, runs of consecutive depolarizations, or episodes of ventricular tachycardia (Calvert et al, 1997).

Gross pathological changes seen in DPs that die with DCM include dilation of the ventricles, particularly the left side (Hazlett et al, 1983). Other commonly reported gross changes are dilation of the atria, pulmonary congestion and edema, ascites, peripheral edema and hepatic congestion and irregular pale fibrotic foci in the myocardium (Calvert, 1986; Hazlett et al, 1983).

Histopathological changes reported in DPs with DCM include interstitial and replacement fibrosis in the myocardium, replacement of myocardial fibers by fat and myocardial fiber degeneration (sarcoplasmic vacuoles) (Hazlett et al, 1983; Calvert, 1986). There is one report of scattered foci of lymphocytes and macrophages in the myocardium of DPs with DCM (Calvert, 1986). Histopathological changes consistent with DCM have been reported in apparently healthy Dobermans that died suddenly. Myocardial necrosis and fibrosis were most severe in the papillary muscles of the Dobermans that died suddenly (Calvert, 1986).

At this stage, the diagnosis of DCM in DPs is made by clinical examination (including echocardiography and electrocardiography) or by the gross pathological changes present at necropsy. A recent study of the urinary lactate to creatinine ratio has revealed a sixfold increase in this ratio in DPs with DCM compared to healthy Dobermans (O'Brien et al, 1993). Further studies are required to determine whether the elevation in the lactate to creatinine ratio could be useful in the early detection of this disease.

Recently it has been established (McCutcheon et al, 1992) that the myocardium of DPs with DCM has a marked reduction in mitochondrial oxidative phosphorylation enzymes, decreased myoglobin concentration, lactacidosis and energy depletion.

The activities of cardiac myofibrillar creatine kinase isoenzyme MM, mitochondrial creatine kinase, and troponin-T concentration were reduced by 25% in Dobermans with DCM compared to normal mixed breed dogs (O'Brien *et al*, 1997). Serum creatine kinase, the isoenzyme creatine kinase MB, myoglobin, lactate dehydrogenase isoenzyme-I and cardiac troponin T have been used as markers of myocardial injury in human medicine (Fitzgerald *et al*, 1996). Serum troponin T may serve as a marker of myocardial injury in Dobermans to assist in the early diagnosis of occult heart disease in this breed.

1.2.13. Rationale for the proposed research

Numerous questions arise from the foregoing research. Can it be confirmed that DCM in Dobermans is an inherited disease? Is the reduced respiratory chain activity in DP's with DCM a consequence of an inherited enzymatic defect? Are the respiratory chain enzyme activities reduced in skeletal muscle of Dobermans with DCM? If the respiratory chain activities are reduced in skeletal muscles of Dobermans with DCM could this deficiency be measured with sufficient accuracy to provide a clinical test for the early recognition of DCM in Dobermans? What are the biochemical changes that occur in the myocardium of Dobermans with DCM to compensate for the reduction in oxidative phosphorylation? Are myocardial anaerobic metabolism and lactate concentration increased? Do Dobermans with echocardiographic changes of occult DCM have biochemical or morphological changes that are significantly different from normal Dobermans? What are the differences in histomorphological changes in the myocardium

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of Dobermans with DCM compared to myocardial changes in other breeds of dogs with DCM?

CHAPTER 2

2.1. INTRODUCTION

Dilated cardiomyopathy in Doberman Pinschers is a chronic, slowly progressive heart disease commonly seen in Australia, Canada and the United States of America (Calvert, 1995; Woodfield, Cove Study Group, 1995). The high prevalence of DCM in Dobermans, the long history of inbreeding and the tendency of the disease to occur in family lines, with multiple littermates affected, suggests a hereditary basis for the disease in Dobermans (Calvert, 1995). Research in this disease is aimed at identifying the molecular defect(s) and the pathogenesis of DCM in Dobermans. A diagnostic test could then be developed to identify affected carrier dogs. Selective breeding of unaffected dogs could be recommended to reduce the prevalence of DCM in Dobermans. An understanding of the molecular defect responsible for DCM in Dobermans may allow the development of gene therapy and more specific medical therapy. Further studies of DCM in Dobermans have been recommended to develop this potential animal model of human dilated cardiomyopathy (Smucker et al, 1990).

Biochemical profile tests revealed marked reduction in respiratory chain enzymes and myoglobin with energy depletion and elevated lactic acid levels in the myocardium of Dobermans with DCM, compared to that of healthy mixed breed dogs (McCutcheon et al, 1992). Nicotinamide adenine dinucleotide dehydrogenase (NADHdh) activity was decreased by 60%, adenosine triphosphate (ATP) synthetase activity was reduced by 45%, total ATP was reduced by 31%, and myoglobin was decreased by 88% in the myocardium of Dobermans with DCM, compared to healthy mixed breed dogs

(McCutcheon *et al*, 1992). While DCM in Dobermans is associated with impaired mitochondrial production of ATP, it is not known if these changes were due to an inherited defect in oxidative phosphorylation or represented secondary changes to some other underlying genetic defect (McCutcheon *et al*, 1992). A comparison of nuclear and mitochondrial genomes amongst normal mixed breed dogs, Dobermans with no echocardiographic abnormalities, and Dobermans with DCM seemed most appropriate. Due to a lack of support and equipment for the molecular biological research, it was decided to verify and extend the knowledge gained from the earlier biochemical studies.

NADHdh activity was chosen as a focus in the current study since the earlier research had established that this enzyme was decreased more than ATP synthetase in the myocardium of Dobermans with DCM compared to mixed breed dogs (McCutcheon *et al*, 1992). NADHdh is the largest of the respiratory chain complexes and is formed by both nuclear and mtDNA (Walker, 1992). These features make an inherited defect in NADHdh a prime candidate to explain an inherited heart defect in Dobermans. Lactate was chosen as a measure of anaerobic metabolism, and a possible early indicator of DCM in Dobermans. Histological studies were performed to compare early morphological changes in Dobermans with occult heart disease with normal dogs, and dogs with DCM.

Three categories of Dobermans (normal, occult heart disease and DCM) were based on previously reported echocardiographic studies (O'Grady and Horne, 1992). The ability to identify Dobermans with occult heart disease facilitated a comparison of the progression of biochemical and histopathological changes in different clinical subcategories of Dobermans, with normal mixed breed dogs.

2.1.1. OBJECTIVES

1. To develop an improved technique for the measurement of NADHdh activity, and re-evaluate NADHdh activity in Dobermans, to address criticism directed at the previous method (Dr P.J.O'Brien, personal communication 1993). NADHdh activity was previously based on standard curves that lacked the highly concentrated myocardial homogenate, and different NADH substrate concentrations were used to measure the total and rotenone-sensitive NADHdh activities (McCutcheon *et al*, 1992; Nowack, *et al* 1992). Secondly, in the previous study, the comparison of NADHdh activity on a wet weight basis did not account for the extensive fibrosis in the myocardium of Dobermans with DCM. Determination of NADHdh activity relative to protein content of the tissues, would provide a better reference for comparison of NADHdh activity in the different groups of dogs.

2. To test the hypothesis that reduced myocardial NADHdh activity is associated with impaired cardiac function by evaluating the relationship between myocardial NADHdh activity and various echocardiographic parameters of cardiac function in healthy mixed breed dogs, normal Dobermans, Dobermans with occult heart disease and Dobermans with DCM.

3. To determine if gastrocnemius muscle NADHdh activity is reduced in Dobermans with the clinical (occult and DCM) categories of cardiac dysfunction. Demonstration of reduced NADHdh activity in both gastrocnemius muscle and myocardium of normal Dobermans would provide evidence that a defect in NADHdh activity is an underlying cause of DCM in Dobermans.

4. To determine if the urinary lactate to creatinine ratio would allow a test of the hypothesis that this ratio is increased in Dobermans with DCM and occult heart disease compared to normal Dobermans, and to determine if this reduction can distinguish Dobermans with occult heart disease from normal Dobermans.

5. To determine if morphological changes (myocardial fibrosis and necrosis) are present in Dobermans with occult heart disease and DCM. but not normal dogs.

2.2. MATERIALS AND METHODS

The proposal, initiated by the author, for the collection of samples from dogs in the study was approved by the Animal Care Committee of the Ontario Veterinary College (OVC) (protocol number 93R094), in accordance with the *Guide to the Care and Use of Experimental Animals* (Canadian Council on Animal Care, Ottawa, Ontario, Canada). Some samples were collected prior to the author's attendance at the University of Guelph.

2.2.1. Tissue source, echocardiography, collection and storage

Canine myocardial samples: All myocardial samples came from dogs where the owners or research groups requested euthanasia. Heart biopsies were collected before death, with general anaesthesia induced by intravenous administration of thiobarbitone sodium. Dogs were breathing room air and were not intubated during the administration of the anaesthetic agent. Myocardial samples were immediately frozen in liquid nitrogen, and later transferred to plastic vials and stored in a freezer at minus 70° C. These samples had been collected prior to the arrival of the author at the University of Guelph.

All of the Dobermans involved in this study were privately owned dogs and had been brought to the Veterinary Teaching Hospital (VTH), at the OVC, for routine medical examination, for ongoing cardiac investigation and therapy by Dr. O'Grady, or the owners had requested euthanasia.

Echocardiography: Echocardiographic measurements were performed by Dr. O'Grady using a Sonos 1000 (Hewlett Packard, Mississauga, Ontario). Dogs were imaged in right lateral recumbency with the transducer directed from beneath. Two-

dimensionally guided M-mode echocardiograms were recorded from the right parasternal long axis view. The categorisation of Dobermans was based on echocardiographic measurements of the left ventricular internal diameter during diastole (LVIDd) and systole (LVIDs). Other echocardiographic measurements made included E point to septal separation (EPSS), left ventricular free wall dimensions in diastole (LVFWd) and systole (LVFWs) (Tables 2.8). These measurements represent the average of 3 observations, and the fractional shortening (FS), and wall stress index in diastole (WSd) and systole (WSs) were calculated (O'Grady and Horne, 1992; Minors 1995).

Normal Dobermans had an average LVIDd of <42 mm and FS of >17%. No ventricular premature contractions were recorded in any of the normal Dobermans. Two Dobermans which had been categorised as normal on echocardiographic studies were found to be hypothyroid and on thyroxin therapy. Both these dogs (H5, H36) were removed from the study. There were 7 female Dobermans in the normal category with ages ranging from 16 to 160 (mean 106.5) months.

Dobermans in the occult category had left ventricular internal dimension at end diastole (LVIDd) of ≥ 46 mm, and a left ventricular internal dimension in systole (LVIDs) of > 35 mm. These cardiac dimensions were recorded on 3 occasions using 2-dimensional M-mode echocardiography from the right parasternal long-axis view of the left ventricle. Ventricular premature contractions were recorded in both Dobermans in the occult category. The male Doberman in the occult group was 91 months of age while the female Doberman was 135 months old (mean age 113 months).

All Dobermans with DCM had been admitted to the VTH with advanced congestive cardiac failure, had detailed echocardiographic histories, and were euthanized

at the owner's request. All Dobermans in the DCM category had left ventricular internal diameter in diastole (LVIDd) of ≥53 mm, and a LVID in systole (LVIDs) of ≥43 mm. The fractional shortening for Dobermans with DCM was < 14%, except for H35, which had a FS of 19.8%. Ventricular premature contractions were recorded in all Dobermans with DCM. There were 7 male and 2 female Dobermans in the DCM category, with ages ranging from 72 to 142 months (mean age 98.1 months).

Normal healthy mixed breed dogs had been part of another project within the University and were euthanized at the end of the studies (Table 2.1). No clinical examination or echocardiographic examination was performed on this group.

Occasionally echocardiographic tests or body weights had not been measured for all dogs. This meant that some dogs could be included in some studies (eg. OG2 included in myocardial NADHdh activity versus WSd), but not other studies (eg. OG2 excluded from myocardial NADHdh activity versus WSs).

Gastrocnemius muscle samples: Muscle samples came from dogs which had been presented to the Ontario Veterinary College, Veterinary Teaching Hospital for clinical cardiological examination by Dr O'Grady, or to the Department of Biomedical Sciences. Most dogs had been admitted with cardiac failure or other diseases where the owners requested euthanasia. Samples were collected before death, under general anesthesia with thiobarbitone sodium (prior to the arrival of the author at the University of Guelph). Gastrocnemius muscle samples were placed immediately in liquid nitrogen and later transferred to plastic vials and stored at −70° C until assayed.

The categorisation of Dobermans as normal, DCM or occult was determined by echocardiologic studies by Dr O'Grady as described earlier. One male and 4 females

were included in the normal Doberman category. Seven males and 1 female constituted the group of Dobermans with DCM. A total of 10 dogs (2 males and 8 females) were present in the normal mixed breed group of dogs.

Urine samples: Urine was collected, routinely, by Dr O'Grady from privately owned Dobermans that had been brought to OVC VTH for clinical examination. Urine was collected from 10 normal mixed breed dogs held in the animal care facilities. Urine was stored in plastic vials at $-70°$ C.

Formalin-fixed tissue: Following the collection of fresh myocardial tissue, the hearts were fixed in 10% neutral buffered formalin. A block of left ventricular papillary muscle, approximately 10 mm by 10 mm by 3 mm, was removed with a number 10 scalpel blade. Tissues were dehydrated, embedded in wax, cut to 3 μm thick, mounted on glass slides and stained with hematoxylin and eosin (Fisher CS400-ID, Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219-4785, USA). Where dogs had been autopsied, tissue sections were retrieved from the OVC Department of Pathobiology filing system. Some heart sections were stained by Masson's trichrome method (Prophet *et al*, 1992).

Dogs included in the different studies (myocardial NADHdh activity, gastrocnemius muscle NADHdh activity, urine and fixed heart) were often different populations.

2.2.2. Pilot study

A pilot study was performed to determine the optimal myocardial and gastrocnemius muscle homogenate concentrations and NADH substrate concentration for determination of NADHdh activity. The myocardial and gastrocnemius muscle

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homogenate concentrations were varied from 0.2 to 4.6 µg homogenate per milliliter of NADH buffer. NADH substrate concentration was varied from 19.2 to 154 mM NADH buffer. The chosen levels of NADH substrate and concentration of muscle homogenate represented a compromise in trying to maximise the recorded NADHdh activity whilst minimising the level of quenching due to NADH and muscle homogenate. All levels were determined in pig and dog heart using a modification of a technique (Table 2.4.) previously described (Nowack et al, 1992; McCutcheon et al, 1992). The following description outlines the modified technique.

Stock solutions and homogenates: A buffer for the assay and homogenizing fluid was made with 80 mM potassium chloride (Sigma P3911, Sigma Chemical Company, St. Louis, MO, USA) and 50 mM Tris ($C_4H_{11}NO_3$, Sigma T1378, Sigma Chemical Company, St. Louis. MO. USA) in distilled. deionized water. The fluid was adjusted to pH 7.0 with 1 N hydrochloric acid (Sigma H1501, Sigma Chemical Company, St. Louis, MO. USA). The pH of the Tris buffer was tested and corrected to pH 7.0 at 37^0 C on each day of experimentation (Passonneau and Lowry, 1993). Plastic tubes with 2.5 ml of the Tris buffer were held at 37°C in a temperature-regulated tube-holder (Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219-4785, USA).

NADH (Sigma N-8129, Sigma Chemical Company, St. Louis, MO, USA) stock solution (1 mM NADH in Tris buffer) was prepared on the day of use and stored on ice between use.

Frozen muscle (myocardium and gastrocnemius) was weighed on a Mettler balance (Delta Range, model AT261 Mettler-Toledo, CH-8606 Greifensee, Switzerland) and placed in a 10 ml plastic tube with Tris buffer. The muscle was finely ground by a

Tekmar homogenizer (Tekmar 7143 East Kemper Rd, Cincinnati, OH 45249, USA) with two 10-second bursts of the rotor separated by 10 seconds of rest. During homogenisation, the muscle was held in a 10 ml plastic tube, which in turn was placed in a cup containing ice.

The solution for inhibiting mitochondrial NADHdh contained 1mM rotenone (Sigma R8875, Sigma Chemical Company, St. Louis, MO, USA) in dimethyl sulphoxide (Fisher D128, Fisher Scientific, Nepean, Ontario, Canada). Rotenone solution was made up on the day of use as its ability to inhibit NADHdh activity was found to decline over a period of days.

Measurement of total NADH oxidation: A small plastic-coated stirring bead was placed in a 3 ml quartz cuvette; 2.5 ml of warm (37°C) Tris buffer was added and the cuvette was set in the warmed (37°C) Perkin Elmer LS50B spectrofluorometer (Perkin-Elmer Co, Norwalk, Connecticut, USA) cuvette holder. The magnetic stirrer speed was set at 7 on an arbitrary scale from 1 to 10. The temperature in the cuvette was checked with a standardised thermometer. The spectrofluorometer was set with an excitation wavelength of 340 nm and corresponding slit width of 5.0 nm; and an emission wavelength of 458 nm with a slit width of 10 nm (Passonneau and Lowry, 1993). The changes in fluorescence were recorded every 0.1 seconds for 2 minutes, with a response time of 0.5 seconds. A Perkin Elmer Fluorescent Data Management software version 3.5 (Perkin-Elmer Co, Norwalk, Connecticut, USA) was used in all NADH analyses.

Recording of fluorescence was commenced immediately after the cuvette was placed in the fluorometer holder. After 10 seconds, 50 μL of 1 mM NADH was added to the cuvette. Once the fluorescence of the mixture had plateaued, 60 μL of the muscle

homogenate (at different known concentrations) was directed into the middle of the cuvette fluid with a Drummond pipette (Drummond Scientific Company, 500 Parkway, Broomall, PA 19008-4293, USA). The formula for line of best fit to the standard curve was used to determine the rate of NADH oxidation (total NADHdh activity), over the first 5 seconds of the reaction between muscle homogenate (total NADHdh) and NADH. The line of best fit was calculated using Sigma Plot version 4 (SPSS Inc., IL, USA), or Microsoft Excel 97 and version 6 (Microsoft Corporation, Redmond, WA, USA). Total NADHdh activity was determined 2 to 4 times on each heart or gastrocnemius muscle sample, and the average value was calculated.

Measurement of rotenone-inhibited NADH oxidation: Rotenone specifically inhibits mitochondrial respiratory complex 1, but not other NADH dehydrogenases in the myocardium. Mitochondrial complex 1 activity can be determined by subtracting rotenone-inhibited NADHdh activity from the total NADHdh activity in myocardial homogenates (Fischer et al, 1986).

Due to the risk of rotenone contamination of the test samples, a separate, marked cuvette and pipette were used to measure the rotenone-inhibited NADHdh activity. The same procedure was followed (3 ml quartz cuvette, magnetic stirring bead, 2.5 ml Tris buffer 37°C, 50 μL 1mM NADH, 60 μL of homogenate). Immediately after the homogenate was added, 30 μL of 1mM rotenone solution was added. The formula for line of best fit to the standard curve was used to determine the rate of NADH oxidation (rotenone-inhibited NADHdh activity) over the first 5 seconds of the reaction between rotenone-inhibited muscle homogenate and NADH as substrate. Rotenone-inhibited

NADHdh activity was determined in duplicate on each heart or gastrocnemius muscle sample, and the average value was calculated.

Standard curve calculation: A similar procedure for sample preparation was used as described above. In this case, 2.4 ml Tris buffer at 37°C, 0.1ml sodium dodecyl sulphate (SDS, laurel suphate) (Sigma L-4509, Sigma Chemical Company, St. Louis, MO, USA) solution, and 60 μL of muscle homogenate were added to a 3 ml quartz cuvette, containing a magnetic stirring bead. In a separate series of studies, the addition of 0.1ml of 10% SDS to the above described standard curve solutions was found to completely inhibit myocardial and gastrocnemius total NADHdh activity without altering the level of NADH fluorescence. The cuvette was let stand for 2 minutes in the heated cuvette holder to ensure complete lysis of the mitochondrial membranes by the SDS. Then 1mM NADH was added in 5 μL aliquots up to a total of 70 nmol of NADH, during continuous recording of the fluorescence. A standard curve was formed by entering the NADH concentration in nmol as the x-axis values and arbitrary fluorescence units were entered as the y-axis values in Microsoft Exel version 7.0 spreadsheet. The curve of best fit to the data was calculated and used as the standard curve (Fig. 2.1. and 2.2.).

Calculation of the mitochondrial NADHdh activity: Fluorescence units determined from the total NADH oxidation at 0 and 5 seconds were plotted on the previously calculated standard curve and the concentration of NADH (in nmol) was calculated. The NADH values at 0 seconds were subtracted from those at 5 seconds. The new calculated value was divided by 5 (seconds) to find the rate of total NADH oxidation per second. The same calculations were performed for the rotenone-inhibited NADH oxidation.

Mitochondrial NADHdh activity was calculated as the difference between the total rate of NADH oxidation and the rate of rotenone-inhibited NADH oxidation, at the same NADH substrate concentration, during the initial 5 seconds of the reaction. This calculated value was then multiplied by the dilution factor for the gastrocnemius or heart homogenate, and then divided by the protein content of the homogenate.

	Old Method	Modified Method
Heart homogenate concentration (µg/L)	9.17	1.3
NADH concentration (mM)	0.092	0.02
Determination of rotenone-sensitive and -insensitive NADHdh activities	Different substrate concentrations	Same substrate concentrations
Standard curve	Heart homogenate	Not included	Included
	Data points	5	> 10
	Data fit	Linear	Curve of best fit
NADHdh activity expressed as per	Gram myocardial wet weight	Gram myocardial protein

Table 2.1. Comparison of old and modified techniques for determination of myocardial NADHdh activity.

2.2.3. Myocardial NADHdh Analysis

Myocardial NADHdh activity: All myocardial NADHdh activities were determined using the modified technique described above. The NADH (1 mM) and myocardium (1:20) concentrations had been determined from the pilot study. The reaction mixture consisted of 19.16 mM NADH, 3 µg of heart in 2.61 ml of 80mM KCl and 50mM Tris at pH 7.0. These recordings were repeated in triplicate for each homogenate. A fourth, marked cuvette, was used to measure the rotenone-inhibited NADHdh activity. The NADH standard curve and calculations of the myocardial mitochondrial NADHdh activity were calculated for each homogenate, as described previously. The dilution factor for calculating the myocardial NADHdh activity was 19.

Protein content determination: A single determination of the protein content was made on all myocardial homogenates. The protein content of each homogenate was determined with a commercially available protein assay kit (Sigma P5656, Sigma Chemical Company, St Louis, MO, USA). Eppendorf 1 ml plastic microcentrifuge tubes (Eppendorf, Postfach 65 06 70, D-2000 Hamburg 65, West Germany) were used throughout the procedure. A protein standard curve was prepared by running solutions containing known concentrations of bovine serum albumin simultaneously with the myocardial homogenates. The blank contained 0.5 ml of distilled water. Myocardial homogenates were mixed with a Vortex mixer (Drummond Scientific Company, 500 Parkway, Broomall, PA 19008-4293, USA), and 20 µL was transferred to a labelled microcentrifuge tube with a Drummond pipette. This solution was made up to 0.5 ml by the addition of 480 µL distilled water. Deoxycholate (0.05 ml, 1.5 mg/ml sodium deoxycholate, Sigma D5525, Sigma Chemical Company, St Louis, MO, USA) was added

to each standard, blank and myocardial homogenate tube. All tubes were vortexed for 3 seconds and left to stand at room temperature for 10 minutes. Trichloracetic acid (0.05 ml, 72% w/v, Sigma F9252, Sigma Chemical Company, St Louis, MO, USA) was pipetted into all tubes, followed by vortex mixing for 3 seconds. All solutions were centrifuged for 10 minutes at 30,000 G in the Eppendorf microcentrifuge (Model 5415c, Eppendorf, Postfach 65 06 70, D-2000 Hamburg 65, West Germany). The supernatant was decanted leaving the solid protein precipitate. This pelleted protein was dissolved in 0.5 ml of the modified Lowry reagent (lauryl sulfate, sodium carbonate, cupric tartrate) (Fisher Scientific), 0.5 ml distilled water was added, and the mixture was vortexed for 10 seconds. All tubes were left standing at room temperature for 20 minutes, followed by the addition of 0.25 ml Folin and Ciocalteus phenol (Sigma[®] proprietary solution, sodium tungstate, sodium molybdate, phosphoric acid, hydrochloric acid, lithium sulfate, bromite, Sigma F9252, Sigma Chemical Company, St Louis, MO, USA). This mixture was vortexed immediately for 10 seconds. Colour was allowed to develop over 30 minutes at 21°C before the absorbance was read in 1 ml plastic cuvettes at 750 nm on a Perkin-Elmer Bio UV/VIS Spectrometer (Perkin Elmer Co, Norwalk, Connecticut, USA). All absorbance readings were completed within 30 minutes.

A calibration curve was constructed by plotting the absorbance of the protein standards (on the y-axis) versus the corresponding known protein concentrations (on the x-axis). Five protein standards were prepared by diluting a known standard (400 μg bovine serum albumin per ml) with distilled water. Sample protein concentrations were determined from the calibration curve. The results were multiplied by the dilution factor of 125 where 10 μL aliquots of the original homogenate were used, and 62.5 for 20 μL

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aliquots. The units of myocardial NADHdh activities were converted from per gram wet weight of myocardium to per gram protein by dividing the former value by the measured grams of protein in each homogenate.

2.2.4. Gastrocnemius muscle NADHdh analysis

NADHdh activity levels: Gastrocnemius muscle NADHdh activities were determined using the modified technique described above. Fluorometric recordings were made on a Perkin Elmer LS50B spectrofluorometer and the Perkin Elmer Fluorescent Data Management software version 3.5. The settings on the fluorometer were the same as those for the measurement of myocardial NADHdh activity. The same sequence of procedures, as established for myocardial NADHdh activity determination, was followed with the exception that a 1 in 12 dilution of gastrocnemius muscle was used in the buffer (80 mM potassium chloride and 50 mM Tris). NADHdh activities in gastrocnemius muscle samples were repeated in quadruplicate or triplicate in the early stages and in duplicate towards the end of the study.

Protein content determination: The protein concentration in the gastrocnemius muscle homogenates was determined with the Sigma Protein Assay Kit (Sigma P5656) as described above.

2.2.5. Lactate analysis of myocardial and gastrocnemius muscle homogenates, and urine

Lactate extraction, and determination of lactate concentration in myocardium and gastrocnemius muscle homogenates: Myocardial and gastrocnemius muscle homogenates were thawed in a shallow plastic tub of ice water and then mixed with a Vortex mixer. Lactic acid was extracted from tissue homogenates using perchloric acid (Gutman and Wahlefeld, 1974). Aliquots of 180 μl of myocardial and gastrocnemius muscle homogenates and lactic acid standard solution (initial concentrations of 2.5, 5, 10, 20, 80, and 120 mg/dl of lactic acid L (+) lactic acid, Sigma L-7022, Sigma Chemical Company, St Louis, MO, USA), were pipetted into 1.5 ml Eppendorf capped microcentrifuge tubes (Eppendorf Model 5415c, Eppendorf, Postfach 65 06 70, D-2000 Hamburg 65, West Germany), and returned to a rack in the ice water tub. The extraction procedure was applied to the lactate standard solutions, on one occasion, to test for the accuracy of the dilution factor, and to determine if the extraction procedure alters the original lactate concentration (separate standard curves were prepared for myocardial and gastrocnemius samples, and these standard solutions were not extracted; see later). Each labelled tube was vortexed for 10 seconds; 20 μl of 5N perchloric acid (Sigma Chemical Company, ST Louis, MO, USA) was added, followed by vortex mixing for 3 seconds and return of the tubes to the ice bath for 10 minutes. All tubes were centrifuged for 30 seconds at 30,000 G in the Eppendorf microcentrifuge. A 150 μl aliquot of the supernatant, from each sample and standard, was pipetted into 1.5 ml Eppendorf plastic microcentrifuge tubes and returned to the ice bath. A 50 μl aliquot of 2N potassium bicarbonate solution (Sigma Chemical Company, St Louis, MO, USA) was added to all

tubes, followed by 2 vortex mixes (3 seconds each time) for each sample. All tubes were spun at 30,000 G for 30 seconds in an Eppendorf microcentrifuge. Aliquots of 100 μl of myocardial extract, or 50 μl of gastrocnemius muscle extract, were added to 1 ml of Sigma Lactate Reagent Solution (Sigma 735-10, lactate oxidase 400 U/L, peroxidase 2400 U/L, chromogen precursor, buffer, Sigma Chemical Company, St Louis, MO, USA). Two separate standard curves and blanks were prepared for the myocardial and gastrocnemius muscle extracts. The standards for the myocardial extracts were prepared with a blank of 100 μl distilled water and the individual standards contained 90 μl of distilled water plus 10 μl of the stock L(+) Lactic acid (Sigma L-7022) (2.5, 5, 10, 20, 80, and 130 mg/dl). Standard solutions for the gastrocnemius muscle extracts contained 90 μl of distilled water plus 10 μl of the stock L(+) Lactic acid (Sigma L-7022) (2.5, 5, 10, 20, 80, and 120 mg/dl) and a 90 μl blank of distilled water. All tubes were left to stand at 25°C for 10 minutes before reading the absorbance at 540 nm in a Shimadzu spectrophotometer (model UV-1601 PC, Mandel Scientific Company, Guelph). A standard curve was constructed and the lactate concentrations were computed. All values were multiplied by the dilution factor of 45 for myocardial muscle extracts and 22.5 for the gastrocnemius muscle extracts. Lactate concentrations for myocardium and gastrocnemius muscle were converted from per gram frozen tissue to per gram protein by dividing the lactate per g frozen tissue by the measured protein per gram of each heart or gastrocnemius muscle homogenate.

Lactic acid in urine: The concentration of lactic acid in urine was determined using a Sigma Diagnostic Lactate kit (Sigma # 735, Sigma Chemical Company, St Louis, MO, USA). Lactic acid concentration was measured in 10 normal mixed breed dogs, 6

normal Dobermans, 4 Dobermans with occult heart disease, and 6 Dobermans with DCM.

Urinary creatinine: Urinary creatinine concentration was measured in the OVC Clinical Pathology Laboratory on an automated clinical biochemical analyser with continuous optical scanning (Dacos 2, Dart reagent system and ion-specific electrodes, Coulter Electronics Incorporated, Hialeah, Florida, USA). Dacos creatinine reagent contains picric acid which forms a red creatinine-picrate complex that is quantified colorimetrically (Dacos 2 Analyser Reference Manual). The instrument was calibrated with known creatinine standards on the day of measurement. Each sample was measured in duplicate and the mean recorded in mmol/l.

2.2.6. Histological Study of Myocardium

Histological sections of left ventricular papillary muscle, or left ventricle (if the papillary muscle was not available), from dogs listed in tables 2.1., 2.2., 2.3., 2.4. and 2.5., were examined at low (20x, and 40x) and high power (100x, and 400x), on an Olympus BX50 microscope (Olympus America Incorporated, 4 Nevada Drive, Lake Success, New York, USA). Histological changes were subjectively graded according to the degree of interstitial and replacement fibrosis, replacement of myocardial fibers by fat, necrosis, inflammation, Purkinje fiber changes and blood vessel morphology. The entire cross-sectional area (csa) of left ventricular papillary muscle, or left ventricular myocardium, on the glass slide, was examined and graded as described below. Two researchers (Drs. L. M. Badcoe and R. Crespo) graded the myocardial changes independently according to the following criteria.

Myocardial fibrosis was graded subjectively as:

0 = not present

+ = patchy interstitial fibrosis or replacement fibrosis affecting up to 10% of

the cross-sectional area (csa) for the tissue section.

++ = interstitial fibrosis ≥ 10%, but ≤ 50% of csa.

+++ = diffuse interstitial fibrosis > 50% csa, replacement fibrosis > 50% of csa

Necrosis was graded as

0 = not present

+ = 1 to 10 foci of necrosis per csa

++ = 11 - 19 foci per csa

+++ = > 20 foci per csa

The presence and type of inflammatory cells were described and graded:

0 = not present

+ = a few scattered foci of inflammatory cells in the entire csa of myocardium.

An inflammatory focus consisted of aggregates of 3 or more inflammatory

cells (lymphocytes, macrophages, neutrophils, eosinophils)

++ = 11 - 19 inflammatory foci

+++ = > 20 inflammatory foci

Fat replacement was graded:

0 = not present

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+ = fat replacement ≤10% of csa

++ = >10, but ≤ 50%

+++ = > 50%

The endocardium, myocyte width and Purkinje fibers were examined and described if abnormal. Where abnormalities were observed in the blood vessel, a "+" symbol was recorded, and these changes were described in the results. The presence (+) or absence (0) of lipofuscin near the nuclear poles of cardiomyocytes was recorded.

2.2.7. Statistical Analysis

Statistical analysis was performed with Statistica® version 5.1 (Statsoft Inc., OK. USA), Sigma Plot version 4, SPSS® version 7.5 and Systat 7.0 (SPSS Inc., IL, USA) software packages. Mean values for canine myocardial NADHdh activity and dog groupings (normal mixed breed, normal Doberman, Doberman occult heart disease and Dobermans with DCM) were compared by a one way analysis of variance (ANOVA). If the ANOVA indicated the groups did not belong to a common population, at the $p ≤ 0.05$ level, the individual groups were compared by Tukey's honest significant difference method (Snedicor and Cochran, 1967). Where the p value for the ANOVA was less than 0.05, and the p value for Tukey's honest significant difference test was not less than 0.05, the protected least significant difference (LSD) test was used (Snedicor and Cochran, 1967).

Linear and non-linear regression were chosen as the statistical methods for comparing the myocardial NADHdh activity with the raw echocardiological data (provided by M. O'Grady). Some echocardiographic data (LVIDs, LVIDd, LVFWs,

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LVFWd) was scaled by dividing the averaged raw data for individual dogs by the body weight to the power of 0.333 (Sisson and Schaeffer, 1991). These scaled echocardiographic data were then compared to myocardial NADHdh activity by linear and non-linear regression. The original raw data provided by M. O'Grady were compared to the myocardial NADHdh activity. Myocardial NADHdh activity was selected as the independent variable (x axis) and the echocardiological data were set as the dependent variable (y axis). Linear and non-linear regression analysis of myocardial NADHdh activity over echocardiological data was performed on the total population of dogs and on separate populations of normal Dobermans and Dobermans with DCM.

Mean gastrocnemius muscle NADHdh activities, as determined per gram wet weight and per milligram muscle protein, were compared among the 4 categories of dogs by a one way ANOVA with the significance level set at $p \leq 0.05$. Post hoc comparisons were made with Tukey's honest significant difference test, the protected least significant difference (LSD) test, and Duncan's test.

The myocardial and gastrocnemius muscle lactate concentrations, and the urinary lactate to creatinine ratio were each analysed by a one way ANOVA for the total population of dogs, and for the separate categories of dogs. The relationship between myocardial NADHdh activity and myocardial lactate was assessed by a one-way ANOVA for the total population of dogs and for the separate populations of normal Dobermans and Dobermans with DCM. The significance level was set at $p \leq 0.05$.

The measurement of agreement between the two pathologists was calculated by a Kappa test, using the computer-based statistical program Kappa (McMaster Programs,

Hamilton, Ontario). A level of $p < 0.05$ was considered significant. Histopathological changes in the left ventricle sections were ranked and statistically tested using the Kruskal-Wallis ANOVA, with a significance level set at $p < 0.05$ (Snedecor and Cochran, 1967).

Dog Identification / O'Grady Pathology	Myocardial NADHdh	Skeletal muscle NADHdh	Myocardial lactate	Skeletal muscle lactate	Urine lactate/ creatinine	Histopathology
OG28	x	x	x	x		
OG29	x			x		
OG31	x		x			
OG32	x					
OG36	x		x			
OG37	x		x			
H14		x				
H30	x	x	x			x
OG30	x		x			
OG27	x		x			
LB2M		x		x		
LB3M		x		x		
LB4M		x		x		
LB5M		x		x		
LB6M		x		x		
LB1H(M)	x	x	x	x		
5045A					x	
8741-KB					x	
9150					x	
5060A					x	
5244					x	
776-P					x	
8387-R					x	
5033-P					x	
9216-P					x	
9314-KB					x	
67068						x
66931						x
66831						x
66571						x
66288						x
66283						x
65331						x
64849						x
64729						x

Table 2.2. Normal mixed breed dogs used in different tests.

Dog Identification		Echocard- iography	Myocardial NADHdh	Skeletal muscle NADHdh	Myocardial lactate	Skeletal muscle lactate	Urine lactate/ creatinine	Histopathology
O'Grady	Pathology							
H42		x	x	x	x	x		
H49		x	x	x	x			
H55		x	x					
H36			x			x		
H32		x	x	x	x	x		
H31		x	x					x
H9		x	x			x		
H17		x	x			x		
H18			x					
1							x	
3							x	
4							x	
5							x	
13							x	
	68056							x
	66301							x
	65636							x
	65619							x
	65570							x
	65479							x
	65320/1							x
	62676/2							x

Table 2.3. Normal Dobermans used in different parts of the study.

Dog Identification		Echocard- iography	Myocardial NADHdh	Myocardial lactate	Skeletal muscle lactate	Urine lactate/ creatinine	Histopathology
O'Grady	Pathology						
H24 (65048)		x	x	x			x
H50		x	x	x			
6						x	
14						x	
18						x	

Table 2.4. Dobermans with occult DCM used in this research.

Dog Identification		Echocard-iography	Myocardial NADHdh	Myocardial lactate	Skeletal muscle NADHdh	Skeletal muscle lactate	Urine lactate/ creatinine	Histopath-ology
O'Grady	Pathology							
OG2		x	x					
OG14		x	x					
OG15		x	x					
OG19		x	x					
OG20		x	x					
OG21		x	x	x				
OG26		x	x	x		x		
H35	65597	x	x	x		x		x
OG25		x	x					
H6M					x	x		
H19M					x	x		
H25M					x	x		
H33M					x	x		
H35M					x			
H40M					x	x		
H41M					x	x		
H24M						x		
H31M						x		
H50M						x		
H18M						x		
H48M					x			
H49M						x		
2							x	
7							x	
8							x	
9							x	
11							x	
	67763							x
	66492							x
	66466							x
	66439							x
	66348							x
	65786							x
	65738							x
	63036							x

Table 2.5. Dobermans with DCM used in different parts of the study.

Dog Identification		Myocardial NADHdh	Skeletal muscle NADHdh	Myocardial lactate	Skeletal muscle lactate	Urine lactate/ creatinine	Histopathology
O'Grady	Pathology						
	67543/1						x
	67107/4						x
	66632/4						x
	66593/7						x
	63690A						x
	63652						x
	63544						x
	63401						x
	63316						x
	61817						x

Table 2.6. Dogs other than Dobermans with DCM that were used in this study.

2.3. RESULTS

2.3.1. Pilot study

From the pilot study, the optimal myocardial homogenate concentration was a dilution of 1 unit mass of myocardium in 19 times this mass of NADH buffer. The optimal concentration for gastrocnemius muscle homogenate was 1 unit mass muscle in 11 times this mass of NADH buffer. NADH substrate concentration for determination of myocardial and gastrocnemius muscles NADHdh activity was 19.16 mM.

Fig. 2.1. illustrates the typical plateauing of fluorescence above 58 mM NADH for one of the myocardial samples.
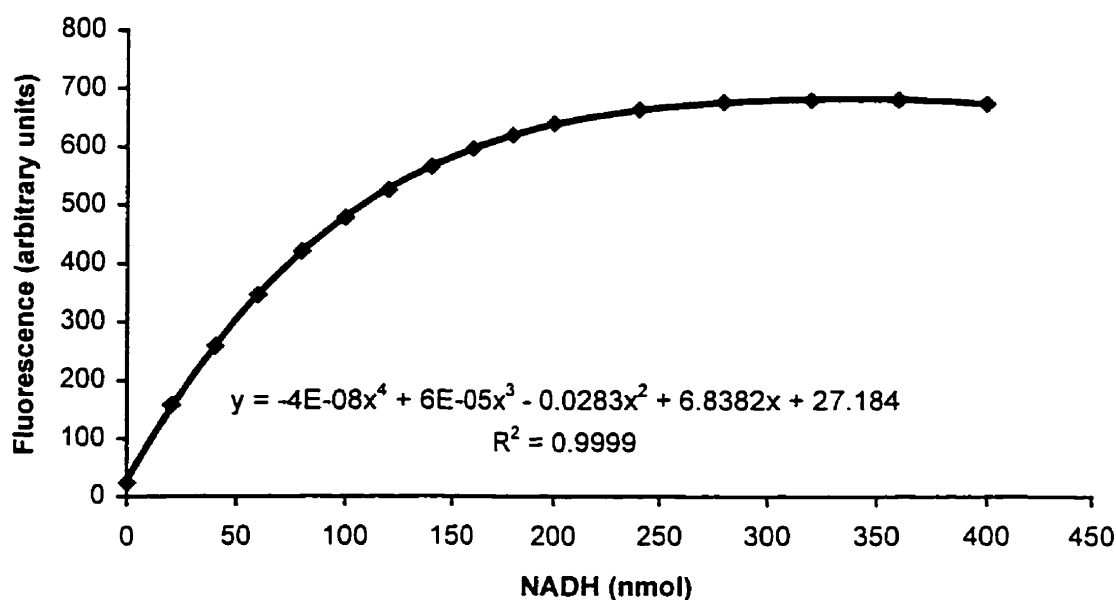

Fig. 2.1. Standard curve for NADH concentration in a reaction mixture containing 3μg of myocardium (LB1H), 2.6ml of 80 mM potassium chloride, 50 mM Tris and 3.85 mM sodium dodecyl sulfate (LB189S01). The equation for the line of best fit has been included.

Table 2.7. illustrates a minimal increase in the calculated myocardial NADHdh activity with NADH substrate concentrations above 19.2 μM (50 ηmol NADH in 2.6 ml 50mM Tris buffer).

Sample number for LB1H	NADH substrate aliquot (ηmol)	Myocardial NADHdh activity LB1H (μmol/min/g ww)
LB189F07	200	30.73
LB189F09	150	28.4
LB189F10	100	26.34
LB189F02	50	26.01

Table 2.7. Myocardial NADHdh activity at different NADH substrate concentrations. The reaction mixture contained 3μg of myocardium (LB1H), 2.6ml of 80 mM potassium chloride, 50 mM Tris and different NADH substrate concentrations.

2.3.2. Myocardial NADHdh activity

All myocardial rotenone-inhibitable NADHdh activities were determined with the revised technique (Table 2.8B.). Since a focal point of this research was the development of a revised technique for myocardial NADHdh activity, and the study of myocardial NADHdh activity in Dobermans, it seemed most appropriate to compare the values for myocardial NADHdh activity that have been determined with the different techniques (Table 2. 8B.).

An example of a standard curve prepared with 2.4 ml of 50 mM Tris (Sigma T1378) 80 mM KCl (Sigma P3911) buffer, 60 μL of myocardial homogenate (one mass of myocardium in 20 times this mass of the same Tris/KCl buffer), 0.1 ml of 10% SDS and 5 μL aliquots of 1 mM NADH (Sigma N-8129) is presented in Fig. 2.2. A graph illustrating the change in fluorescence with the addition of 40 μL of 1 mM NADH. 60 μL 1 in 20 myocardial homogenate, with and without 30 μL of 1 mM rotenone in dimethyl sulphoxide (DMSO) is presented in Fig. 2.3., for a normal Doberman (H31).

An analysis of variance on the myocardial NADHdh activity (both as μmol/min/g frozen heart and ηmol/min/mg protein) for the 4 categories of dogs demonstrated that there was a significant difference ($p < 0.001$, $n = 28$). Based on the differences in myocardial NADHdh activities, there is a 95% probability that the 4 groups of dogs are not from a common population. A comparison of the mean myocardial NADHdh activity (ηmol/min/mg protein) for the 4 dog categories using Tukey's test revealed a significant difference between the means for Dobermans with DCM compared to normal mixed breed dogs ($p < 0.001$), and Dobermans with DCM compared to normal Dobermans ($p =$

0.006) (Fig. 2.4.). Dobermans with DCM had a 68% reduction in myocardial NADHdh activity (17.0±7.4 ηmol/min/mg protein) when compared to normal mixed breed dogs (54±16.5 ηmol/min/mg protein) and a 61.4% reduction when compared to normal Dobermans (44.1±19 ηmol/min/mg protein). The mean myocardial NADHdh activity (44.1 ± 19 ηmol/min/mg protein) in normal Dobermans was 18.3% lower than that for normal mixed breed dogs. Two Dobermans with occult heart disease had the mean myocardial NADHdh activity (36.7 ± 8 ηmol/min/mg protein) reduced by 32.2 % compared to normal mixed breed dogs. However this mean myocardial NADHdh activity in Dobermans with occult heart disease was not significantly different from the other 3 groups of dogs (p = 0.33).

A comparison among the three categories of Dobermans (normal, occult cardiac dysfunction and DCM) with respect to echocardiographic measurements (FS, aLVIDd, aLVIDs, EPSS, aLVFWd, aLVFWs, WSd and WSs) is presented in Tables 2.9. and 2.10. EPSS showed no significant difference among the three categories of Dobermans, and consequently this value was not compared with the biochemical data.

Figures 2.5., 2.6., 2.7., 2.8., 2.9., 2.10., and 2.11. represent the regression of the myocardial NADHdh activity on the different echocardiological data. A significant exponential relationship was demonstrated between myocardial NADHdh activity and $LVIDd/BW^{0.333}$, $LVIDs/BW^{0.333}$, and FS (Figs 2.5., 2.6. and 2.7.). Linear (and nonlinear) regression analysis of myocardial NADHdh activity versus LVFWs, LVFWd, WSd, and WSs for the total population of Dobermans, and separate populations of normal Dobermans and Dobermans with DCM, revealed no significance (Figs. 2.8., 2.9., 2.10., and 2.11.). Small values for the regression coefficient (R^2) observed here (Figs. 2.8., 2.9.,

2.10., and 2.11.) indicate a poor fit of the data to the line of best fit for myocardial

NADHdh activity and echocardiological parameter.

A)	Normal Mixed Breed Dogs	Dobermans with DCM
NADHdh activity (μmol/min/g frozen heart)	26.9 ± 3.4 M=4, F=4	10.7 ± 4.5 M=6, F=4

B)	Normal Mixed Breed Dogs	Dobermans with DCM
NADHdh activity (μmol/min/g frozen heart)	12.5 ± 5.9 M=7, F=1, U=2	3.7 ± 1.4 M=7, F=2
NADHdh activity (ηmol/min/mg protein)	54 ± 16.5 M=7, F=1, U=2	17 ± 7.4 M=7, F=1

Table 2.8. Myocardial NADHdh activity in normal mixed breed dogs and Dobermans with DCM. A) Values obtained by old method (McCutcheon *et al*, 1992). B) Values obtained by modified technique. M indicates the number of males, F indicates the number of females and U indicates the number of dogs of unknown gender.
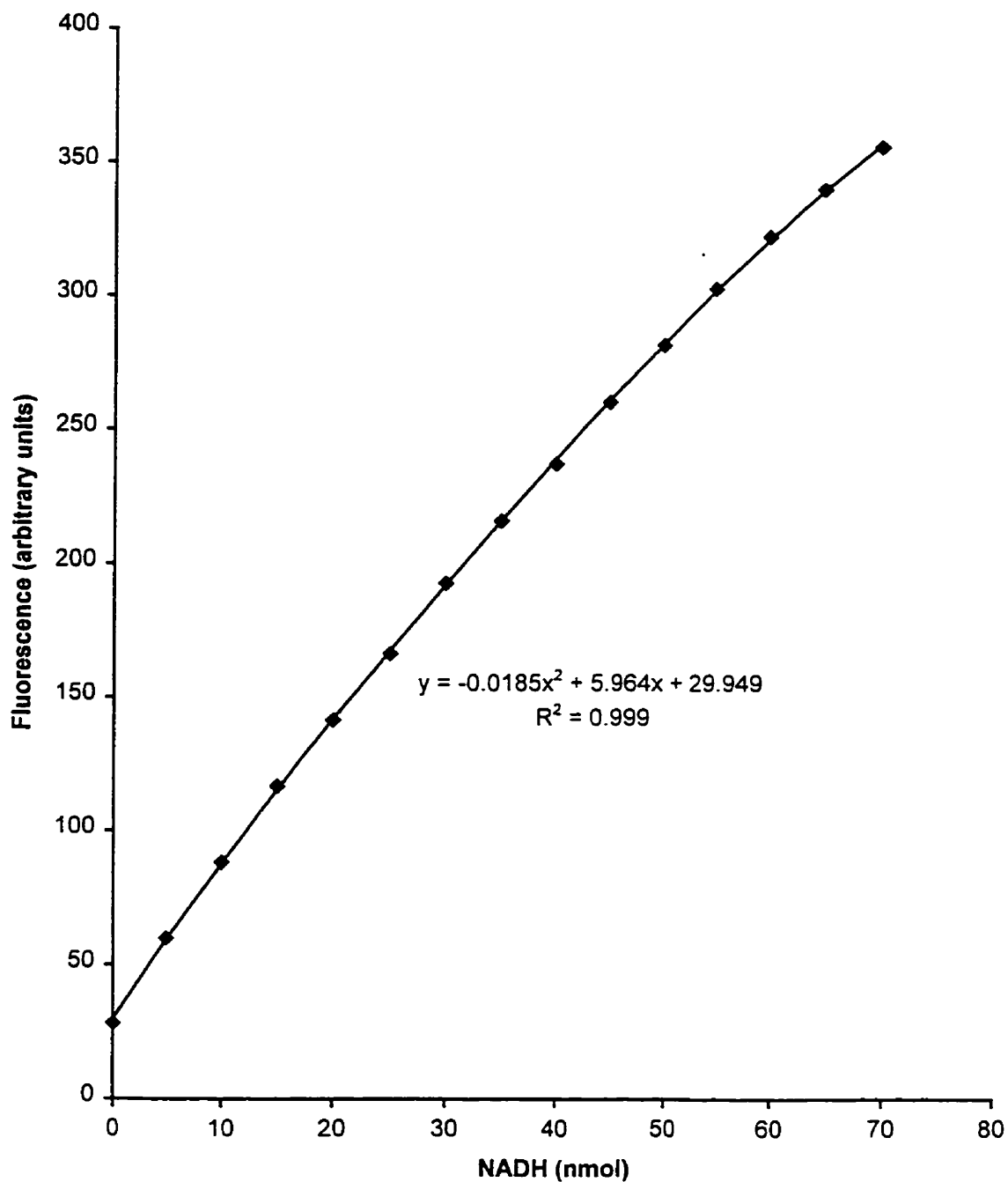
Fig. 2.2. Standard curve for NADH in myocardial homogenate (sample number LB238S1). The quadratic equation for line of best fit and the regression coefficient (R^2) have been included with the graph.
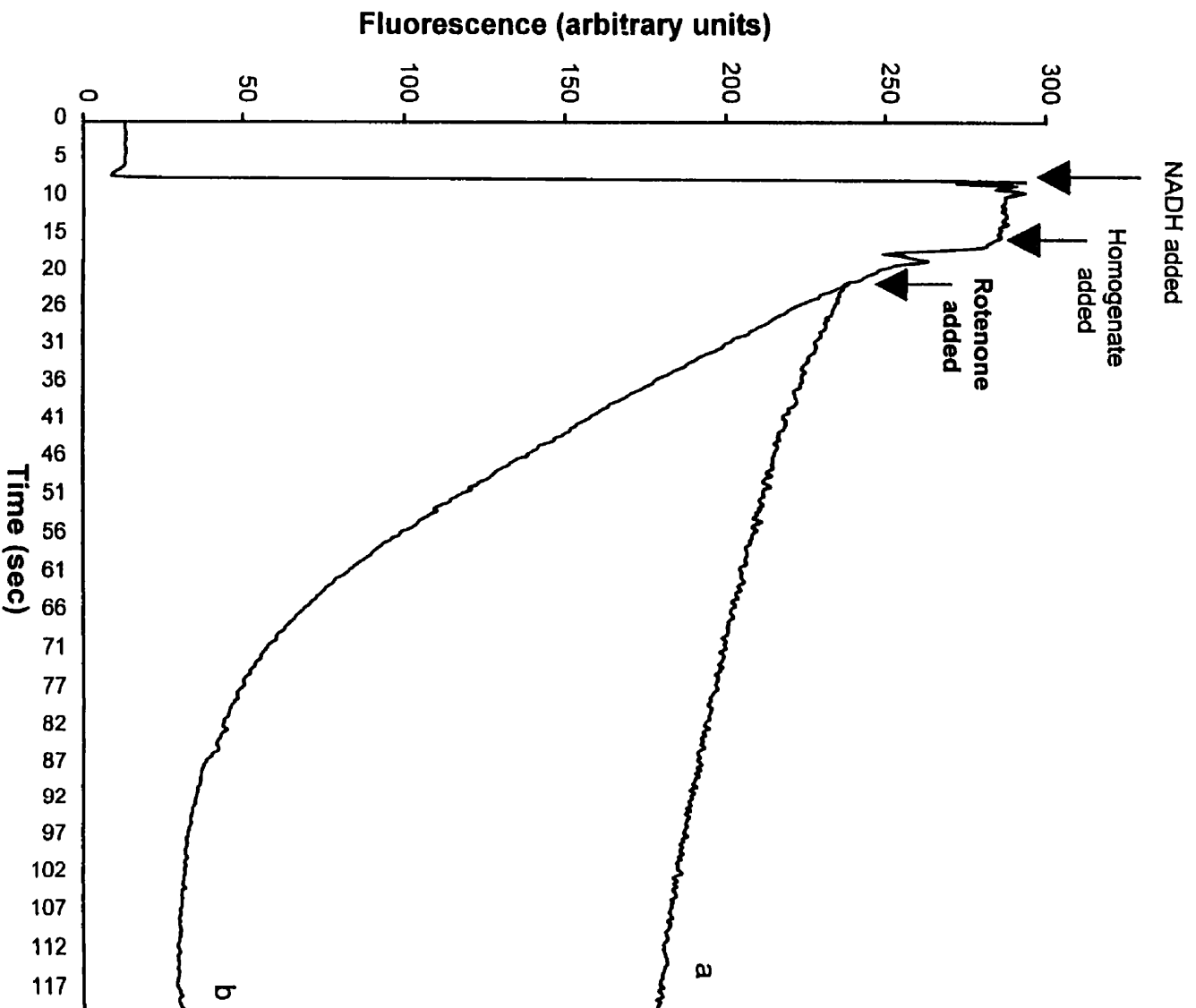
Fig. 2.3. Comparison of myocardial NADHdh activity with (a), and without (b) the addition of rotenone (dog number H31). Reaction mixtures for both a and b contained 3 μg heart and 19 mM NADH in 2.6 ml of 80 mM KCl and 50mM Tris at pH 7.0. In (a), 30 ηmoles of rotenone in 99.9% dimethyl sulphoxide were added immediately after the myocardial homogenate.
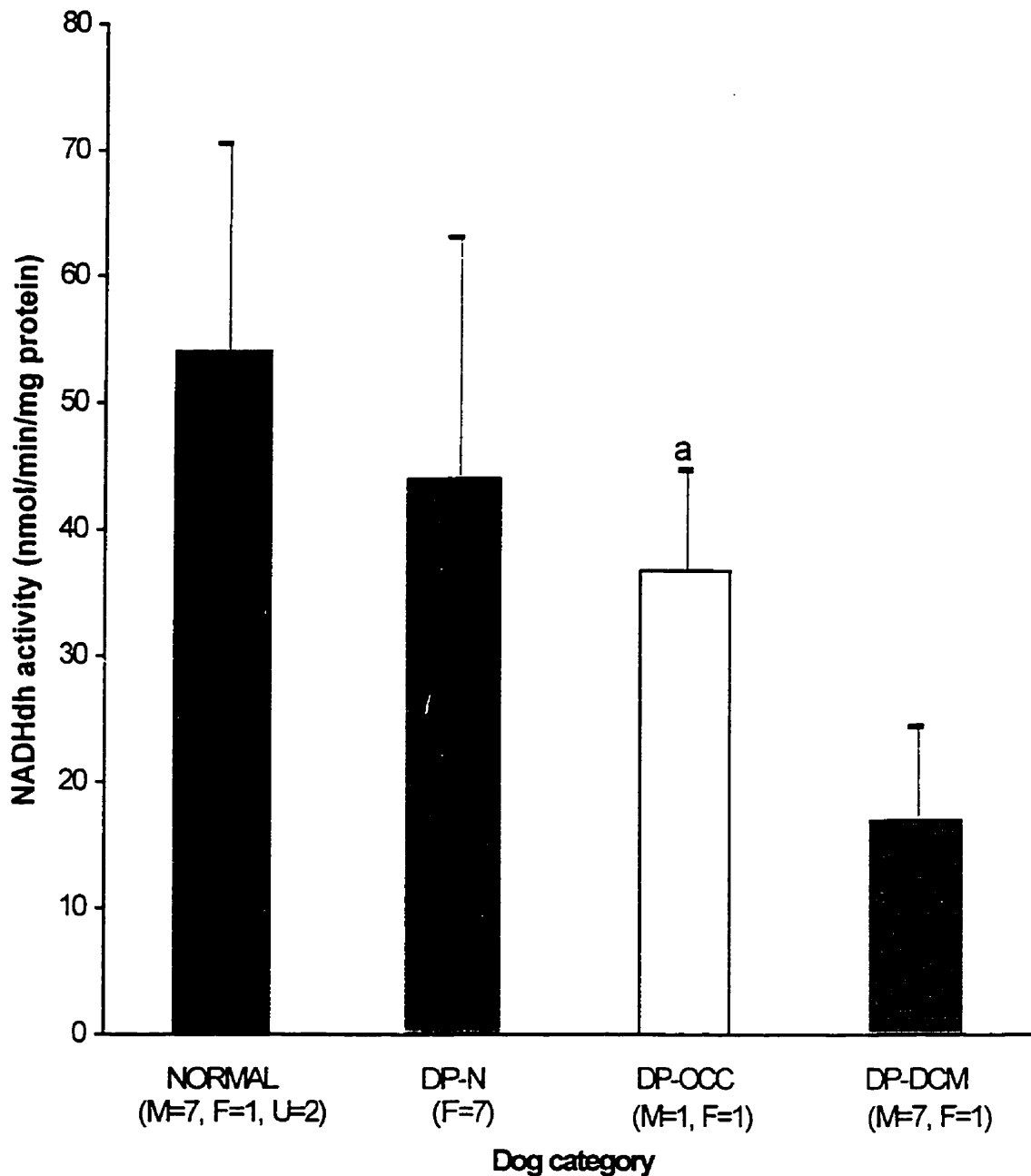
Fig. 2.4. Mean myocardial NADHdh activity for each dog category. The lines over the bars indicate the standard deviation. Different letters above the bars indicate a statistically significant difference between the means at p < 0.001. The number in brackets, below DP-N (normal Doberman Pinschers), DP-OCC (Doberman Pinschers with occult disease) and DP-DCM (Doberman Pinscher with dilated cardiomyopathy), indicates the number of dogs in each category.

	DP-N (Mean ± SE)	DP-OCC (Mean ± SE)	DP-DCM (Mean ± SE)	F	p	Power
FS (%)	23.9 ± 1.9 [a]	19.7 ± 4.1 [a]	7.7 ± 2.2 [b]	15.6	< 0.001	0.997
LVIDd (mm)	36.3 ± 1.3 [a]	47.5 ± 2.8 [b]	57.8 ± 1.3 [c]	58.0	< 0.001	1.000
LVIDs (mm)	27.7 ± 1.8 [a]	38.2 ± 3.9 [b]	53.6 ± 2.1 [c]	43.4	< 0.001	1.000
EPSS (mm)	3.1 ± 1.5	6.6 ± 3.2	7.0 ± 1.7	1.6	0.238	0.283
LVFWd (mm)	7.9 ± 0.4	6.5 ± 0.9	7.8 ± 0.5	1.0	0.391	0.192
LVFWs (mm)	11.4 ± 0.5 [a]	8.7 ± 1.1 [ab]	8.0 ± 0.6 [b]	9.3	0.002	0.945
WSd	4.7 ± 0.4 [a]	7.6 ± 0.9 [b]	7.6 ± 0.5 [b]	11.6	0.001	0.979
WSs	2.5 ± 0.5 [a]	4.5 ± 1.0 [ab]	7.0 ± 0.6 [b]	17.8	< 0.001	0.999

Table 2.9. Statistical analysis of echocardiography data, including 9 (all female) normal Dobermans (DP-N), 2 (1 female, 1 male) Dobermans with occult heart disease (DP-OCC), and 8 (7 females, 1 male) Dobermans with DCM (DP-DCM). Echocardiographic parameters included average fractional shortening (FS), average left ventricular internal diameter during diastole (LVIDd), average left ventricular internal diameter during systole (LVIDs), average E point to septal separation (EPSS), average left ventricular free wall stress during diastole (LVFWd), average left ventricular free wall stress during systole (LVFWs), and average wall stress during diastole (WSd), and systole (WSs). The mean, plus or minus the standard error, has been included for each echocardiographic parameter. Superscripts [a], [b], and [c], denote differences among the groups of dogs for each echocardiographic parameter. The F and p values, and the power of the test have been included for the analysis of variance between the three groups of dogs.

	DP-N (Mean ± SE)	DP-OCC (Mean ± SE)	DP-DCM (Mean ± SE)	F	p	Power
LVIDd /BW$^{0.333}$ (mm/kg)	11.8 ± 0.4 [a]	14.3 ± 0.6 [a]	18.6 ± 0.7 [b]	33.1	< 0.001	1.000
LVIDs /BW$^{0.333}$ (mm/kg)	9.0 ± 0.4[a]	11.5 ± 0.8 [a]	17.4 ± 0.8 [b]	42.7	< 0.001	1.000
EPSS /BW$^{0.333}$ (mm/kg)	1.0 ± 0.3	2.0 ± 0.1	2.4 ± 0.9	1.2	0.328	0.215
LVFWd /BW$^{0.333}$ (mm/kg)	2.7 ± 0.1	2.0 ± 0.4	2.4 ± 0.1	3.2	0.076	0.503
LVFWs /BW$^{0.333}$ (mm/kg)	3.7 ± 0.2 [a]	2.6 ± 0.3 [ab]	2.4 ± 0.2 [b]	8.1	0.007	0.884
WSd	4.5 ± 0.3 [a]	7.6 ± 1.7 [ab]	7.9 ± 0.7 [b]	9.5	0.003	0.938
WSs	2.5 ± 0.2 [a]	4.5 ± 0.9 [ab]	7.2 ± 1.0 [b]	11.7	0.002	0.97

Table 2.10. Statistical analysis of echocardiography data, scaled for body weight, to the power of 0.333. The dog categories contained 6 (all female) normal Dobermans (DP-N), 2 (1 female, 1 male) Dobermans with occult heart disease (DP-OCC), and 8 (1 female, 7 male) Dobermans with DCM (DP-DCM). Echocardiographic parameters included average left ventricular internal diameter during diastole (LVIDd), average left ventricular internal diameter during systole (LVIDs), average E point to septal separation (EPSS), average left ventricular free wall stress during diastole (LVFWd), average left ventricular free wall stress during systole (LVFWs), and average wall stress during diastole (WSd). The mean, plus or minus the standard error, has been included for each echocardiographic parameter. Superscripts [a], [b], and [c], denote differences among the groups of dogs for each echocardiographic parameter. The F and p values, and the power of the test have been included for the analysis of variance among the three groups of dogs.
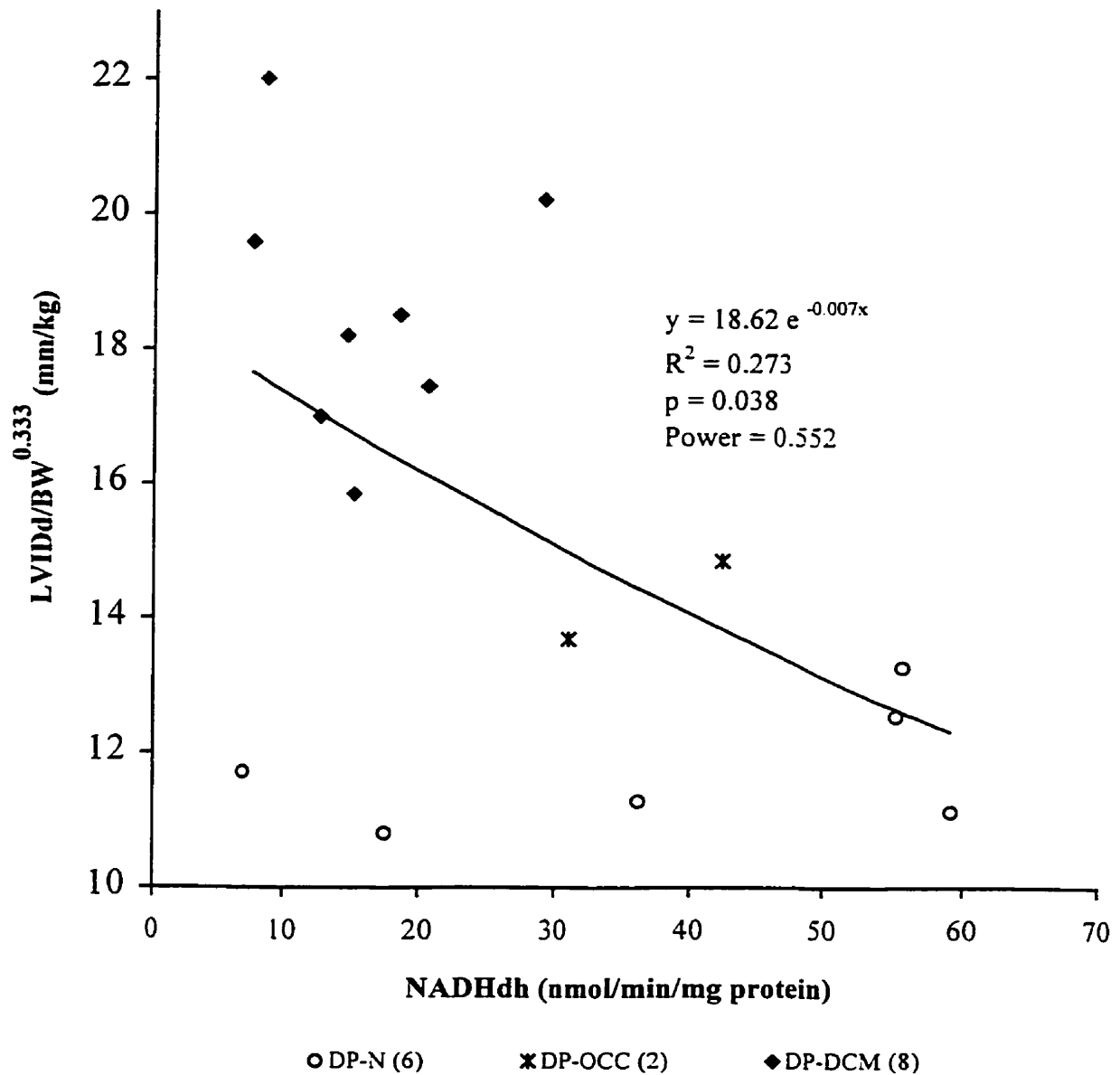
Fig. 2.5. Nonlinear regression of myocardial NADHdh activity over average left ventricular internal dimension during diastole (LVIDd), scaled to the 0.333 power of the body weight. The equation for line of best fit, the regression coefficient (R^2), the p value, and the power, have been included with the graph. The number in brackets, beside DP-N (normal Doberman Pinschers), DP-OCC (Doberman Pinschers with occult disease) and DP-DCM (Doberman Pinscher with dilated cardiomyopathy), indicates the number of dogs in each category.
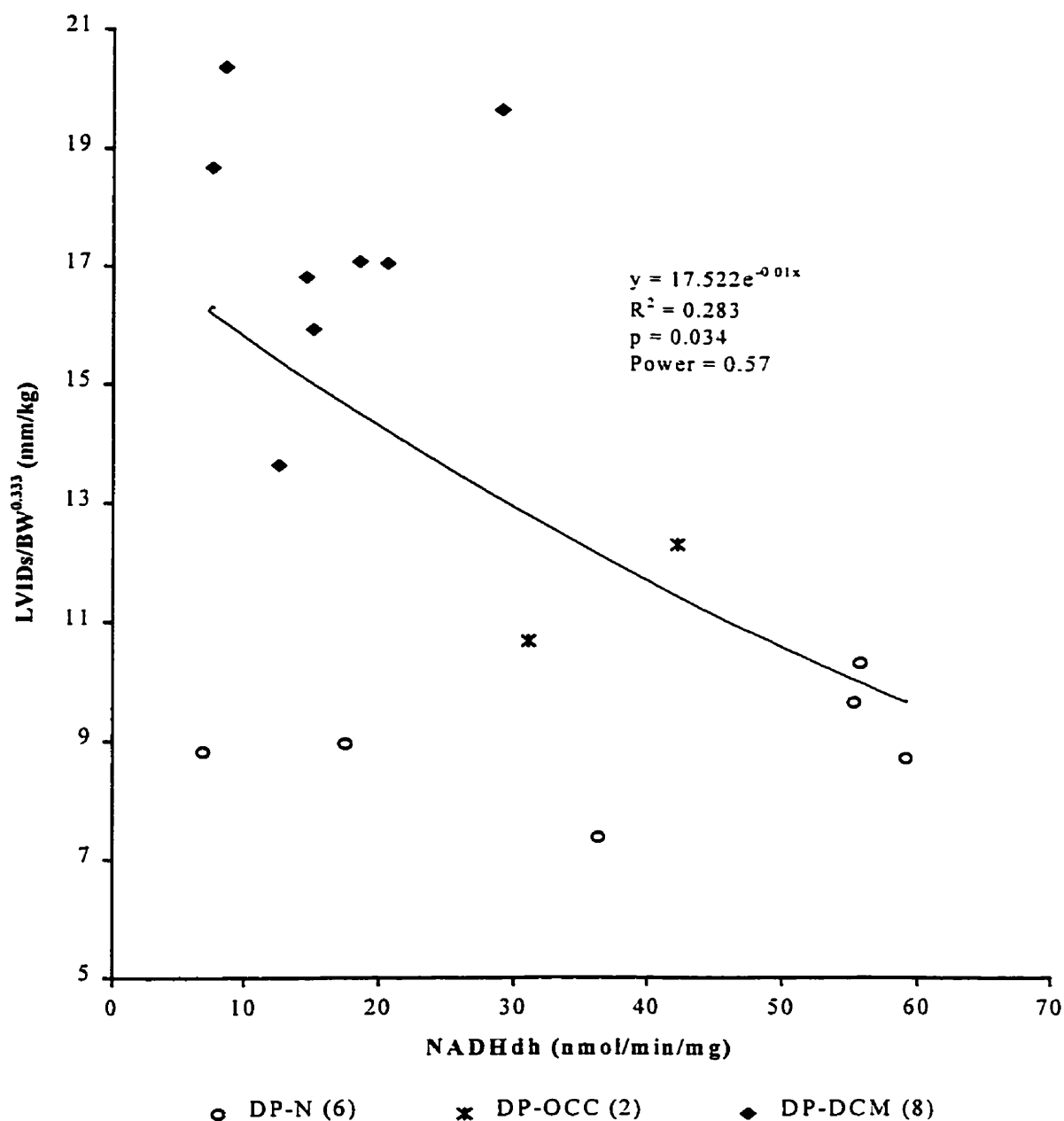
$$y = 17.522e^{-0.01x}$$
$$R^2 = 0.283$$
$$p = 0.034$$
$$\text{Power} = 0.57$$

LVIDs/BW$^{0.333}$ (mm/kg)

NADHdh (nmol/min/mg)

o DP-N (6)   x DP-OCC (2)   ◆ DP-DCM (8)

Fig. 2.6. Nonlinear regression of myocardial NADHdh activity over average left internal dimension during systole (LVIDs), scaled for body weight to the power of 0.333. The equation for line of best fit, the regression coefficient (R^2), the p value, and the power have been included with the graph for normal Doberman Pinschers (DP-N), Dobermans with occult heart disease (DP-OCC), and Doberman Pinscher dogs with dilated cardiomyopathy (DP-DCM). The number of dogs in each category has been indicated in brackets beside the corresponding category.
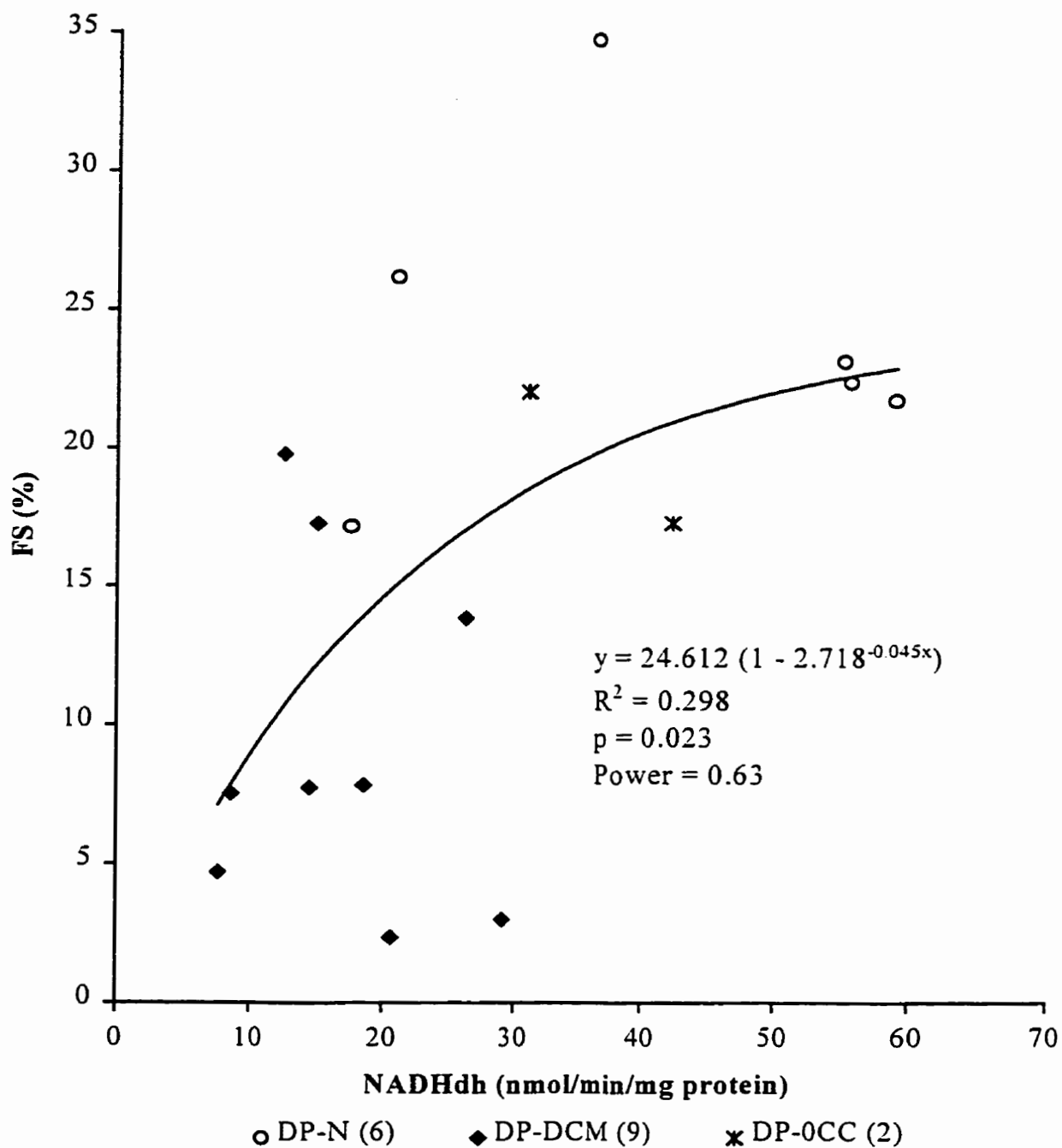
Fig. 2.7. Nonlinear regression of myocardial NADHdh activity on fractional shortening (FS). The equation for line of best fit, the regression coefficient (R^2), p value, and power, have been included with the graph for three types of Doberman Pinscher dogs: normal (DP-N), occult (DP-OCC) and those with dilated cardiomyopathy (DP-DCM). The number of dogs in each category is indicated in brackets beside the corresponding category.
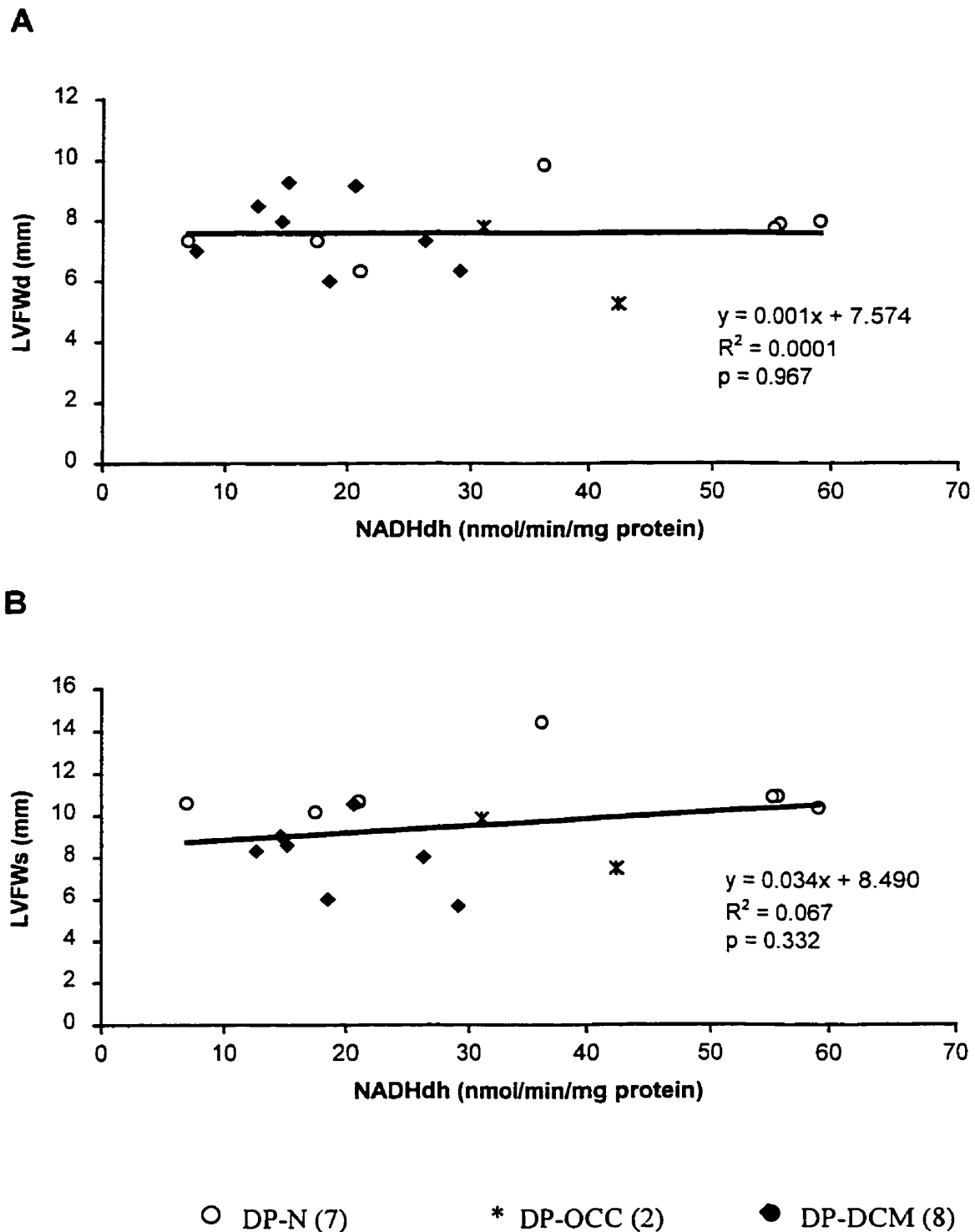
A

B

O DP-N (7)   * DP-OCC (2)   ● DP-DCM (8)

Fig. 2.8. Regressions of myocardial NADHdh activity over average left ventricular free wall dimension (LVFW) during A) diastole (LVFWd), and B) systole (LVFWs). The equation for line of best fit, the regression coefficient (R^2), and the p value have been included with both graphs. The number of normal Doberman Pinschers (DP-N), Doberman Pinscher with occult cardiomyopathy, and Doberman Pinscher dogs with dilated cardiomyopathy (DP-DCM) are indicated in brackets beside each category.
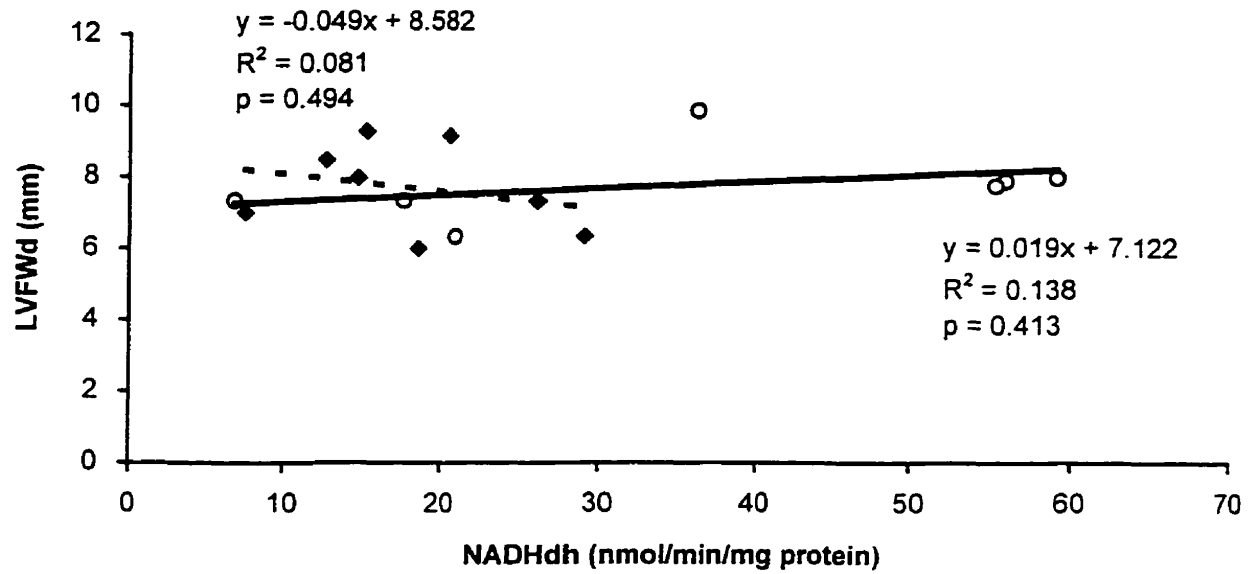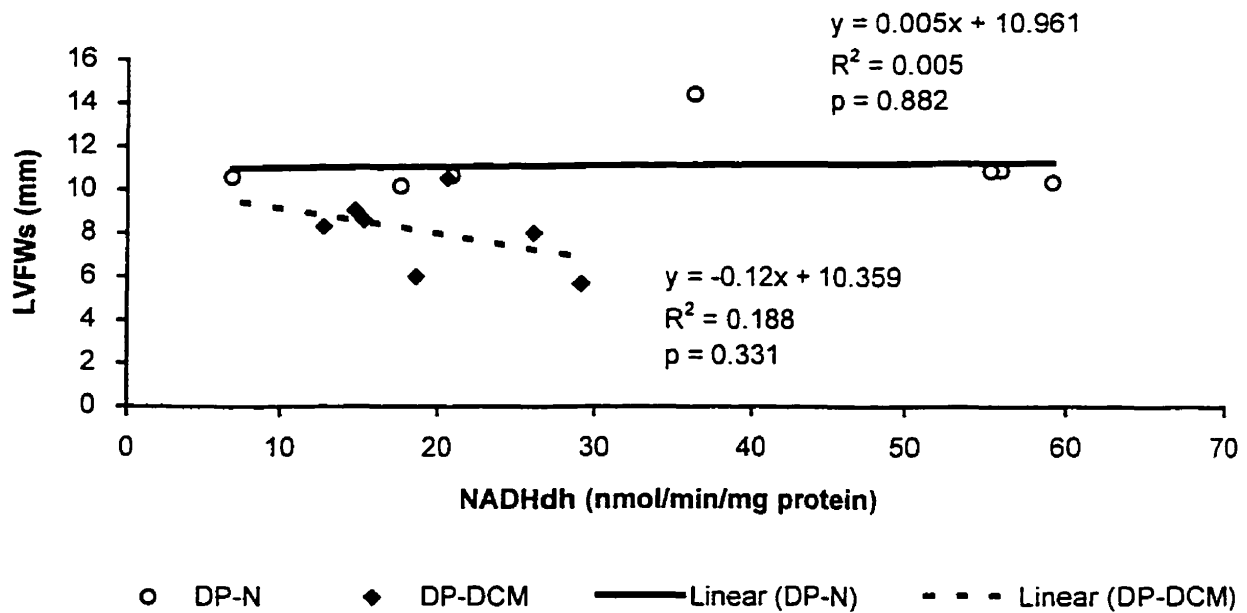
A

$y = -0.049x + 8.582$
$R^2 = 0.081$
$p = 0.494$

$y = 0.019x + 7.122$
$R^2 = 0.138$
$p = 0.413$

B

$y = 0.005x + 10.961$
$R^2 = 0.005$
$p = 0.882$

$y = -0.12x + 10.359$
$R^2 = 0.188$
$p = 0.331$

○ DP-N   ◆ DP-DCM   ──── Linear (DP-N)   ‑ ‑ ‑ Linear (DP-DCM)

Fig. 2.9. Regressions of myocardial NADHdh activity over average left ventricular free wall dimension (LVFW) during A) diastole (LVFWd), and B) systole (LVFWs). The equation for line of best fit, the regression coefficient (R^2), and the p value have been included with the graph for both normal Doberman Pinschers (DP-N), and Doberman Pinscher dogs with dilated cardiomyopathy (DP-DCM).

A

B
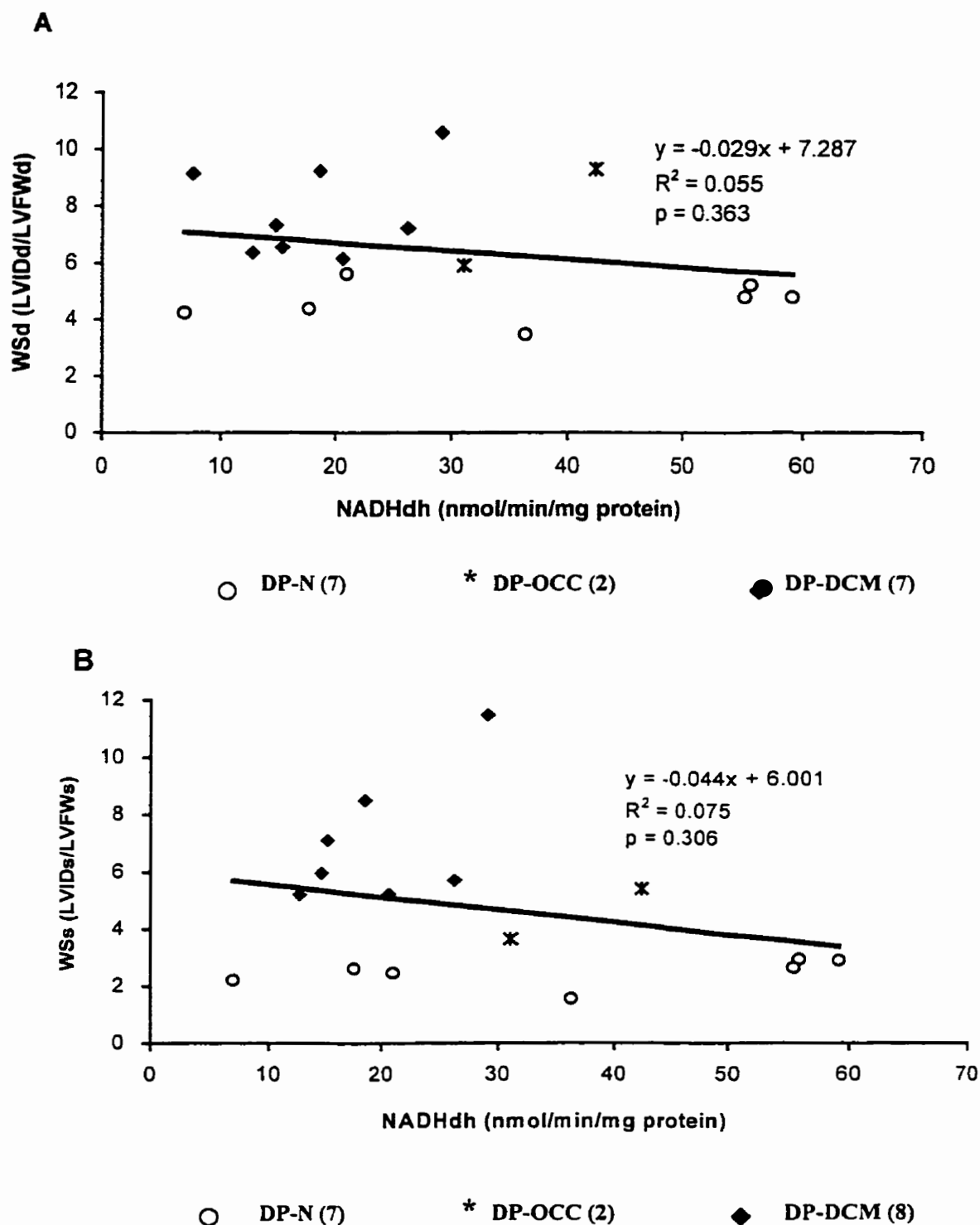
Fig. 2.10. Regressions of myocardial NADHdh activity over average wall stress (WS) during A) diastole (WSd), and B) systole (WSs). The equation for line of best fit, the regression coefficient (R^2), and the p value have been included with the graph for all dog types. The number of normal Doberman Pinschers (DP-N), Doberman Pinchers with occult disease and Doberman Pinscher dogs with dilated cardiomyopathy (DP-DCM) are indicated in brackets, beside each category.

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Fig. 2.11. Regressions of myocardial NADHdh activity over average wall stress (WS) during A) diastole (WSd), and B) systole (WSs). The equation for line of best fit, the regression coefficient (R^2), and the p value have been included with the graph for both normal Doberman Pinschers (DP-N), and Doberman Pinscher dogs with dilated cardiomyopathy (DP-DCM).
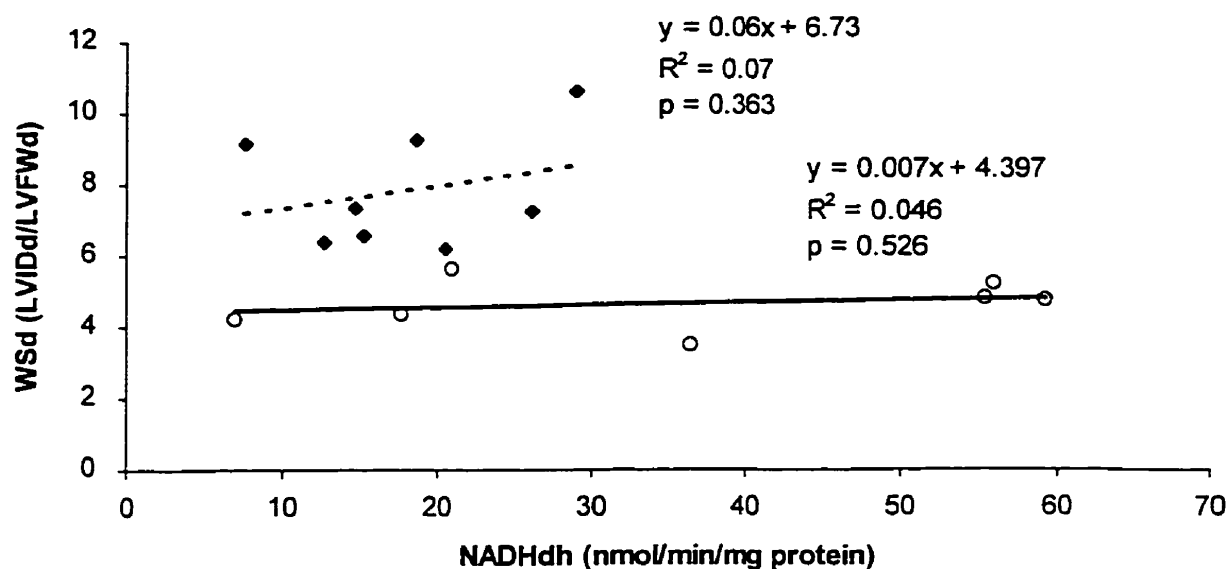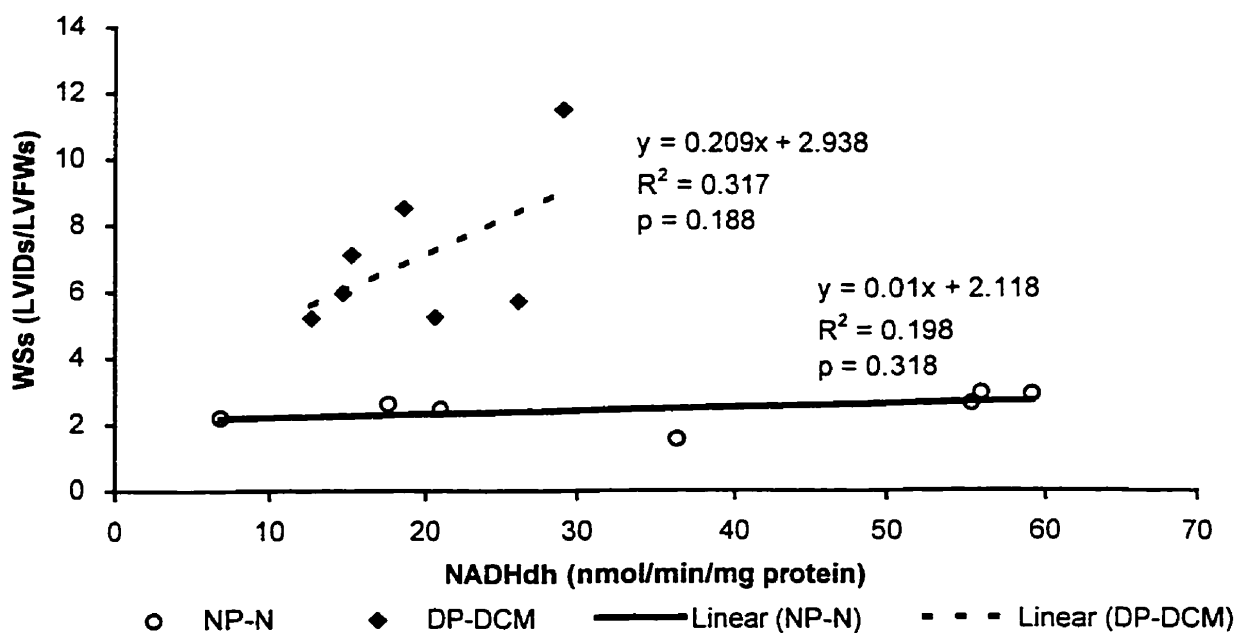
2.3.3. Gastrocnemius muscle NADHdh activity

When gastrocnemius muscle NADHdh activity was measured using the revised technique that had been developed for myocardial NADHdh determination, only small differences in total NADHdh activity and rotenone-inhibitable NADHdh activity were observed results. A higher concentration of muscle in the homogenate (1 mass of muscle to 11 times this mass in buffer) was chosen as a compromise between the need for a higher total NADHdh activity per unit volume of buffer and the consequent increase in quenching of fluorescence (Fig. 2.13.). The standard curve was prepared with 2.4 ml buffer (50 mM Tris/80 mM KCl), 60 μl of gastrocnemius muscle homogenate, 0.1 ml of 10% SDS and 5 μl aliquots of 1 mM NADH (Fig. 2.12.). There was a good fit of the data to the quadratic curve ($R^2 = 0.9999$) (Fig. 2.12.). A comparison of the total and rotenone-insensitive NADHdh activities can be seen in Fig. 2.13. Mean gastrocnemius muscle NADHdh activity for the different categories of dogs is plotted in Fig. 2.14. and Fig. 2.15. The ANOVA of gastronemius muscle NADHdh activities in normal mixed breed dogs, normal Dobermans and Dobermans with DCM, on a wet weight of gastrocnemius muscle, was significant (p = 0.035). In post hoc tests, a comparison of gastronemius muscle NADHdh activities, by the protected LSD method, yielded significant differences between the gastrocnemius muscle NADHdh activities of Dobermans with DCM compared to normal mixed breed dogs (p = 0.027), and between normal Dobermans compared to normal mixed breed dogs (p = 0.029) (Fig. 2.14). Duncan's test also demonstrated a significant difference between the gastrocnemius muscle NADHdh activities of Dobermans with DCM compared to normal mixed breed dogs (p = 0.045),

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and between normal Dobermans compared to normal mixed breed dogs (p = 0.031). Tukey's honest significant difference test for groups with unequal number of samples gave a probability value of 0.077 for the comparison of gastrocnemius muscle NADHdh activities of Dobermans with DCM compared to normal mixed breed dogs.

No significant difference could be demonstrated between the mean gastrocnemius muscle NADHdh activity, expressed per milligram muscle protein, in normal mixed breed dogs (19.8 ± 11 ηmol/min/mg protein), normal Dobermans (9.3 ± 4.3 ηmol/min/mg protein) and Dobermans with DCM (12.9 ± 8.4 ηmol/min/mg protein) (Fig. 2.15). The ANOVA for this comparison was p = 0.103. There was an increase in the variance, when gastrocnemius muscle NADHdh activity was determined per wet weight of muscle (mean plus and minus the standard deviation for all dogs = 3.84 ± 1.85 μmol/min/g muscle wet weight), compared to when expressed in terms of the protein content of the muscle (14.65 ± 9.82 ηmol/r.:in/mg protein).
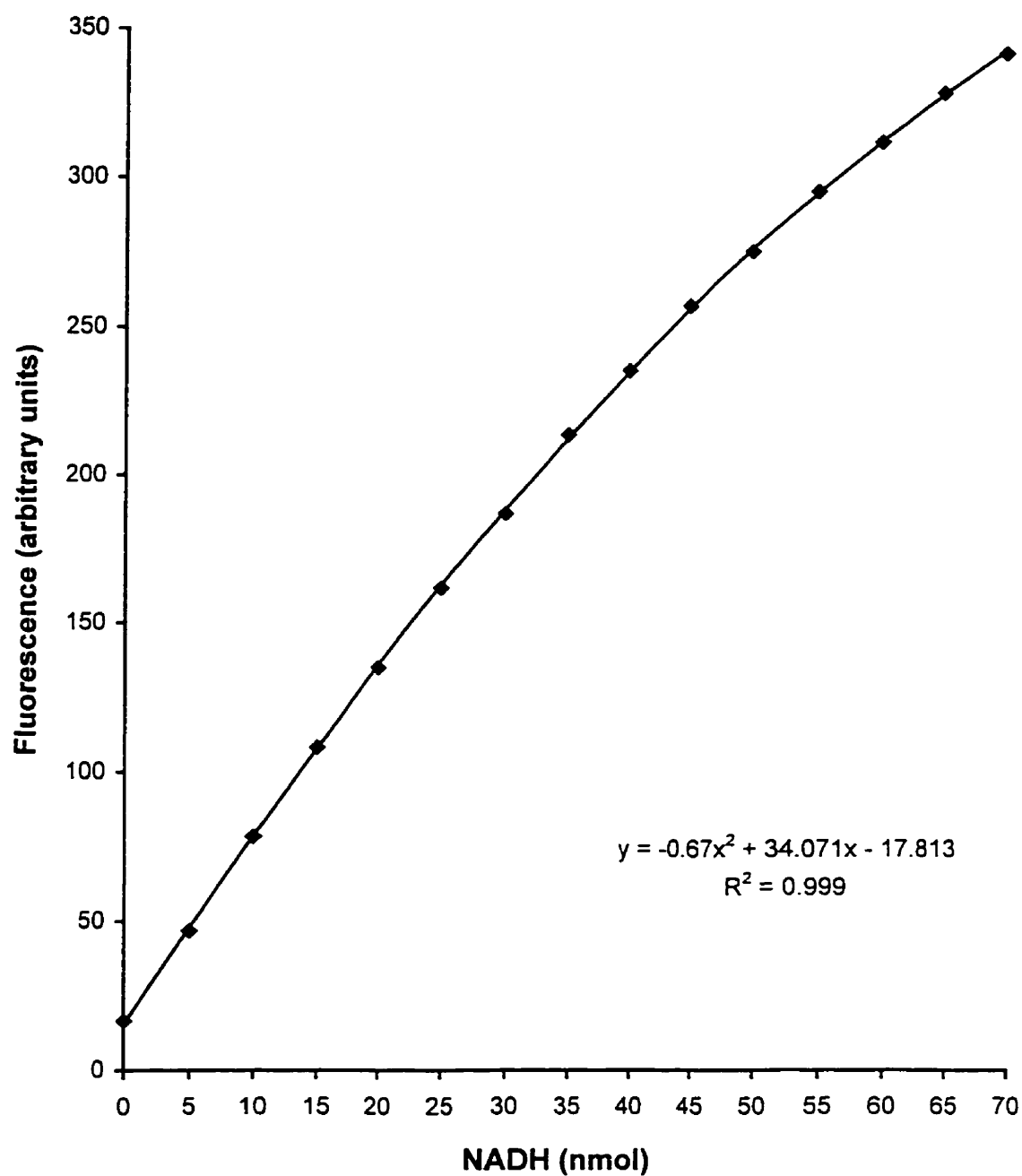
Fig. 2.12. Standard curve for NADH in gastrocnemius muscle homogenate (sample number LB246S1). The quadratic equation for line of best fit and the regression coefficient (R^2) have been included with the graph.
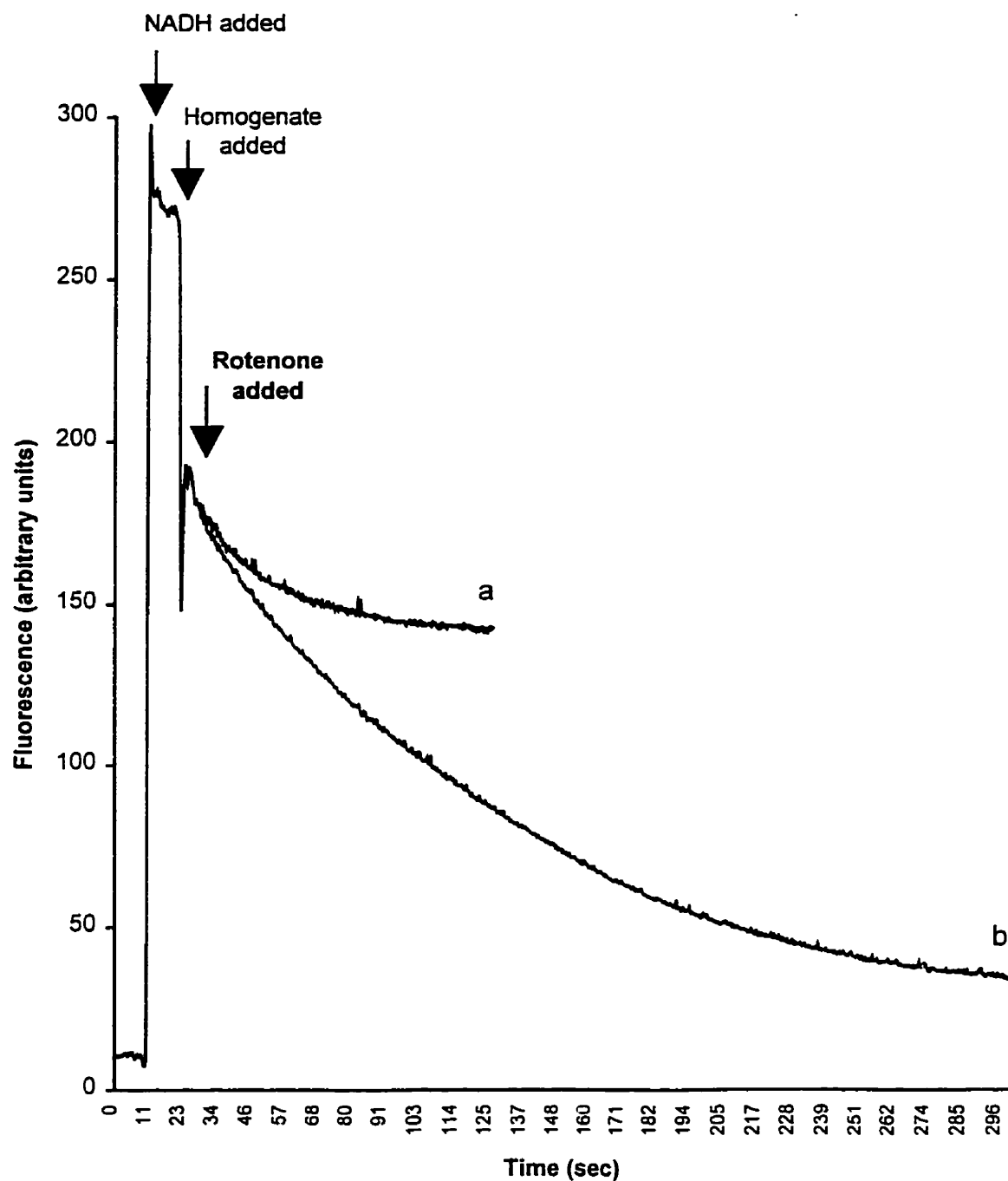
Fig. 2.13. Comparison of gastrocnemius muscle NADHdh activity with (a), and without (b) the addition of rotenone (dog number H25). The reaction mixtures for a and b contained 2.5 ml of 80 mM potassium chloride, 50mM Tris, 5 μg gastrocnemius muscle and 19 mM NADH at pH 7.0. In a, 30 ηmoles of rotenone in 99% dimethyl sulfoxide were added immediately after the muscle homogenate.
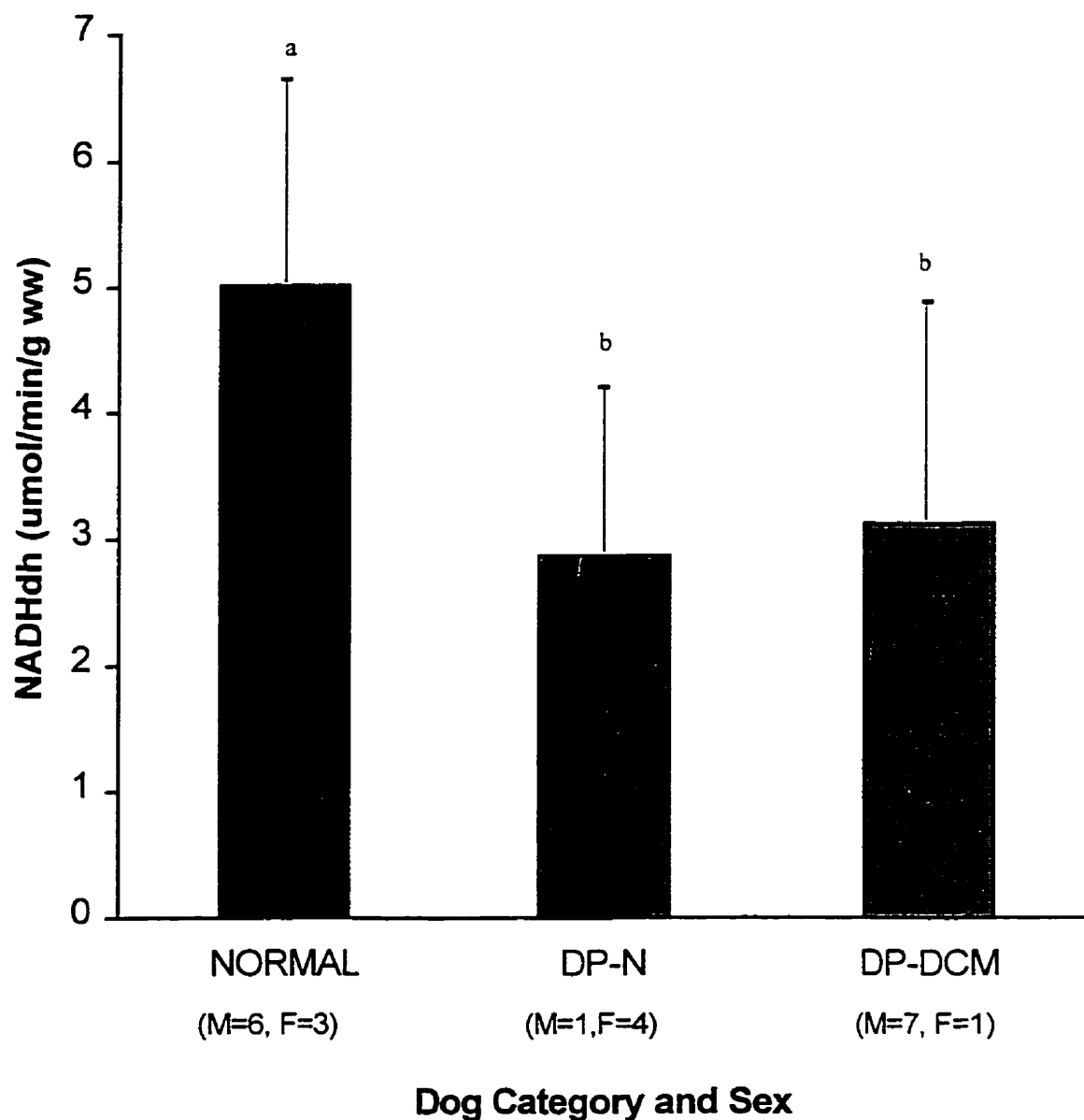
Fig. 2.14. Mean gastrocnemius muscle NADHdh activity, per gram wet weight (ww) of muscle, for each dog category. The lines above the bars indicate the standard deviation. The p value for ANOVA was 0.035. Different letters above the bars indicates a significant (p < 0.05) difference between dog categories by the protected LSD test. The number in brackets, below Normal (normal mixed breed dogs), DP-N (normal Doberman Pinschers), and DP-DCM (Doberman Pinscher with dilated cardiomyopathy), indicates the number of male (M) and female (F) dogs.

Fig. 2.15. Mean gastrocnemius muscle NADHdh activity, per milligram of muscle protein, for each dog category. Lines above the bars indicate the standard deviation. The p value for the ANOVA was 0.103. The number in brackets, below Normal (normal mixed breed dogs), DP-N (normal Doberman Pinschers), and DP-DCM (Doberman Pinscher with dilated cardiomyopathy), indicates number of male (M) and female (F) dogs.
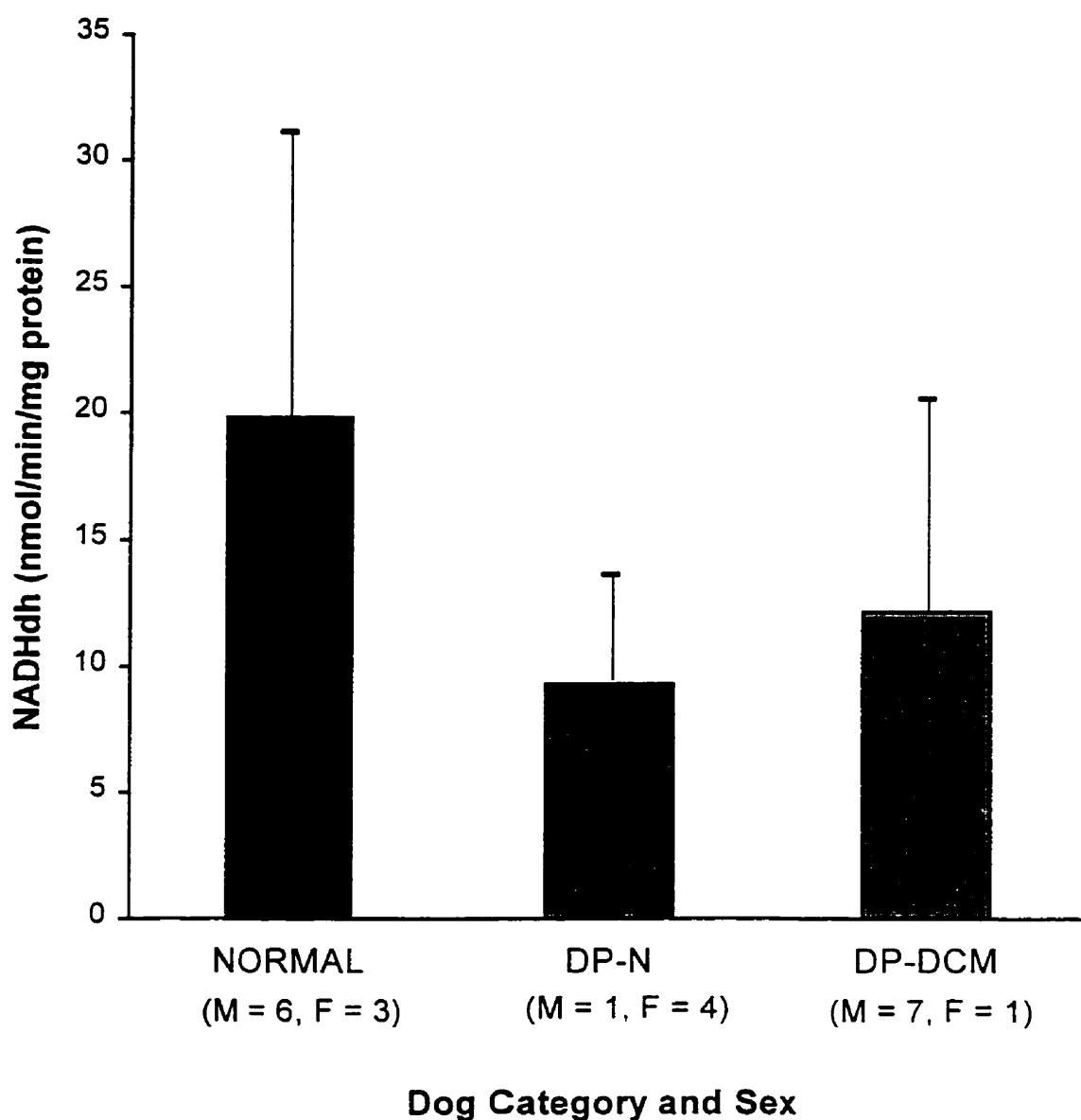
2.3.4. Myocardial and gastrocnemius muscle lactic acid concentration and urinary lactate to creatinine ratio

Mean myocardial lactate concentration in Dobermans with DCM was significantly higher (188 ± 83 µmol/g protein) ($p < 0.05$) than normal mixed breed dogs (109 ± 26 µmol/g protein) and normal Dobermans (109 ± 36 µmol/g protein) (Fig. 2.16.).

No significant difference was measured in the mean gastrocnemius muscle lactate concentration per gram frozen tissue or per gram protein for the 4 categories of dogs (Fig. 2.18.).

The mean urinary lactate to creatinine ratio for Dobermans with DCM was significantly higher than the ratio for the normal mixed breed dogs (Fig. 2.20.). While the urinary lactate to creatinine ratio increased progressively from normal mixed breed dogs (0.98 ± 0.71%), to normal Dobermans (4.0 ± 3.5%), and then Dobermans with occult heart disease (5.52 ± 6.66%), this difference was not significant (Fig. 2.20.).

A third order polynomial relationship (Fig. 2.17.) and an exponential decay function (not presented) were demonstrated between myocardial NADHdh activity and myocardial lactic acid concentration.

No significant linear (Fig. 2.19.) or nonlinear (not presented) relationship could be demonstrated for the population of normal mixed breed, normal Dobermans, or Dobermans with DCM (Fig. 2.19.).

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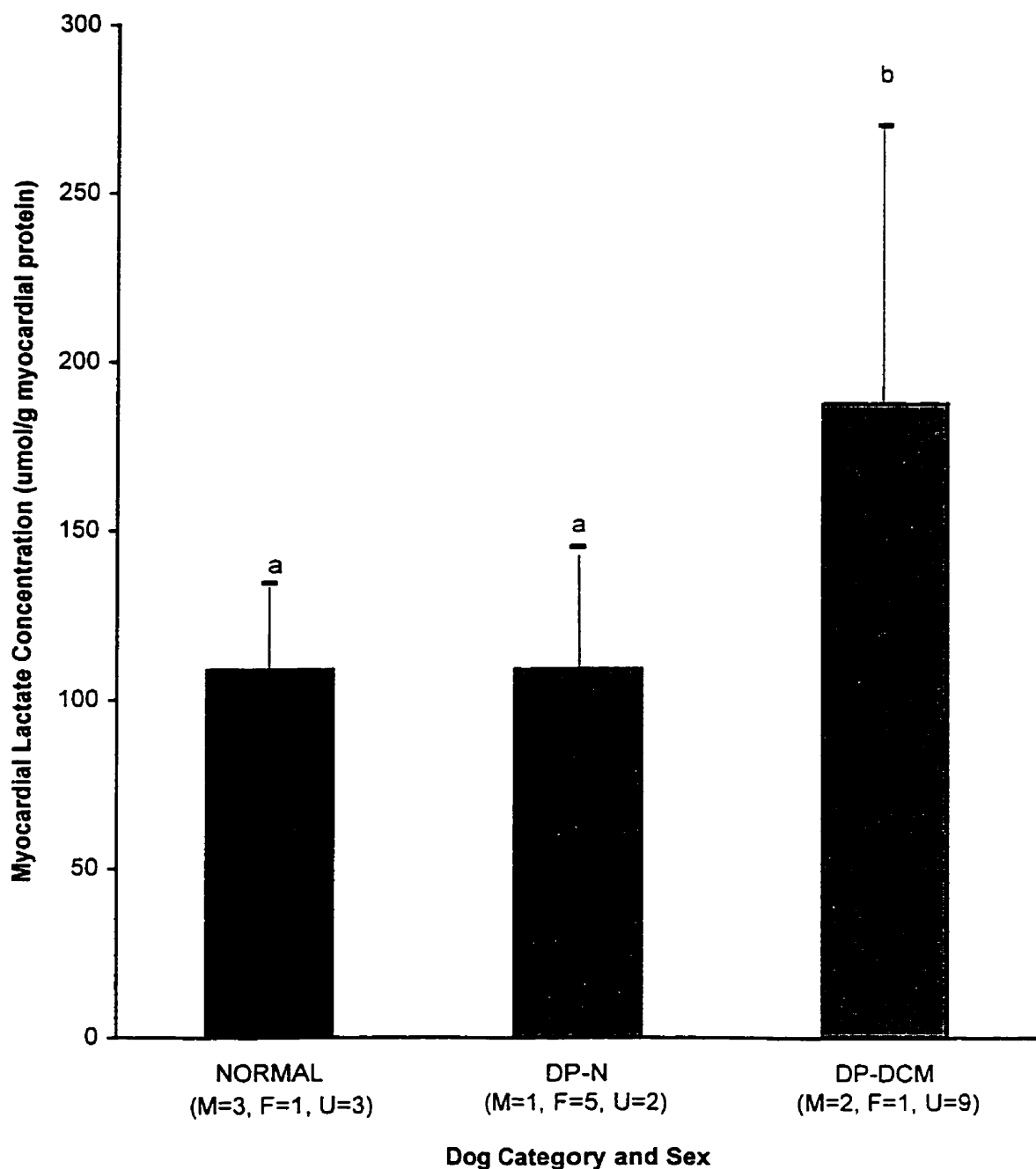
Fig. 2.16. Mean myocardial lactate concentration for each dog category. The lines over the bars indicate the standard deviation. Different letters above the bars indicates a statistically significant difference between means at p < 0.05 by Tukey's honest significant difference test. The number in brackets, below Normal (normal mixed breed dogs), DP-N (normal Doberman Pinschers), and DP-DCM (Doberman Pinscher with dilated cardiomyopathy), indicates the number of males (M), females (F) or unknown (U) sex of the dogs.

$$y = 3.68 + (11624.02/x) + (-371272.36/x^2) + (3636739/x^3)$$
$$R^2 = 0.77$$
$$p = 0.0008$$
$$\text{Power (at } \alpha = 0.05) = 0.997$$
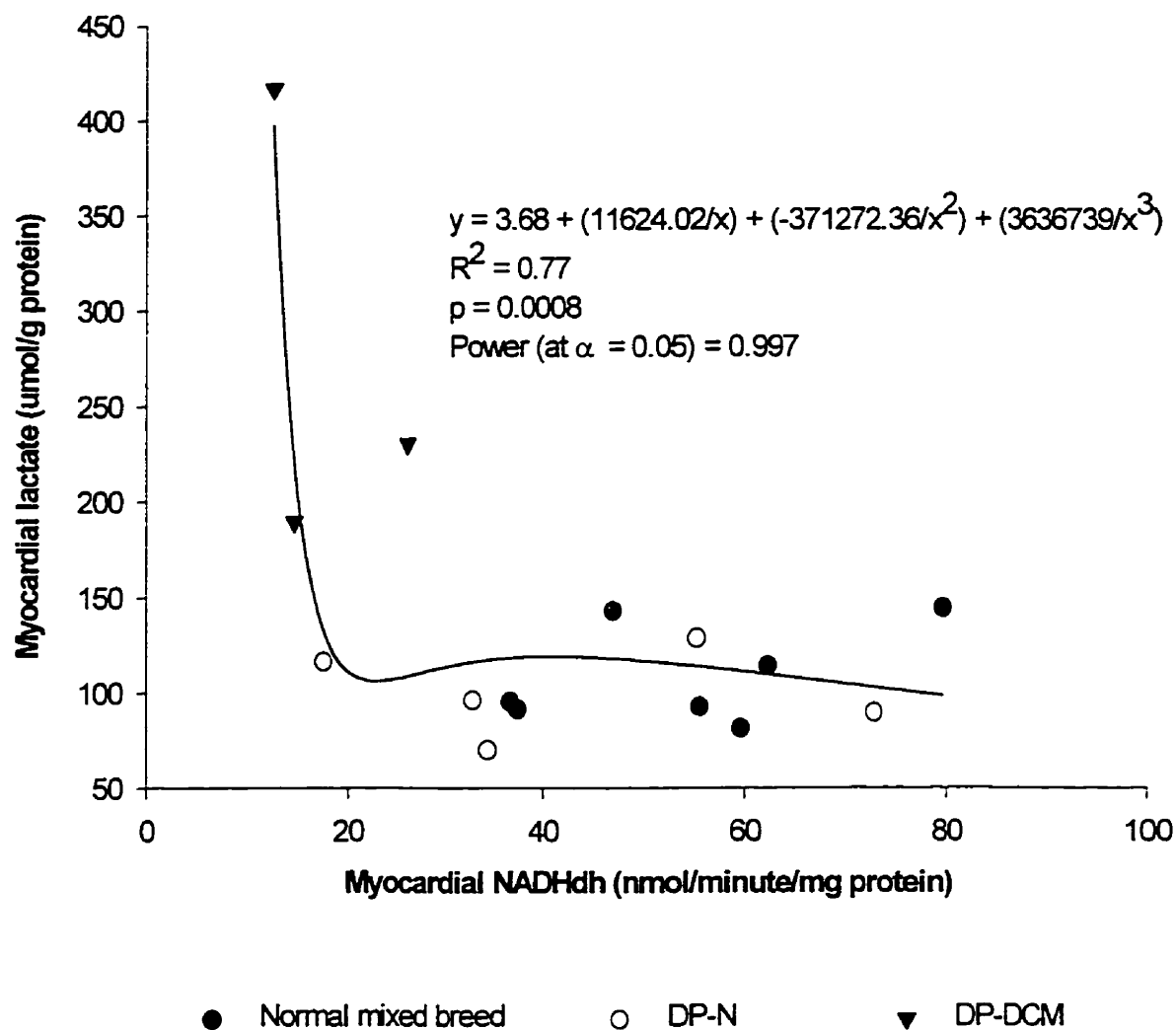
Fig. 2.17. Nonlinear regression of myocardial NADHdh activity over myocardial lactate for total population of normal mixed breed dogs, normal Dobermans (DP-N) and Dobermans with DCM (DP-DCM). The equation for line of best fit, the regression coefficient (R^2), p value and power of the test have been included with the graph.
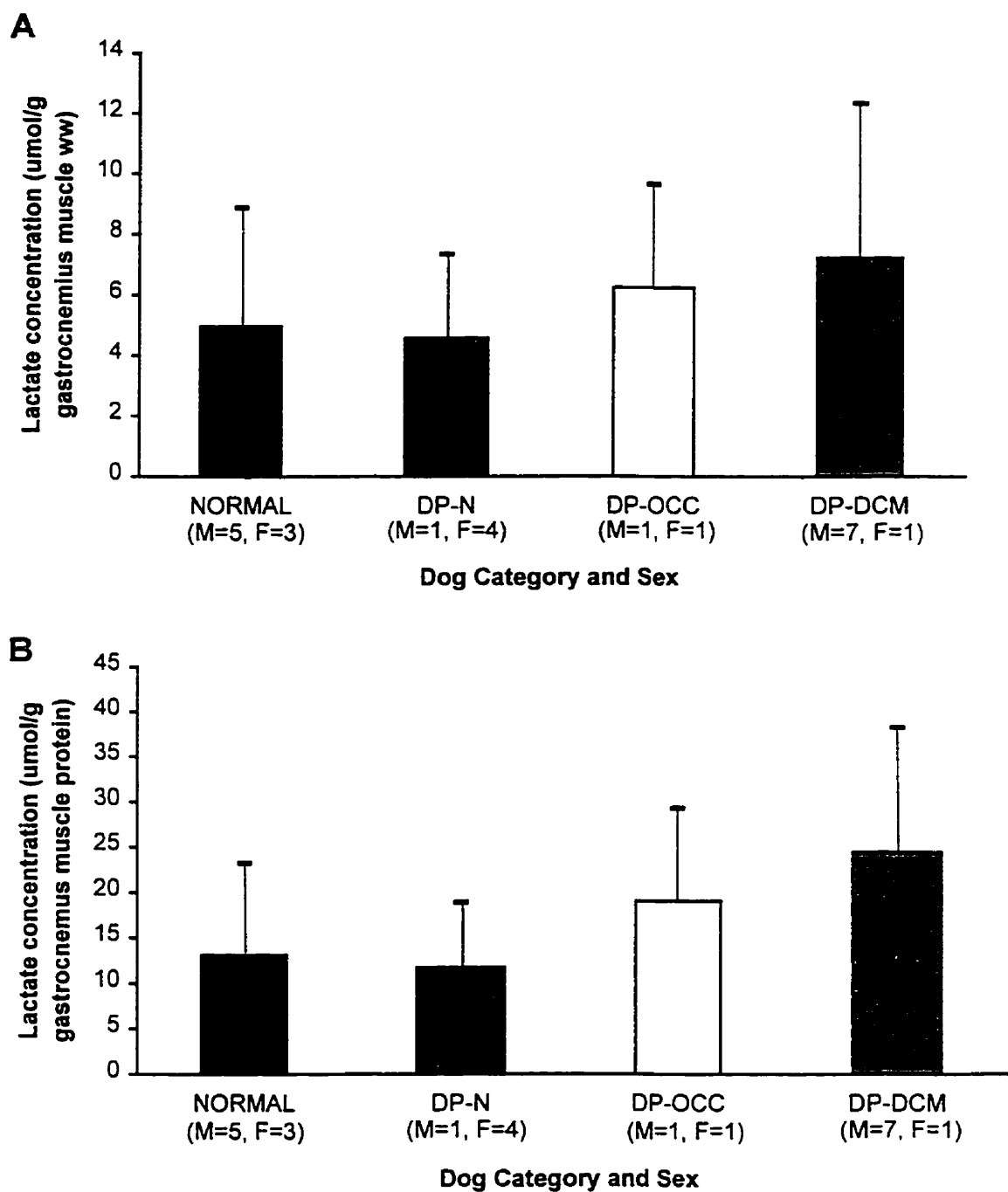
Fig. 2.18. Mean lactate concentration in gastrocnemius muscle for each dog category expressed in A) per gram wet weight (ww) of muscle, and B) per gram muscle protein. The lines over the bars indicate the standard deviation. No significant differences were present between the means for the different dog categories. The number in brackets, below Normal (normal mixed breed dogs), DP-N (normal Doberman Pinschers), and DP-DCM (Doberman Pinscher with dilated cardiomyopathy), indicates the number of male (M) and female (F) dogs.
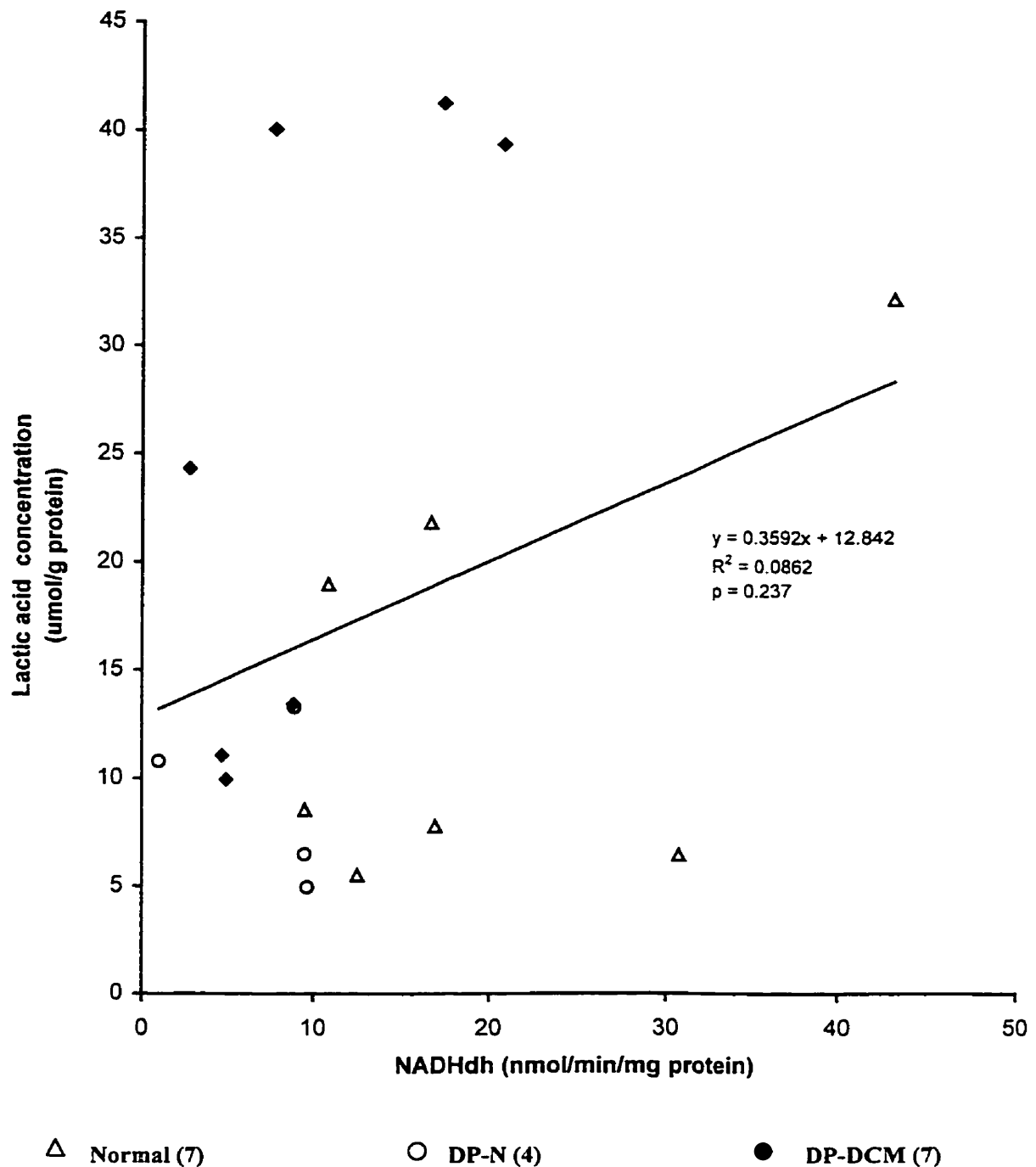
Fig. 2.19. Regression of gastrocnemius muscle NADHdh activity over muscle lactic acid concentration for the total population of dogs including normal mixed breed (normal), normal Dobermans (DP-N), and Dobermans with DCM (DP-DCM). Numbers in the brackets beside normal, DP-N, and DP-DCM indicate the number of dogs in each category. The equation for line of best fit, regression coefficient (R^2) and p value have been included with the graph.

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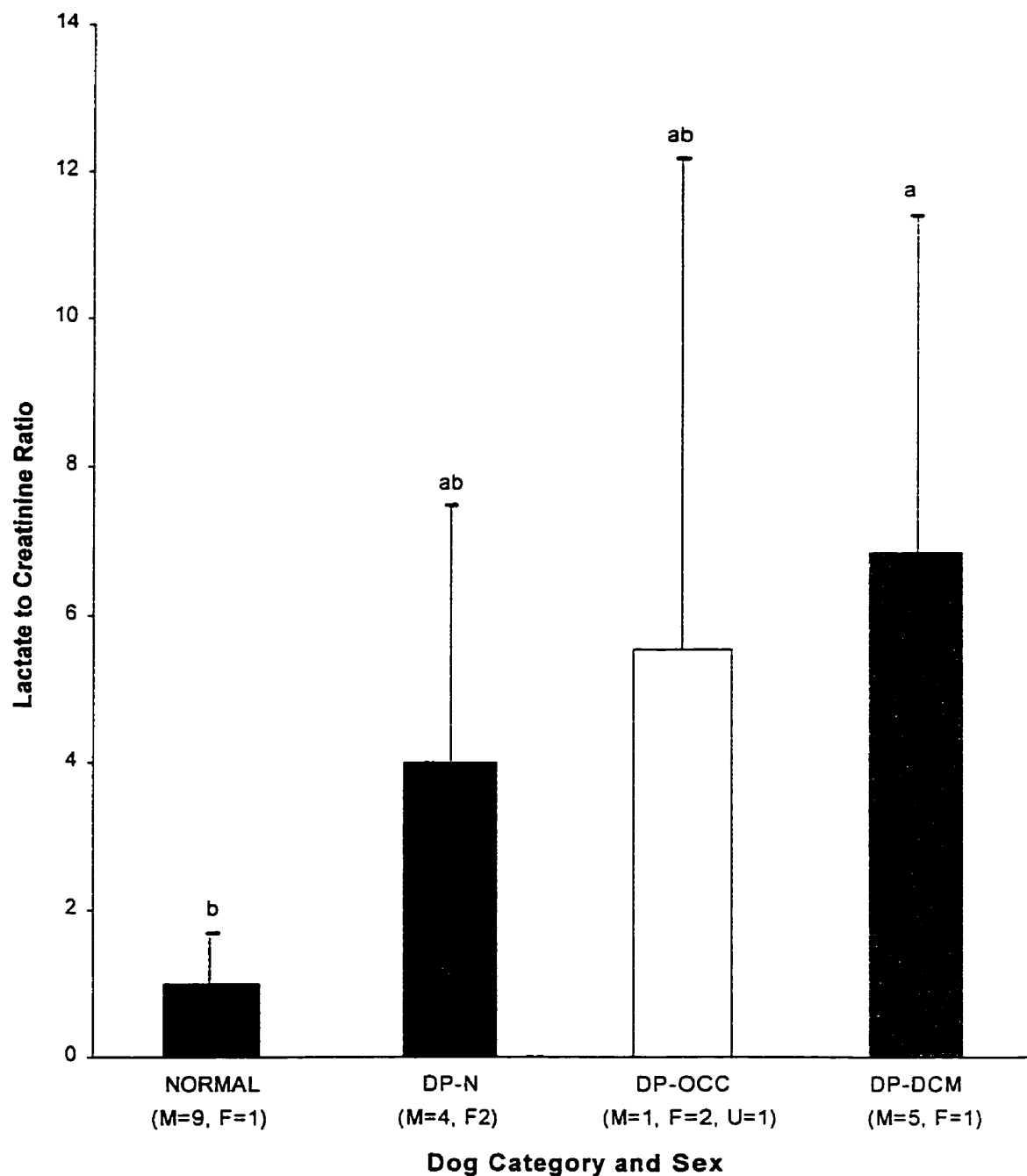
Fig. 2.20. Mean urinary lactate to creatinine ratio for each dog category. The lines over the bars indicate the standard deviation. Different letters above the bars indicates a statistically significant difference between the means at p = 0.05 by Tukey's honest significant difference test. The numbers in brackets indicate the number of male (M) and female (F) dogs.

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2.3.5. Histological study of myocardium

Formalin fixed myocardial tissue was only available from 1 normal mixed breed dog, 2 normal Dobermans, 1 occult Doberman and 1 Doberman with DCM, of those dogs included in the biochemical studies. Other cases with myocardium from normal mixed breed dogs, normal Dobermans, Dobermans with DCM and other breeds with DCM were included in the description from the Department of Pathobiology, Ontario Veterinary College files. A summary of histological changes present in the heart of the different dogs is shown in tables 2.11., 2.12., and 2.13.

2.3.5.1. Normal Mixed Breed Dog

Mild fatty replacement and a single inflammatory focus, containing small numbers of lymphocytes and macrophages, were seen in the section of left ventricular papillary muscle from one normal mixed breed dog (64849) (Table 2.11.). A single medium-sized muscular artery in the myocardium of dog 65331/8 had circumferential thickening of the tunica intima due to the deposition of amorphous eosinophilic material (possibly amyloid), up to 75 μm thick, and rare macrophages.

Brown pigment granules (lipofuscin) were found near the poles of cardiomyocyte nuclei in 6 out of 8 normal mixed breed dogs over 3.5 years of age.

2.3.5.2. Normal Dobermans

H31 was a 10 year old spayed female Doberman (VTH # 181889). One medium-sized muscular artery had an eccentric subintimal accumulation of amorphous

eosinophilic material and macrophages. This change in the vessel wall extended for 2.5 mm in the section (Table 2.11.).

Four apparently normal Dobermans (66469, 65320, 65479 and 66301) had mild, multifocal interstitial fibrosis. Mild multifocal interstitial myocarditis was observed in five apparently normal Dobermans (62676, 65479, 65570, 65636, and 66469) (Table 2.11.). Very mild focal accumulation of eosinophilic amorphous material was present beneath in the tunica intima of a few small muscular arteries of one apparently normal Doberman (66301) (Table 2.11.).

Histopathological changes were reported in the kidneys from 4 out of the 9 "normal Dobermans", and included glomerulonephritis (65479, 66469) and renal tubular necrosis (62676, 66301). Renal changes (chronic diffuse interstitial fibrosis, OVC case number 66288) were reported in only 1 out of 10 normal mixed breed dogs. Six (OVC case numbers 63036, 65597, 665786, 66466, 66492, and 67763) out of 9 Dobermans with DCM had renal histopathological changes, and 6 (61817, 63544, 63652, 63690, 66632, 67543) out of 10 other breeds of dogs with DCM had renal histopathological changes.

2.3.5.3. Occult DCM Doberman

Tissue for histological examination was available from only one of the Dobermans categorised as having occult heart disease from echocardiography (H24, VTH # 175540, OVC# 65048). This 8.5 year old castrated male Doberman had a history of a serosanguineous, right nasal discharge for 4 to 6 weeks. A nasal osteosarcoma was diagnosed on biopsy and the owners requested euthanasia. Interstitial and replacement fibrosis and fatty replacement in the myocardium were severe and extensive, accounting

for approximately 30% of the cross-sectional area of this slide. Five medium-sized muscular arteries within the myocardium had thickened tunica media. Four muscular arteries had foamy lipid-like material immediately beneath the endothelium along with irregular thickening of the tunica intima. Two small foci of necrosis were accompanied by a focal infiltrate of lymphocytes and macrophages. Two other small focal infiltrates of lymphocytes and macrophages were present between muscle fibers in the regions of interstitial and replacement fibrosis. Numerous cardiomyocytes had large vacuoles in the sarcoplasm. In lung sections, there was extensive hemorrhage into alveoli, erythrophagocytosis, bronchiolitis obliterans and patchy areas of interstitial fibrosis.

2.3.5.4. Dobermans with DCM

Formalin-fixed myocardial tissue was available from only one (H35) of the Dobermans included in the biochemical studies. Two other cases of Dobermans with DCM which had been necropsied by the author were included. Doberman H35 (VTH # 184600, OVC# 65597) was a 9 year old castrated male which developed a cough and had paroxysmal ventricular tachycardia, left ventricular dilation, reduced left ventricular contractility and mitral regurgitation, when the owners requested euthanasia. There was patchy interstitial fibrosis, occasional perivascular foci of replacement fibrosis and mild fatty replacement in the left ventricular myocardium. Small numbers of lymphocytes were present around blood vessels in areas of myocardial fibrosis and fat replacement.

Case number 66348 was a Doberman with DCM that had been submitted for routine necropsy at the owner's request. Myocardial interstitial fibrosis was severe and diffuse accounting for up to approximately 15% of the cross-sectional area in some fields.

Fibrosis and fatty replacement of the myocardium accounted for up to 20% of the cross-sectional area in a few left ventricular myocardial fields. Occasionally, segments of myofibers had lost the cross-striations and the sarcoplasm was coarsely clumped. Adjacent parts of the same muscle fiber had cross striations but no pyknosis or karyorrhexis (suggesting that the cell was still viable at the time of euthanasia). Small numbers of lymphocytes and macrophages surrounded the focal cardiomyocyte necrosis. Numerous medium-sized muscular arteries in the left ventricular myocardium had foci of amorphous eosinophilic material and large well-defined clear spaces (suggesting lipid) beneath the endothelium. The tunica media of these medium-sized muscular arteries was mildly thickened.

OVC case # 66492 (VTH# 199850) was a Doberman with DCM, ventricular premature contractions and acute oliguric renal failure that was submitted for necropsy. Multiple foci of cardiomyocyte necrosis were surrounded by small numbers of macrophages and occasional lymphocytes. A few small muscular arteries in the left ventricular myocardium had amorphous eosinophilic material and large well-defined, clear vacuoles (suggesting lipid), immediately beneath the endothelium. One small muscular artery with a focal deposit of eosinophilic material in the tunica intima, also had small numbers of lymphocytes in the adventitia.

2.3.5.5. Other breeds of dogs with DCM

Ventricular dilation was present at necropsy in all other breeds of dogs with DCM, however the cause of DCM was not demonstrated in any case. The range and severity of myocardial lesions in other breeds of dogs with DCM was similar to that in

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Dobermans. There was increased severity of the myocardial intersitial fibrosis in Dobermans with DCM compared to the other breeds of dogs with DCM (Tables 2.12. and 2.13.).

Multifocal large clear vacuoles, up to 25 μm in diameter, were present in the left ventricular Purkinje fiber sarcoplasm of a 3.5 year old Rottweiler (63401) (Table 2.13.).

2.3.5.6. Statistical analysis of myocardial histopathological changes

The Kruskall-Wallis ANOVA test of ranks for histopathological changes in left ventricular heart muscle demonstrated significant differences between normal Dobermans and Dobermans with DCM for interstitial fibrosis, replacement fibrosis, myocardial inflammation, and necrosis (Table 2.13.). Similarly significant differences were evident for normal mixed breeds and other breeds of dogs with DCM in interstitial fibrosis. replacement fibrosis, fatty replacement, myocardial inflammation and necrosis (Table 2.13.).

The Kappa test demonstrated strong agreement in the classification of myocardial lesions by the two pathologists as shown in tables 2.15 and 2.16.

DOG CATEGORY Age, Sex	DOG#	Interstitial Fibrosis	Replacement Fibrosis	Fat Replacement	Inflammation	Myocyte Necrosis	Purkinje Fibres	Blood Vessels	Myocyte Lipofuscin
Normal Mixed Breed									
	H30	0	0	0	0	0	0	0	0
Golden Retriever, MC, 1yr 4m	67068	0	0	0	0	0	0	0	0
Lakeland Terrier, 3yr 11m	66931/7	0	0	0	0	0	0	0	0
Golden Retriever, MC, 7yr 8m	66831	0	0	0	0	0	0	0	0
Mixed Breed, FS, 8yr	66571	0	0	0	0	0	0	0	+
Cocker Spaniel, FS, 8yr	66288	0	0	0	0	0	0	0	+
Golden Retriever, MC, 3yr 10m	66283	0	0	0	0	0	0	0	+
Mixed Breed, M, 11yr 3m	65331/8	0	0	0	0	0	0	+	+
Golden Retriever, M, 10yr 5m	64849	0	0	+	+	0	0	0	+
Yorkshire Terrier, M, 3.5yr	64729	0	0	0	0	0	0	0	+
Normal Dobermans									
FS, 10yr 4m	H31 (424/96)	0	0	0	0	0	0	+	+
F, 7m	68056/2	0	0	0	0	0	0	0	0
F, 3m	66469/4	+	+	0	+	+	0	+2	0
M, 8yr 6m	66301/2	+	+	+	0	0	0	+	+
MC, 8yr 9m	65636/1	0	0	+	+	0	0	0	+
F, 3yr	65619/4	0	0	+	0	0	0	0	+
M,2yr 3m	65570/2	0	0	0	+	+	0	0	+
MC, 3yr 8m	65479/4	+	0	0	+	+	0	0	+
F, 4yr 10m	65320/1	+	0	0	0	0	NA	+1	+
MC, 9yr 1m	62676/2	0	0	0	+	+	0	+1	+

Table 2.11. Histopathological changes observed in left ventricle of normal dogs. F = female, FS = spayed female, M = male, MC = castrated male, NA = not present in the section examined. 1 = focal hyperplasia of smooth muscle cells in the tunica intima (intimal cushion). 2 = fibrinoid vasculitis.

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Table 2.12. Histopathological changes observed in left ventricle of Dobermans with cardiac dysfunction.

DOG CATEGORY Age, Sex	DOG#	Interstitial Fibrosis	Replacement Fibrosis	Fat Replacement	Inflammation	Myocyte Necrosis	Purkinje Fibres	Blood Vessels	Myocyte Lipofuscin
Doberman Occult DCM									
MC, 7.5 yr	H24(65048)	++	++	++	+	+	0	+	+
Dobermans with DCM									
M	67763	+	+	0	+	0	0	0	+
F, 10yr	66492	++	++	++	+	+	0	+	+
M, mature	66466	++	0	+	+	+	0	$+_i$	+
F, 12yr 3m	66439	+	+	+	+	0	0	$+_i$	+
M, 8yr	66348	++	++	++	+	+	0	+	+
M, 5.5yr	65786	++	+	++	+	+	0	$+_i$	+
M, 9yr	65738	++	+	+	++	+	0	$+_i$	0
MC, 10yr	1135(65597)	+	+	+	+	+	0	0	+
FS, 11yr 4m	..6	++	++	++	+	+	0	+	+

Table 2.12. Histopathological changes observed in left ventricle of Dobermans with cardiac dysfunction. Where: F = female, FS = spayed female, M = male, MC = castrated male. $_i$ = focal hyperplasia of smooth muscle cells in the tunica intima (intimal cushion).

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DOG CATEGORY Age, Sex	DOG#	Interstitial Fibrosis	Replacement Fibrosis	Fat Replacement	Inflammation	Myocyte Necrosis	Purkinje Fibres	Blood Vessels	Myocyte Lipofuscin
Other Breeds with DCM									
Newfoundland	67543/1	+	+	+	0	0	0	+	+
Great Pyrenees, F, 5yr 8m	67107/4	++	++	+	++	+++	0	+$_4$	+
Bernese Mountain, F, 5y 6m	66632/4	+	+	+	+	+	0	0	+
Great Dane, MC, 4yr 9m	66593/7	+	0	0	+	+	0	0	+
Irish Wolfhound, M, 8yr 11m	63690A	+	+	+	+	0	0	+$_3$	+
Boxer, M, 6yr 10m	63652	+	+	+	+	+	0	0	+
Great Dane, M, 2yr	63544	+	+	0	0	0	0	0	+
Rottweiler, FS, 3.5yr	63401	+	0	0	+++	+	+	+$_1$	+
German Shepherd, M, 8yr	63316	0	0	+	+	0	0	0	+
Labrador Cross, FS, 6yr	61817	0	0	0	0	0	0	+$_1$	+

Table 2.13. Histopathological changes observed in left ventricle of other breeds of dogs with cardiac dysfunction. Where: F = female, FS = spayed female, M = male, MC = castrated male. $_1$ = focal hyperplasia of smooth muscle cells in the tunica intima (intimal cushion); $_2$ = contraction band necrosis in Purkinje fibres; $_3$ = atrophy and vacuolar degeneration of tunica media smooth muscle cells in medium-sized muscular arteries; $_4$ = necrosis of tunica media in medium-sized muscular arteries of the myocardium.

DOG CATEGORY	Interstitial Fibrosis	Replacement Fibrosis	Fat Replacement	Inflammation	Myocyte Necrosis	Purkinje Fibres	Blood Vessels	Myocyte Lipofuscin
ANOVA for all breeds	<0.001**	<0.001**	0.001**	0.001**	0.009**	0.424	0.033*	0.045*
Normal Mixed Breed vs Normal Dobermans	0.029*	0.146	0.276	0.057	0.029*		0.057	0.342
Normal Dobermans vs Dobermans with DCM	0.0007**	0.025*	0.004*	0.012*	0.105		0.223	0.167
Normal Mixed Breed vs other Breeds with DCM	0.0005***	0.005***	0.022*	0.007***	0.012*		0.057	0.029*
Dobermans with DCM vs other Breeds with DCM	0.01*	0.107	0.025*	0.457	0.399		0.225	---

Table 2.14. Statistical comparison of histopathological changes in the left ventricular myocardium of normal dogs and dogs with cardiac dysfunction using Kruskal-Wallis ANOVA test by ranks. Numbers in the boxes correspond to the p values. The symbol * means $p < 0.05$, and ** corresponds to $p < 0.01$.

| | Pathologist 1 | | | |
	0	1	2	3
0		1		
1	2	6		
2		3		
3				

(Rows labelled Pathologist 2: 0, 1, 2, 3)

Table 2.15. Coben's Kappa test illustrating low agreement, but no significant difference in the histological grading determined by 2 different pathologists, for myocardial necrosis in 12 Dobermans with DCM and occult DCM. Kappa = -0.161, p = 0.84.

| | Pathologist 1 | | | |
	0	1	2	3
0		1		
1		9	1	
2		1		
3				

(Rows labelled Pathologist 2: 0, 1, 2, 3)

Table 2.16. Coben's Kappa test demonstrating a low level of agreement, but no significant difference in the histological grading determined by 2 different pathologists, for myocardial inflammation in 12 Doberman's with DCM and occult DCM. Kappa = - 0.091, p = 0.67.
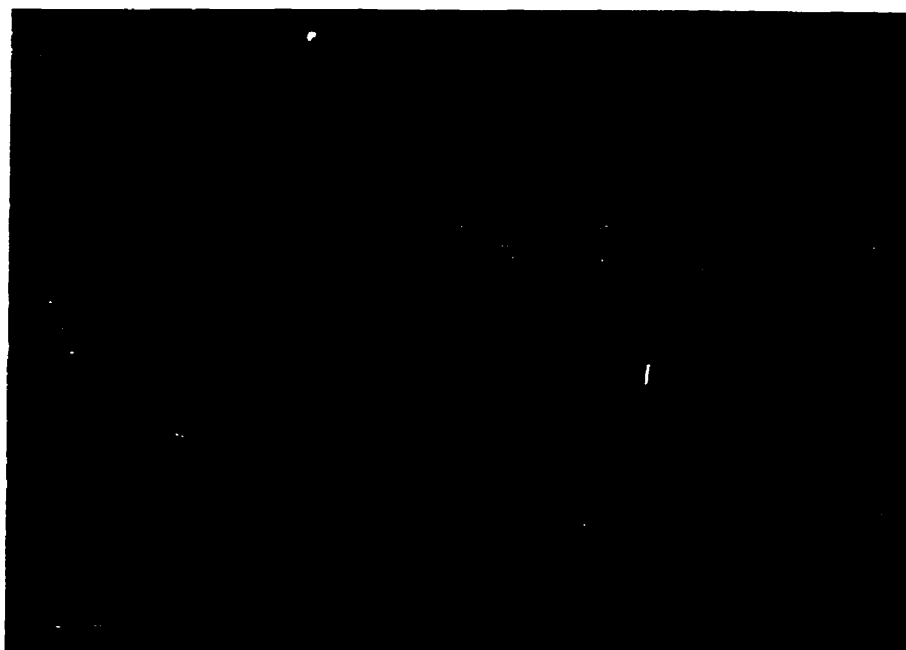
Fig. 2.21. Photomicrograph of focal myocardial necrosis in a Doberman with DCM. hematoxylin and eosin stain (66492/1). Bar = 50 μm.

2.4. DISCUSSION

A significant reduction of myocardial NADHdh activity in Dobermans with DCM (Fig. 2.4.), by 68.5% compared to normal mixed breed dogs, and by 61.4% compared to normal Dobermans, could be explained by a number of different mechanisms. First, it is possible that some Dobermans have an inherited defect in the nuclear and/or mitochondrial genes that give rise to a defect in one or more of the 41 subunits, or proteins involved in the transport and assembly, of NADHdh in myocardial mitochondria. A defect in myocardial NADHdh activity could lead to reduced mitochondrial ATP production (Robinson, 1994), leading to reduced rate and force of cardiac contraction, and reduced rate in myocardial relaxation in diastole (Fozzard *et al*, 1992). Reduced myocardial NADHdh activity could also interfere with the efficiency of electron transfer from NADH to ubiquinol (coenzyme Q), leading to increased levels of free radicals (superoxide radical, hydroxyl radical and hydrogen peroxide). Free radicals are known to damage mitochondrial DNA, leading to a cumulative increase in somatic mutations in mtDNA, reduced efficiency of oxidative phosphorylation and decreased ATP production (Ozawa 1995).

A second explanation for decreased levels of myocardial NADHdh activity in Dobermans with DCM is that some other primary defect has led to reduced gene expression for the subunits of respiratory complex I. Experimental models of canine congestive cardiac failure, induced by rapid ventricular pacing, have produced significant reduction in myocardial ATP concentration (O'Brien *et al*, 1994; Montgomery *et al*, 1992), and decreased sarcoplasmic reticulum Ca-release channel activity (O'Brien *et al*, 1994). Down regulation of gene expression for Ca-release channel proteins was

proposed to reduce the total amount of Ca^{++} cycled and slow the rise and fall of Ca^{++} (O'Brien *et al*, 1994). An inherited defect in myocardial myoglobin, myocardial ATP synthetase, cytochrome oxidase or mitochondrial ATP antiporter could reduce the efficiency of oxidative phosphorylation leading to reduced mitochondrial NADHdh activity as a secondary consequence.

Defective myocardial structural components, such as cardiac alpha-actin, desmin, and titin, may predispose to cardiac failure with reduced respiratory chain activity occurring as a final common pathway for failing cardiomyocytes.

It was postulated that the myocardial NADHdh activity in Dobermans with asymptomatic left ventricular dysfunction (occult category) might provide a link in the sequence of changes leading to DCM. Myocardial samples from only 2 Dobermans with occult heart disease were available. The mean myocardial NADHdh activity in the 2 Dobermans from the occult category (36.7 ± 8 ηmol/min/mg protein) was lower than the mean value for normal Dobermans (44.1 ± 19 ηmol/min/mg protein), and normal mixed breed dogs (54.0 ± 17 ηmol/min/mg protein), however this difference was not significant for the small number of dogs studied (Fig. 2.4.). A larger sample of Dobermans with occult heart disease would be required to test a hypothesis that the myocardial NADHdh activity was lower in Dobermans with occult heart disease compared to normal Dobermans and mixed breed dogs.

The myocardial NADHdh activity in the normal Dobermans had the greatest range in values (17.6 to 72.8, mean 44.1 ± 19 ηmol/min/mg protein) of all dog categories. The small number of dogs in the normal Doberman category does not allow a

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meaningful statistical analysis of the population for normal distribution of myocardial NADHdh activity.

A trend of progressive reduction in myocardial NADHdh activity with increasing severity of myocardial disease (from normal mixed breed to DP-N, and DP-OCC, followed by DP-DCM) may represent down regulation of gene expression for mitochondrial respiratory complex I (NADHdh) as a consequence of some other cardiac defect.

A statistical analysis of both the raw and scaled echocardiographic data confirmed the significant differences ($p < 0.01$) between the 3 categories of Dobermans (DP-N, DP-OCC, and DP-DCM) in FS, LVIDd, LVIDs, LVFWs, WSs, and WSd (Tables 2.9. and 2.10.). However there was no significant difference among these 3 groups of dogs for EPSS or LVFWd (Tables 2.9. and 2.10.). The relatively high correlation coefficients and significant p values suggest that there is a valid relationship between myocardial NADHdh activity and scaled aLVIDd, aLVIDs and FS. Nonlinear regression analysis of myocardial mitochondrial NADHdh activity versus the scaled echocardiographic parameters revealed a significant correlation between myocardial NADHdh activity and LVIDd, LVIDs and FS, for the total population of Dobermans (Figs. 2.5., 2.6., 2.7.). An exponential decay in FS with decreasing mitochondrial NADHdh activity could be explained in part by reduced levels of myocardial ATP leading to reduced myocardial contractility. Earlier research has found significant reduction in myocardial total ATP and mitochondrial NADHdh activity in Dobermans with DCM compared to normal mixed breed dogs (McCutcheon et al, 1992). Modest decreases in myocardial ATP concentration in the failing heart are believed to be significant (Fozzard et al, 1992).

Reduced mitochondrial NADHdh activity, and decreased myocardial ATP would interfere with the normal Frank-Starling mechanism for intrinsic regulation of heart pumping in two ways. Myocardial ATP has an allosteric effect, such that higher levels of myocardial ATP allow for increased rate, and force of contraction, and more rapid initiation of relaxation (Fozzard *et al*, 1992). With a reduction in mitochondrial NADHdh activity, and decreased myocardial ATP, there would be reduced contractility and decreased FS. Myocardial relaxation is dependent on ATP for the active sequestration of sarcoplasmic Ca^{++} into the sarcoplasmic reticulum by the SR Ca^{+}-ATPase (Lytton and MacLennan, 1993). Reduced levels of myocardial ATP would reduce myocardial relaxation and interfere with the stretching of myocardial fibers necessary to increase cardiac output via the Frank-Starling mechanism.

A long term study of echocardiographic changes in normal Dobermans with low myocardial NADHdh activity would be required to determine if this biochemical abnormality predisposed to progressive deterioration in cardiac function. Such a study would require serial endomyocardial biopsy and assay for mitochondrial NADHdh activity, or nuclear magnetic resonance studies for phosphorus-31, with subsequent comparison of myocardial phosphate metabolism and clinical cardiovascular parameters. Analysis of the weights of whole hearts and the individual components (including the left ventricle), compared to myocardial NADHdh activity and aLVIDd, would provide another way of studying the effect of myocardial biochemical changes on morphology, and function of the heart. Data on the weights of components of the hearts, were not available.

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In the regression of myocardial NADHdh activity on the clinical parameters, it was noted that the normal Dobermans and Dobermans with DCM formed 2 distinct populations (Figs. 2.9. and 2.11.). This may be attributed to the Dobermans with DCM being in a state of cardiac dysfunction, where the normal functional inter-relationships between measures of cardiac function and biochemical parameters have been distorted by compensatory mechanisms in the failing heart.

The ANOVA of gastrocnemius muscle NADHdh activity in normal mixed breed dogs, normal Dobermans and Dobermans with DCM was significant when compared per gram wet weight of myocardium (p = 0.04) (Fig. 2.14.), but not significant when determined per milligram of gastrocnemius muscle protein (p = 0.1) (Fig. 2.15.). In post hoc tests, gastrocnemius muscle NADHdh activity per unit wet weight of gastrocnemius muscle, was significantly lower in normal Dobermans and Dobermans with DCM, than in normal mixed breed dogs, when compared by both the least significant difference (LSD) pairwise comparison test, and Duncan's test (p < 0.04) (Fig. 2.14.). It is possible that additional variation introduced with the calculation of protein content of the gastrocnemius muscle samples reduced the significance of this measurement. The observed power of 0.64 for the ANOVA test on the gastronemius muscle NADHdh activity per unit wet weight, is regarded as low (Glantz 1998; Zar 1996). Some statisticians prefer the power for the test to be in the order of 0.8. or higher (Glantz 1998; Zar 1996). In view of the low sample numbers, the significance of this finding should not be overemphasised. The LSD test is regarded as less conservative than Tukey's honest significant difference test. However, the use of the LSD test in post hoc data analysis, following a significant ANOVA, is regarded as acceptable (Snedecor and Cochran,

1967). Further testing on the gastrocnemius muscle NADHdh activity should be attempted in the future when larger numbers of samples from dogs in each group are available.

The normal range for skeletal muscle (biceps, gastrocnemius, and quadriceps) NADHdh activity has been reported in humans to be 3.19 ± 0.26 μmol/min/g wet weight using a similar technique to that used in the current study (Scholte *et al*, 1987). These values are similar to the measured levels of gastrocnemius NADHdh activity in normal mixed breed dogs in the current study (5 ± 1.6 μmol/min/g wet weight).

In the current research, a higher concentration of gastrocnemius muscle homogenate (1 mass of muscle in 11 times this mass of 50 mM Tris/80 mMKCl buffer) and relatively high level of NADH in the cuvette (20 μM or 50 nmol in 2.5 ml), were used, when compared to the technique used here for measurement of NADHdh activity in myocardial samples. Despite the higher level of fluorescence quenching, by the muscle homogenate, there was a good fit of the standard curve data to the quadratic curve (Fig. 2.12.). This implies that the equation can be used to accurately predict the NADH level and determine the rate of utilization of substrate.

In homogenising the skeletal muscle, a variable amount of fibrous connective tissue was observed. Often this fibrous connective tissue would remain tightly wrapped around the shaft of the Tekmar homogeniser. Expression of the NADHdh activity per gram of protein tended to remove variability created by the fibrous connective tissue, but incorporated an additional random error to the measurement of NADHdh activity.

A modified technique was used for determination of myocardial NADHdh activity. Many problems were identified in the original technique (Nowack *et al*, 1992,

McCutcheon *et al*, 1992): 1) The standard curve had been constructed with a small number of data points (4) and did not include the heart homogenate. 2) A computer software package had been used incorrectly to approximate the standard curve to a straight line and force this line to pass through the origin in some measurements. 3) Rotenone-inhibited NADHdh activity, and total myocardial NADHdh activity (rotenone-inhibited NADHdh activity, plus rotenone-insensitive NADHdh activity) were measured at different NADH substrate concentrations. A further improvement on the technique used in the current study would be to restrict the measurement of NADHdh activity to isolated mitochondria.

The modified technique used low concentration of heart homogenate and NADH, which reduced substantially the degree of quenching compared to the original technique (Nowack *et al*, 1992, McCutcheon *et al*. 1992). Quenching is the loss of fluorescence due to an interaction between the fluorophor (NADH in this case) with the solvent or solute dissolved in the solvent (Tietz, 1986). Any component that absorbs light (muscle homogenate for example) will reduce the intensity of light passing through the solution, and diminish the emitted light (i. e., quench the fluorescence). The emitted light is proportional to the concentration of the substance to be measured only when the absorption of the exciting light is negligible (Passonneau and Lowry, 1993). The relationship between fluorescence emission and concentration of a fluorophor is nonlinear because light is absorbed by the fluorophor (John, 1992).

An important difference between the revised and original techniques for myocardial NADHdh determination was that, in the revised technique, when rotenone was added to the cuvette reaction mixture, the NADH substrate was near maximal

concentration. In the original technique, rotenone was added when the level of substrate (NADH) was down to about 10% of the initial substrate level. The measured rotenone-sensitive NADHdh activity, with the original method, was variable and low depending on the amount of NADH remaining when rotenone was added. The Commission on Enzymes has recommended that enzyme assays should be based on measurement of the initial rate of reaction unless it is known that the velocity of reaction remains constant as the amount of substrate changes (International Union of Biochemistry, 1965). Total NADHdh activity and rotenone-insensitive NADHdh activity, were observed to change dramatically as the substrate concentration decreased. The inclusion of heart homogenate in the solution for preparing an NADH standard curve and the determination of rotenone-insensitive NADHdh activities at higher substrate (NADH) levels in the revised technique tend to decrease the measured myocardial NADHdh activity in the revised compared to the previous techniques (Table 2.8).

Due to a variation in the amount of myocardial fibrosis, edema and fatty replacement in Dobermans with DCM, the determination of NADHdh activity per gram of myocardial protein provides a better indicator of myocardial NADHdh activity in cardiomyocytes than estimates based on NADHdh activity per gram frozen heart (Table 2.8). Previous reports of myocardial NADHdh activity in dogs have been related to the mass of frozen heart tissue (Nowack et al, 1992; McCutcheon et al, 1992). McCutcheon et al (1992) have reported NADHdh activity in μmol/min/g frozen heart to be reduced by 60% in Dobermans with DCM compared to normal mixed breed dogs. In the current study a reduction in the mean myocardial NADHdh activity, measured in both

μmol/min/g frozen heart and protein, was observed in Dobermans with DCM compared to normal mixed breed dogs (Table 2.8.).

The fact that mean myocardial lactate concentration in Dobermans with DCM was significantly higher (188 ± 83 μmol/g protein) than the concentration in normal mixed breed dogs (109 ± 26 μmol/g protein) and normal Dobermans (109 ± 36 μmol/g protein) was an anticipated finding. It is reasonable that reduced myocardial NADHdh activity produces a compensatory increase in anaerobic metabolism, with increased concentration of myocardial lactate in Dobermans with DCM. This study also provided evidence that the slight reduction in mean myocardial NADHdh activity that was observed in normal Dobermans (18.3% reduced compared to normal mixed breed dogs), was not associated with a measurable increase in mean myocardial lactate concentration. These current findings are in agreement with earlier studies, where the concentration of myocardial lactate was significantly (p< 0.05) higher in Dobermans with DCM (11.6 ± 2.1 mM wet weight of myocardium, n=10) compared to healthy mixed breed dogs (7.9 ± 2.7 mM, n= 8) (McCutcheon *et al*, 1992). A significant third order polynomial relationship was observed when the myocardial NADHdh activities of normal mixed breed dogs, normal Dobermans, and Dobermans with DCM were plotted against myocardial lactate concentration (Fig. 2.17.). This graph illustrates that when myocardial NADHdh activity is reduced below 30 ηmol/min/mg protein in Dobermans with DCM, there is a marked increase in myocardial lactate concentration (Fig. 2.17.). One normal Doberman had a mean myocardial NADHdh activity reduced below 30 ηmol/min/mg protein, however, this dog did not have an elevated myocardial lactate concentration.

The gastrocnemius muscle lactate tended to increase from normal mixed breed dogs and normal Doberman, to Dobermans with occult heart disease, and finally Dobermans with DCM (Fig. 2.18.). Raised lactate concentrations in gastrocnemius muscle of Dobermans with DCM, may be in part due to lower levels of NADHdh activity (and increased anaerobic metabolism) in these dogs, compared to healthy mixed breed dogs.

Myocardial lactate concentration had a significant, third order inverse polynomial relationship with myocardial NADHdh activity in the population of normal mixed breed dogs, normal Dobermans, and Dobermans with DCM (Fig. 2.17.). This suggests that it is not until myocardial NADHdh activity is reduced below half normal (for normal Dobermans) that the myocardial lactate (and anaerobic metabolism) increases dramatically (Fig. 2.17.).

No significant relationship could be demonstrated between gastrocnemius muscle NADHdh activity and gastrocnemius muscle lactate concentration (Fig. 2.19.).

While the mean concentration of lactic acid in gastrocnemius muscle of Dobermans with DCM (24.4 ± 14 µmol/g protein) was higher than that of normal mixed breed dogs (13 ± 10 µmol/g protein), this difference was not significant (Fig. 2.18.).

The urinary lactate to creatinine ratio in Dobermans with DCM (6.9 ± 4.6 %) was significantly higher than that of normal mixed breed dogs (1 ± 0.7 %) (Fig. 2.20.), as was reported previously (O'Brien *et al*, 1993). While the urinary lactate to creatinine ratio in Dobermans with occult heart disease (5.5 ± 6.6 %) was higher than that of normal Dobermans (4 ± 3.4 %) this difference was not significant (Fig. 2.20.), and so this ratio would not serve as a nonspecific indicator of occult heart disease. Large variances in

these readings, and small number of samples, reduced the significance of the differences in the means for the different categories of dogs. An important finding in this earlier paper was that the lactate to creatinine ratio in sick dogs of other breeds was higher than the mean value ratio in Dobermans with DCM (O'Brien *et al.* 1993). The higher mean values for urinary lactate concentration for the 3 dog groups in the former study may reflect the use of a Model 23L Lactate Analyzer (Yellow Springs Instrument Company, Yellow Springs, Ohio, USA), compared to the use of a Sigma Diagnostics Kit in the present study.

Increased lactate concentration in body fluids is one sign of mitochondrial respiratory chain disorders (Scholte *et al*, 1987). Significantly increased urinary lactate to creatinine ratio in Dobermans with DCM, compared to normal mixed breed dogs, strongly suggests increased blood lactate. Serum was not available to confirm the elevated concentration of lactate.

One of the key findings in the histopathology study was the focal myocardial necrosis, surrounded by small numbers of lymphocytes and macrophages. Often there was a single focus of myocardial necrosis per $1cm^2$ block of left ventricular papillary muscle, that occurred separate from the interstitial and replacement fibrosis (Fig. 2.20.). Myocardial necrosis has been reported in the left ventricular papillary muscle of Dobermans with dilated cardiomyopathy (Sandusky *et al*, 1984) and in comprehensive investigations of human dilated cardiomyopathy (Roberts *et al*, 1987). Necrosis of individual cardiomyocytes in Dobermans with DCM may represent single cell failure associated with inadequate ATP for maintenance of normal cell functions; increased levels of free radicals associated with respiratory chain failure, and rising levels of lactic

acid. The release of free radicals into the myocardial interstitium could stimulate the inflammatory response, and initiate interstitial fibrosis. Research in human cardiomyopathy has led some authors to believe that the first event is injury to myocytes, followed by initial hypertrophy, and later atrophy of myocytes, and increased fibrotic tissue (Schaper *et al*, 1995). It has not been definitively established if the interstitial fibrosis that occurs in cardiomyopathy is a secondary consequence of cardiomyocyte necrosis, or is a primary phenomenon (Schaper *et al*, 1995). Myocardial interstitial fibrosis may be a result of scattered cardiomyocyte necrosis stimulating fibroblast proliferation and collagen deposition. Congestive cardiac failure leads to reduced cardiac output, and activation of the renin-angiotensin-aldosterone humoral system. Interstitial fibrosis in the myocardium of dogs with DCM may be due to the release of angiotensin II and/or aldosterone (Campbell *et al*, 1995).

A higher prevalence of myocyte necrosis, myocarditis and myocardial interstitial fibrosis in normal Dobermans, compared to other breeds of normal dogs, might have been associated with abnormal renal function in 4 of the normal Dobermans. It is unlikely that the chronic interstitial myocardial fibrosis observed in the 2 normal Dobermans (66301 and 66469) could be a consequence of the mild, acute renal tubular necrosis in these 2 dogs. However, chronic activation of the renin-angiotensin-aldosterone system, which can occur with unilateral renal ischemia, has been associated with perivascular and interstitial fibrosis in both the left and right ventricles of experimental male Sprague-Dawley rats (Campbell *et al*, 1995).

Glomerulonephritis was present in 2 normal Dobermans (65479, 66469) and renal tubular necrosis occurred in 2 other normal Dobermans (62676, 66301). "Renal

tubulopathy" with cardiomyopathy has recently been described in a person with complex I deficiency (Pitkanen *et al*, 1996). Angiotensin II has been demonstrated to produce focal myocardial necrosis, and aldosterone infusion has been shown to cause myocardial fibrosis (Campbell *et al*, 1995). Thus an association between renal disease and dilated cardiomyopathy could be considered in future investigations of heart disease in Dobermans.

Dobermans in the occult and DCM categories had similar histopathological changes in the left ventricle. These findings of moderately extensive interstitial and replacement myocardial fibrosis with multiple foci of myocyte necrosis were observed in the single Doberman with occult heart disease where fixed tissue was available. An important finding in this Doberman with occult heart disease (H24), was the presence of severe histopathological changes in the myocardium, yet mild echocardiographic changes (LVIDd of 46.1 mm and FS of 22) and myocardial NADHdh activity (42.4 ηmol/min/mg protein) that was not significantly different from that of normal Dobermans (mean of 36.1 ± 21.3 ηmol/min/mg protein). In this individual dog, severe histopathological changes in the myocardium preceded a change in myocardial NADHdh activity from the mean level in normal Dobermans.

One normal Doberman (62676), and one Doberman with DCM (66466) had myocardial interstitial fibrosis without replacement fibrosis in the sections examined (Tables 2.11. and 2.12.). However there were no cases with myocardial replacement fibrosis without interstitial fibrosis (Table 2.11. and 2.12.). This suggests that the interstitial fibrosis arises from a different process than replacement fibrosis, and that interstitial fibrosis may precede replacement fibrosis in the myocardium of Dobermans

with DCM. Myocardial necrosis is believed to lead to replacement fibrosis in the left ventricular wall (Beltrami *et al*, 1994). Experimental studies with hormone infusions in mice have demonstrated that both angiotensin II and aldosterone can produce myocardial necrosis and fibrosis by different pathogenic mechanisms and with a different time course (Campbell *et al*, 1995). Angiotensin II is believed to produce early myocyte necrosis through the release of catecholamines from the adrenal medulla. Aldosterone is thought to cause myocyte necrosis weeks after its elevation through increased urinary excretion of potassium (Campbell *et al*, 1995). The separation of cardiomyocytes by interstitial fibrosis, and replacement fibrosis, are likely to interfere with the transfer of electrical stimuli between myocytes, and also in the exchange of oxygen and metabolites (Schaper *et al*, 1995).

There are reports of "appreciable regeneration" in the myocardium (Field, 1960; Hermanson and Evans, 1993). However, the progression of coagulative myocytolysis through macrophage absorption of sarcoplasm leaving an empty space within a single myocardial cell and subsequent loss of the nucleus has been described (Baroldi, 1975).

No history or clinical information was available on dogs used in the biochemical study until the last stages of the research. Since fixed tissue samples were available from only 3 of the dogs included in the biochemical study, it was necessary to do a computer search of VTH records for other samples. This computer search of clinical and pathological records revealed that two of the dogs (H5, H36) that had been included in the initial biochemical study as normal Dobermans had been diagnosed in a clinical pathology study to be hypothyroid. Both dogs had been on long-term thyroxin therapy. Lymphocytic and plasmacytic thyroiditis with thyroid atrophy was confirmed at necropsy

in both cases. Lymphocytic and plasmacytic thyroiditis leads to destruction of thyroid acini and reduced levels of thyroxine (T4) and triiodothyronine (T3) (Williams and Braunwald, 1992). Limited information is available on the effects of hypothyroidism on the cardiovascular system of dogs. Long standing hypothyroidism is recognised to produce hypercholesterolemia and atherosclerosis of coronary, cerebral and other blood vessels (Capen, 1993; Robinson and Maxie, 1993). Hypothyroidism in people can cause cardiac enlargement, cardiac dilation and congestive heart failure (Williams and Braunwald, 1992). Myocardial changes in people with hypothyroidism include myofibrillar swelling, loss of striations and interstitial fibrosis. Thyroid hormones are believed to mediate effects on the myocardium by change of protein synthesis (e.g.. increased myosin synthesis) and alteration in intracellular calcium handling (Williams and Braunwald, 1992). Both of these hypothyroid dogs were excluded from the study.

The pattern and severity of histopathological changes in Dobermans with DCM and other breeds of dogs with DCM was similar. Interstitial myocardial fibrosis was significantly more severe in the left ventricle of Dobermans with DCM than other breeds of dogs with DCM, possibly because of the greater mean age of Dobermans with DCM (9 years and 4 months) compared to the other breeds of dogs with DCM (mean age 5 years and 8 months) (Tables 2.11., 2.12. and 2.13.).

2.5. LIMITATIONS OF THIS STUDY

2.5.1. Small sample size

Low numbers of dogs, particularly in the group of Dobermans with occult heart disease, limited the potential contribution of this research. It was not possible to perform meaningful statistical analyses on a group of 2 dogs. An attempt was made to add these 2 dogs in the occult category to the normal Dobermans, or Dobermans with DCM categories, and the regression analysis was recomputed.

The sex distribution of the 4 categories of dogs in the myocardial NADHdh activity study was not uniform. There were 7 males, 1 female and 2 unknown sex in the normal mixed breed dogs; 7 female normal Dobermans; 1 male and 1 female Doberman with occult heart disease; and 7 male and 2 female Dobermans with DCM (Fig. 2.4).

DCM in Dobermans is recognised to be an insidious, progressive disease, affecting dogs ranging from puppies up to 14 years, but mostly between the ages of 7 to 8 years (Calvert, 1995). In order to study the biochemical progression of DCM, and identify the primary biochemical changes, it would be necessary to study young, normal Dobermans. Normal Dobermans in the current study ranged from 1.3 up to 13.3 (mean 8.9) years of age. While the echocardiographic measurements for these normal Dobermans were within the range of normal when measured, it is possible (from the high prevalence of this disease in Dobermans) that some may have progressed to DCM (Minors, 1995).

Further refinements could have been made to the method for myocardial NADHdh determination. If more 3 ml quartz cuvettes had been available, the rotenone-

inhibited NADHdh could have been determined with each homogenate. Only one cuvette was dedicated for use with rotenone. The concentration of NADH could have been reduced to 10 μM (25 nmol in 2.5 ml) and the quantity of myocardium could have been halved. This would have reduced the amount of myocardium required and further reduced the quenching of NADH fluorescence. With the reduction in myocardium the amount of Tris/KCl buffer could have been decreased.

2.5.2. Myocardial and gastrocnemius muscle lactate concentration and urinary lactate to creatinine ratio

A decision was made by the research committee, in the 10th semester of this study, to measure lactate concentration in myocardial and gastrocnemius muscle homogenates which had been frozen for 8 months by that time. The determination of myocardial and gastrocnemius muscle protein (for NADHdh activity) necessitated the thawing and re-thawing of all homogenates on 2 occasions before commencement of lactate analysis. The results of this first lactate analysis were unsatisfactory, and the lactate concentrations and protein contents had to be completely repeated on fresh samples. Sigma Chemical Company (St Louis, MO, USA) suggests that lactate concentrations are not stable in whole blood, and plasma lactate is stable for up to at least 1 month of storage at -20°C.

2.6. CONCLUSIONS

Myocardial NADHdh activity per milligram muscle protein was significantly ($p \leq$ 0.05) lower in Dobermans with DCM compared to normal mixed breed dogs (Fig. 2.4.). Myocardial NADHdh activity declined from normal mixed breed dogs (54 $=$ 16.5 ηmol/min/mg protein) to normal Dobermans (44.1 $\pm$ 19 ηmol/min/mg protein), and then Dobermans with occult heart disease (36.7 $\pm$ 8 ηmol/min/mg protein); but, this difference was not significant (Fig. 2.4.). Gastrocnemius muscle NADHdh activity in normal Dobermans and Dobermans with DCM was significantly reduced when compared to normal mixed breed dogs per gram wet weight of muscle by the protected LSD method (Fig. 2.14.), but not when compared per milligram muscle protein (Fig. 2.15.). Reduced myocardial and gastrocnemius muscle NADHdh activity in Dobermans with DCM, compared to normal mixed breed dogs, may represent a deficiency of this mitochondrial enzyme that predisposes this breed to cardiac failure. Alternatively, reduced myocardial NADHdh activity in normal Dobermans and Dobermans with DCM may be secondary to some other underlying genetic defect.

A significant exponential relationship between myocardial NADHdh activity and LVIDd, LVIDs and FS, in normal Dobermans and Dobermans with cardiac dysfunction, established a link between this mitochondrial respiratory chain enzyme, left ventricular internal dimensions, during diastole and systole, and a measure of cardiac contractility (FS) (Figs. 2.5., 2.6., and 2.7.). Reduced myocardial NADHdh activity in normal Dobermans and those with cardiac dysfunction was accompanied by an exponential increase in left ventricular internal dimension during diastole (Fig. 2.5.) and systole (Fig. 2.6.), and an exponential decline from maximum in the fractional shortening (Fig. 2.7.).

The significant exponential correlation between myocardial NADHdh activity and LVIDd, LVIDs and FS in normal Dobermans and Dobermans with cardiac dysfunction could be explained, partially, by low myocardial NADHdh activity producing reduced concentrations of myocardial ATP. Decreased ATP would impair the force and rate of myocyte contraction, and the rate of myocyte relaxation, which is one of the first abnormalities to appear in the energy deprived heart (Fozzard *et al*, 1992).

No significant correlation could be demonstrated between myocardial NADHdh activity and echocardiographic parameters EPSS, LVFW, and WS in the total population of normal Dobermans, Dobermans with occult heart disease and Dobermans with DCM. When the categories of dogs were investigated separately, no significant correlation could be established between myocardial NADHdh activity and EPSS, LVFW, and WS in Dobermans with DCM.

Dobermans with DCM had significantly ($p < 0.05$) higher myocardial lactate concentration (188 ± 83 µmol/g protein) than normal mixed breed dogs (109 ± 26 µmol/g protein), and normal Dobermans (109 ± 36 µmol/g protein) (Fig. 2.16.), indicating decreased aerobic and increased anaerobic metabolism in these tissues. A significant, inverse, third order polynomial relationship between myocardial NADHdh activity and myocardial lactic acid concentration suggested that myocardial NADHdh activity had to fall to half the mean level for normal Dobermans before myocardial lactic acid concentration rose steeply (Fig. 2.17.). On the other hand, gastrocnemius muscle lactic acid concentrations in normal mixed breed dogs, normal Dobermans, and Dobermans with DCM were not significantly different (Fig. 2.18.).

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Increased anaerobic metabolism in Dobermans with DCM was further supported by a significantly higher urinary lactate to creatinine ratio than in normal mixed breed dogs and normal Dobermans (Fig. 2.20.). However, measurement of urinary lactate to creatinine ratio would not serve as a definitive indicator of early cardiac dysfunction in Dobermans because there were not significant differences in the urinary lactate to creatinine ratio between normal mixed dogs, normal Dobermans and Dobermans with occult heart disease.

Histologically, myocyte necrosis accompanied by a localised inflammatory response was seen in the left ventricular myocardium of the one Doberman with occult heart disease, 78% of Dobermans with DCM and 40% of normal Dobermans (Tables. 2.11. and 2.12.). Although myocardial histopathological changes were severe and extensive in a single Doberman with occult heart disease (Table 2.12.), myocardial NADHdh activity in this Doberman was not significantly different from that of normal mixed breed dogs or normal Dobermans (Fig. 2.4.).

The hypothesis that myocardial fibrosis and necrosis were present in normal Dobermans, Dobermans with DCM, and Dobermans with occult heart disease, but not in normal mixed breed dogs was confirmed. Reduction in myocardial NADHdh activity and myocardial ATP, combined with diffuse interstitial myocardial fibrosis, in Dobermans with DCM would all contribute to impaired relaxation, increased cardiac stiffness, and cardiac diastolic dysfunction. This research confirmed the reduced myocardial NADHdh activity in Dobermans with DCM which (along with findings of decreased myocardial ATP in earlier research (McCutcheon et al, 1992) would explain reduced myocardial contractility, and systolic dysfunction.

Further research on the pathogenesis of DCM in Dobermans should be directed in three general fields. Firstly, an hereditary basis for DCM in Dobermans has been suggested on the basis of the high prevalence of DCM in Dobermans, the long history of inbreeding within the breed and the occurrence of DCM along family lines with multiple littermates affected (Calvert, 1995). An investigation of the section of genome controlling complex I of the respiratory chain in Dobermans with DCM and compared to normal mixed breed dogs seems warranted.

Second, further biochemical studies of myocardium from young Dobermans bred from affected parents may help narrow the field of genetic investigation. Because dilated cardiomyopathy is associated with a decrease in aerobic metabolism, a biochemical investigation that tests all components of the respiratory chain such as concentration of myoglobin, and the activities of NADHdh, succinate dehydrogenase, cytochrome oxidase, F_1 ATP synthetase, citrate synthetase, lactate dehydrogenase, β-hydroxylacyl coenzyme A dehydrogenase and adenine nucleotide carrier may be reasonable.

Third, an investigation of the possible link between renal disease in Dobermans and DCM is warranted based on the recent report of "renal tubulopathy" with cardiomyopathy in people with mitochondrial respiratory complex 1 deficiency (Pitkanen et al, 1995), the presence of histopathological changes in the kidneys from 4 normal Dobermans in the current study, and the demonstration of focal myocardial necrosis due to angiotensin II, and myocardial fibrosis following aldosterone infusion in rats (Campbell et al, 1995).

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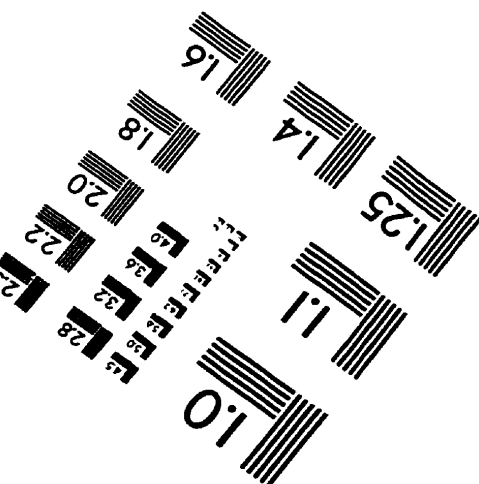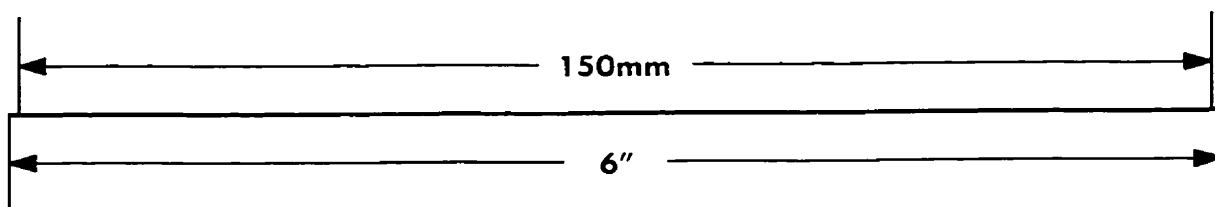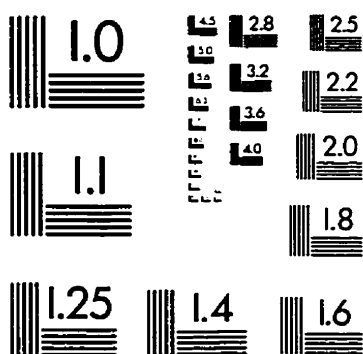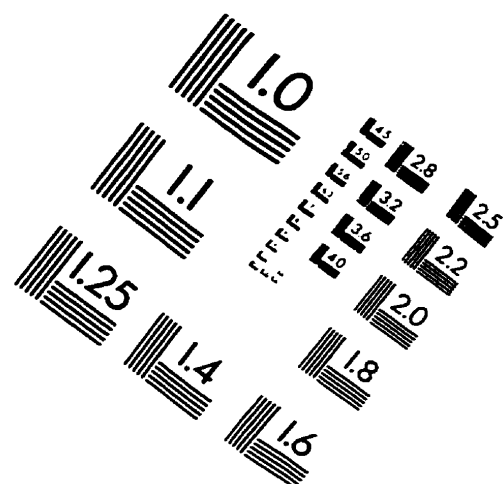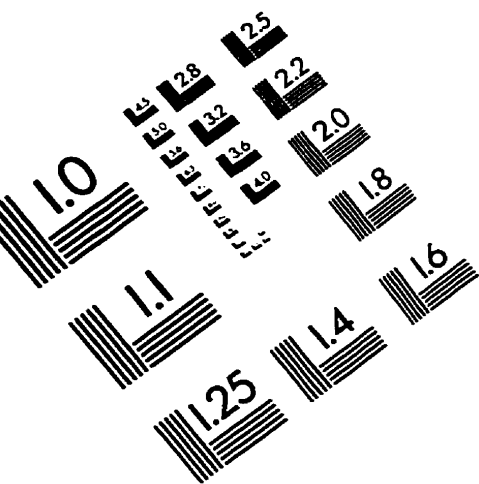
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IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"