British Heart Journal, 1977, **39**, 1333–1339

Enzymic analysis of endomyocardial biopsy specimens from patients with cardiomyopathies

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Myocardial biopsies have been obtained from patients with hypertrophic or congestive cardiomyopathies.

Marker enzymes for the principal subcellular organelles of the myocardium were estimated using highly sensitive assay procedures. The results were compared with those obtained in tissue from patients with valvular heart disease with good or poor left ventricular function.

Left ventricular myocardial tissue from patients with hypertrophic cardiomyopathy showed essentially normal levels of enzymic activities.

In congestive cardiomyopathy, right ventricular tissue showed reduced levels of mitochondrial enzymes with increased levels of lactate dehydrogenase.

Left ventricular tissue from patients with congestive cardiomyopathy showed reduced levels of mitochondrial and myofibril enzymes but high levels of lactate dehydrogenase. The reduced levels of myofibril Ca^{++}-activated ATP in congestive cardiomyopathy is similar to that found in patients with impaired left ventricular function secondary to valvular disease.

It is suggested that defective mitochondrial function is a characteristic feature of congestive cardiomyopathy and that the increased levels of lactate dehydrogenase reflect a compensatory response.

Although extensively studied both by the examination of postmortem tissue and by clinical studies, little is known of the underlying nature of the group of diseases known as the primary cardiomyopathies. On clinical criteria two main subgroups have been distinguished (Goodwin and Oakley, 1972). The hypertrophic cardiomyopathies form a distinct clinical and pathological entity. Bands of hypertrophied muscle are present which may obstruct the outflow tract of either ventricle, though the left ventricle is more commonly affected than the right. Histologically this tissue shows a characteristic pattern of muscle fibre arrangement but the remainder of the myocardium is essentially normal (Maron *et al.*, 1975). The congestive cardiomyopathies form a heterogeneous group of diseases, usually of unknown aetiology but sometimes due to viruses or toxins, such as ethanol, with similar clinical and pathological features (Goodwin and Oakley, 1972).

The introduction of the bioptome has provided a safe and reliable method of obtaining endomyocardial tissue from both the left and right ventricles (Konno *et al.*, 1971; Brooksby *et al.*, 1974).

Received for publication 14 January 1977

Examination of the tissue by morphological techniques can provide information of diagnostic value (Olsen, 1974) but these studies have thrown little light on the mechanism of the myocardial damage. The use of enzymic analysis of milligram quantities of tissue has proved of value in the understanding of certain experimental diseases of the cardiovascular system in animals (Peters *et al.*, 1972; Peters and de Duve, 1974; Welman and Peters, 1976, 1977). The application of a similar approach to various human diseases has similarly proved rewarding (Kane and Peters, 1975; Peters *et al.*, 1975; Peters and Seymour, 1976; Segal and Peters, 1976). In the present paper this approach is applied to tissue obtained by endomyocardial biopsy from patients with cardiomyopathy, in an attempt to gain insight into the disease processes in this group of disorders.

Subjects and methods

SUBJECTS

Patients with cardiomyopathies or with valvular heart disease secondary to either previous rheumatic fever or to bacterial endocarditis were investigated by routine diagnostic catheterisation.

The patients were assigned to the various diagnostic groups after reviewing the clinical features, catheter studies, and histological data. Left ventricular function was assessed as described previously (Peters *et al.*, 1976) and left ventricular biopsies were obtained by the technique of Brooksby *et al.* (1974) at St. Thomas' Hospital. Right ventricular biopsies were obtained with the Olympus bioptome by a modification of Konno's method (Konno *et al.*, 1971) at Hammersmith Hospital. The material was processed by conventional techniques for both light and electron microscopy. A separate biopsy for enzymic analysis was immediately placed in ice-cold 0·25 mol/l sucrose containing 1 mmol/l Na_2 EDTA *p*H 7·2 and stored at –20° until assay. The investigations reported in this publication have been approved by the relevant ethical committees.

ENZYMIC ANALYSES

Because of the small size of the biopsies, approximately 5 mg, highly sensitive enzyme assays using fluorigenic and radio-labelled substrates have been employed (Peters *et al.*, 1972). The tissue was homogenised in 2 ml isotonic sucrose with 10 strokes of a tight fitting pestle (type B) in a Dounce homogeniser (Kontes Glass Co., Vineland, N.J., U.S.A.). A single biopsy was assayed, in duplicate, from each patient. Table 1 shows the assay procedure used for the principal marker enzymes. In order to assess the optimum *p*H, optimal assay conditions, linear kinetics with respect to time of incubation, and amount of enzyme and variability of assay procedures (Seymour and Peters, 1977), preliminary studies were made for each enzyme with material obtained at the time of open heart surgery.

Ca^{++}-activated ATPase was assayed with $\gamma[^{32}P]$ ATP as described by Avruch and Wallach (1971). The incubation medium contained 2 mmol/l ATP, 10 mmol/l Ca^{++}, 100 mmol/l Tris-HCl buffer, *p*H 7·4 in a total volume of 0·5 ml. After incubation for up to 30 minutes at 37°C the reaction was stopped by the addition of 1 ml of solution containing 4 per cent activated charcoal and 4 per cent celite in 0·1 mol/l HCl containing 10 mmol/l NaH_2PO_4. After standing for 30 minutes at 0°C the mixture was centrifuged at 2000 g for 20 minutes. 0·5 ml of the supernatant was mixed with 10 ml Triton-toluene scintillant (Lazarow and de Duve, 1971). Appropriate blanks and standards were assayed with all samples. Corrections for basal ATPase activity were made by parallel assays in which the calcium ions were replaced by 1 mmol/l EDTA. In order to enhance the specificity of the Ca^{++}-activated ATPase for the myofibrillary component, assays were performed in which 10 mmol/l sodium azide or 0·1 mmol/l oligomycin was added to the incubation medium. These compounds are selective inhibitors of mitochondrial ATPase activity (Fanbury and Gergely, 1965; Harigaya and Schwartz, 1969; Bloomfield and Peters, 1974; Leclercq and Swynghedauw, 1976). Enzyme activity associated with the myofibrils (Chandler *et al.*, 1967) and with the sarcolemma (Bloomfield and Peters, 1974) are relatively unaffected.

Enzyme activities were expressed as milliunits of activity/mg protein where 1 milliunit corresponds to the hydrolysis of 1 nmol substrate. Protein was

Table 1 *Enzyme assay conditions*

Enzyme	*Substrate*	*pH*	*Incubation medium*	*Reference*
Lactate dehydrogenase	Pyruvate; NADH	7·4	0·1 mol/l sodium phosphate buffer*; 1 mmol/l dithiothreitol	Reeves and Fimognari (1966); Lowry *et al.* (1957)
Malate dehydrogenase	Oxaloacetate; NADH	7·4	0·1 mol/l sodium phosphate buffer*; 1 mmol/l dithiothreitol	Dupourque and Kun (1969); Lowry *et al.* (1957)
Glutamate dehydrogenase	α-ketoglutarate, NADH $(NH_4)_2SO_4$	7·4	0·07 mol/l triethanolamine-HCl buffer*	Ellis and Goldberg (1972); Lowry *et al.* (1957)
Monoamine oxidase	^{14}C tryptamine	7·4	0·3 mol/l Na phosphate buffer	Wurtman and Axelrod (1963)
5′ Nucleotidase	[^3H]adenosine 5′ monophosphate	9·0	60 mmol/l piperazine-HCl buffer 24 mmol/l $MgCl_2$; 12 mmol/l 2-glycerophosphate	Seymour and Peters (1977)
α-glucosidase	4-methylumbelliferyl α-glucopyranoside	7·5	0·1 mol/l sodium acetate buffer†	Peters *et al.* (1972)
Acid phosphatase	4-methylumbelliferyl phosphate	4·0	0·1 mol/l sodium acetate buffer†	Peters *et al.* (1972)
N-acetyl-β-glucosaminidase	4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucopyranoside	5·8	0·1 mol/l sodium acetate buffer	Peters *et al.* (1972)
β-glucuronidase	4-methylumbelliferyl-β-D-glucuronide trihydrate	3·5	0·1 mol/l sodium acetate buffer†	Peters *et al.* (1972)
Cathepsin D	[^{14}C]-cyanate labelled haemoglobin	3·5	0·1 mol/l sodium acetate buffer†	Roth *et al.* (1971)

*Incubation medium contained 0·01 per cent Triton ×100.
†Incubation medium contained 0·1 per cent Triton ×100.

assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

DNA was assayed by the fluorometric technique of Le Pecq and Paoletti (1966) with calf thymus DNA (Sigma) as standard.

Results

HYPERTROPHIC OBSTRUCTIVE CARDIOMYOPATHY

Table 2 shows specific activities of marker enzymes in the cardiac biopsies from patients with hypertrophic cardiomyopathy and from patients previously reported (Peters *et al.*, 1976) with valvular heart disease with good left ventricular function. The levels of the enzymes in the myopathic tissue were consistently higher than in the valvular heart disease tissue, though this increase only reaches statistical significance for three enzymes. The azide-resistant Ca^{++}-activated ATPase was significantly higher in the myopathic tissue though the total Ca^{++}-activated ATPase failed to reach a statistically significant increase. Malate and lactate dehydrogenases were significantly different between the two groups. For the other marker enzymes there was no difference between the two patient categories.

A limited number of preliminary experiments have been performed in which tissue obtained at open heart surgery, from patients with hypertrophic obstructive cardiomyopathy, was assayed for marker enzyme activities and the levels compared with left ventricular tissue from patients with congenital or acquired valve disease also obtained at open heart surgery. Though the specific activities of the enzymes in the tissue obtained by open biopsy were higher than those in tissue obtained by endomyocardial biopsy, the mitochondrial and myofibril marker enzyme activities were higher in the hypertrophic cardiomyopathy tissue than in the valvular heart disease tissue.

CONGESTIVE CARDIOMYOPATHY

Table 3 compares the specific enzyme activities in left ventricular muscle from the congestive cardiomyopathy patients and the two valvular heart disease groups. The levels of Ca^{++}–ATPase activity in the myopathic group were intermediate between the two valvular heart disease groups whether assayed in the presence or absence of azide. The levels of lactate dehydrogenase in the congestive cardiomyopathy tissue were significantly raised compared with both the control groups. Malate and glutamate dehydrogenases, however, were present at lower concentrations in the myopathic group than in the control tissue. Monoamine oxidase, 5′ nucleotidase, α-glucosidase, the acid hydrolases, and DNA content of the tissue were not significantly different between any of the patient groups.

Table 4 shows the results of assays of marker enzymes in right ventricular biopsies from valvular heart disease and myopathic patients. The pattern of results is similar to that found with the left ventricu-

Table 2 *Enzymic analysis of left ventricular biopsies (mean ± SEM milliunits/mg protein; number of samples assayed between parentheses)*

Enzyme	*Valvular heart disease* with good LV function*	*Hypertrophic cardiomyopathy*	*Statistical significance*
Ca^{++}-dependent ATPase	48·9 ±4·6 (16)	62·1 ±8·4 (10)	$P < 0.05$
Oligomycin-insensitive Ca^{++}-dependent ATPase	40·4 ±4·0 (15)	50·4 ±13·6 (4)	$P > 0.05$
Azide-insensitive Ca^{++}-dependent ATPase	42·1 ±4·8 (12)	57·0 ±8·7 (5)	$P < 0.05$
Lactate dehydrogenase	232 ±35 (23)	395 ±58 (12)	$P < 0.025$
Malate dehydrogenase	4250 ±290 (20)	5880 ±150 (11)	$P < 0.005$
Glutamate dehydrogenase	6·24 ±1·34 (21)	7·95 ±1·99 (5)	$P > 0.05$
Monoamine oxidase	1·41 ±0·56 (13)	2·42 ±0·45 (13)	$P > 0.05$
5′ Nucleotidase	10·9 ±2·25 (16)	11·9 ±1·3 (10)	$P > 0.05$
Neutral α-glucosidase	0·043 ±0·010 (11)	0·176 ±0·037 (9)	$P > 0.05$
Acid phosphatase	1·71 ±0·34 (14)	2·92 ±0·51 (8)	$P > 0.05$
N-acetyl-β-glucosaminidase	1·050 ±0·35 (13)	1·99 ±0·33 (11)	$P > 0.05$

*Peters *et al.* (1976).

lar biopsies with significantly reduced levels of Ca^{++}-dependent ATPase and glutamate and malate dehydrogenase but with much increased levels of lactate dehydrogenase. The levels of monoamine oxidase and the acid and neutral hydrolases were not significantly different between the two groups.

Discussion

The data in this paper represent the first attempt to investigate the subcellular pathology with biochemical techniques in the myopathic human heart using endomycardial biopsy material. Certain

Table 3 *Enzymic analysis of left ventricular biopsies (mean ± SEM milliunits/mg protem number of samples assayed between parentheses)*

Enzyme	*Valvular heart disease**		*Congestive cardiomyopathy*	*Statistical significance*	
	Good function (G)	*Poor function (P)*		*G*	*P*
Ca^{++}-activated ATPase	48·9 ±4·6 (16)	23·4 ±4·4 (16)	28·6 ±2·9 (8)	P < 0·05	P > 0·05
Azide-insensitive Ca^{++}-activated ATPase	42·1 ±4·8 (12)	10·4 ±1·9 (15)	20·4 ±4·9 (7)	P < 0·05	P < 0·05
Lactate dehydrogenase	232 ±35 (23)	421 ±58 (20)	1390 ±300 (8)	P < 0·005	P < 0·01
Malate dehydrogenase	4250 ±290 (20)	3110 ±350 (18)	1320 ±190 (8)	P < 0·0005	P < 0·01
Glutamate dehydrogenase	6·24 ±1·3 (21)	4·25 ±1·51 (14)	1·62 ±0·31 (6)	P < 0·05	P > 0·05
Monoamine oxidase	1·41 ±0·56 (13)	2·37 ±0·58 (15)	1·42 ±0·06 (5)	P > 0·05	P > 0·05
5′ Nucleotidase	10·9 ±2·25 (16)	9·76 ±1·93 (14)	8·89 ±2·1 (7)	P > 0·05	P > 0·05
Neutral α-glucosidase	0·043 ±0·010 (11)	0·053 ±0·010 (9)	0·050 ±0·021 (7)	P > 0·05	P > 0·05
Acid phosphatase	1·71 ±0·31 (14)	1·61 ±0·30 (12)	2·01 ±0·51 (8)	P > 0·05	P > 0·05
N-Acetyl-β-glucosaminidase	1·05 ±0·35 (13)	0·734 ±0·103 (16)	0·99 ±0·21 (7)	P > 0·05	P > 0·05
Cathepsin D	74·5 ±13·0 (10)	59·2 ±8·7 (8)	49·9 ±13·2 (5)	P > 0·05	P < 0·05
β-glucuronidase	1·09 ±0·26 (5)	0·91 ±0·37 (7)	0·819 ±0·27 (4)	P > 0·05	P > 0·05
DNA	18·0 ±1·64 (13)	16·4 ±2·7 (8)	17·0 ±4·2 (7)	P > 0·05	P > 0·05

*Peters *et al.* (1976).

Table 4 *Enzymic analysis of right ventricular biopsies (mean ± SEM milliunits of activity/mg protein; number of samples assayed between parentheses)*

Enzyme	*Valvular heart disease**	*Congestive cardiomyopathy*	*Statistical significance*
Ca^{++}-dependent ATPase	39·9 ±4·1 (8)	20·8 ±4·9 (8)	P < 0·05
Lactate dehydrogenase	336 ±69 (10)	1530 ±260 (8)	P < 0·005
Malate dehydrogenase	4030 ±106 (8)	2540 ±700 (8)	P < 0·01
Glutamate dehydrogenase	4·50 ±0·20 (7)	1·04 ±0·04 (8)	P < 0·05
Monoamine oxidase	3·78 ±0·83 (8)	2·08 ±0·54 (8)	P > 0·05
5′ Nucleotidase	8·10 ±1·12 (8)	6·10 ±1·21 (9)	P > 0·05
Neutral α-glucosidase	0·073 ±0·019 (8)	0·078 ±0·020 (9)	P > 0·05
Acid phosphatase	2·39 ±0·32 (8)	2·00 ±0·32 (9)	P > 0·05
N-acetyl-β-glucosaminidase	0·49 ±0·12 (10)	0·71 ±0·12 (8)	P > 0·05

*Peters *et al.* (1976).

technical problems had to be overcome before these studies could be undertaken. Because of the small size of the tissue fragments obtained and the large number of different marker enzymes to be assayed, highly sensitive techniques using fluorigenic and radiolabelled substrates were necessary. The variation between duplicate estimations was within 5 per cent but the variation between separate biopsies from the same patient could be up to 20 per cent. This variability is the result of the different amounts of endocardium and myocardium in the individual biopsies. This fact, together with the wide scatter of results in the patient groups and the technical difficulties in the measurements, makes the routine use of these estimations in the diagnosis of cardiomyopathy unlikely. Nevertheless, by comparing pooled data from groups of patients, it is hoped that clues to the pathogenesis of the disease could be obtained.

One problem encountered in the present work and common to most biochemical studies on tissue fragments is the nature of the reference used in expressing the results. In the present study the enzyme activities have been expressed in terms of total tissue protein. Analysis of the DNA content of tissues from valvular heart disease and myopathic heart disease indicates no significant differences in the DNA/protein ratio, suggesting that enzyme changes per cell are similar to the changes per mg protein. The use of wet or dry tissue weights as reference indices is not practicable.

The finding of increased levels of enzymes associated with the myofibrils in patients with hypertrophic cardiomyopathy may be associated with the histological findings of grossly hypertrophied and irregularly arranged myocardial fibres (Maron *et al.*, 1975). The increased myofibril ATPase activity is probably a reflection of hypertrophy without cardiac failure (Thyrum *et al.*, 1971). The increased levels of certain mitochondrial enzyme activities presumably reflect the increased energy requirements of the hypertrophic tissue. Morphological studies have commented on the increased numbers of this organelle, 'mitochondriosis' in the myocardium in hypertrophic cardiomyopathy (van Noorden *et al.*, 1971; Sekiguchi, 1974). However, the enzyme which shows the greatest increase, malate dehydrogenase, also has a significant localisation to the cytosol of the myocardium, and it is not clear in which of these components it is increased. Increased numbers of lipofuscin-containing bodies, presumed to be lysosomes, have been described in hypertrophic cardiomyopathy (van Noorden *et al.*, 1971). Though the two lysosomal enzymes, acid phosphatase and N-acetyl-β-glucosaminidase, assayed in the present studies were raised compared with the control tissue, the difference did not reach statistical significance. These studies do not, therefore, indicate a pronounced defect in any of the organelles of the myocardial cell in hypertrophic cardiomyopathy.

The studies on the tissue from the patients with congestive cardiomyopathy do show abnormalities in the levels of certain marker enzymes. The levels of myofibril associated Ca^{++}-dependent ATPase are reduced, but it is likely that this is associated with impaired ventricular function (Berson and Swynghedauw, 1973; Conway *et al.*, 1975; Peters *et al.*, 1976). The decreased levels of mitochondrial enzymes, particularly those associated with the inner mitochondrial matrix, are of interest. It is unlikely that this simply reflects a reduction in the total number of mitochondria, as monoamine oxidase, associated with the outer mitochondrial membrane (Schnaitman and Greenawalt, 1968; Parsons *et al.*, 1966), is not significantly altered. Electron microscopical examination of the myocardium from patients with congestive cardiomyopathy has disclosed increased numbers of mitochondria, many of which show cristolysis (Olsen, 1975). The present studies suggest an abnormality in the properties or function of mitochondria in tissue from patients with congestive cardiomyopathy. Studies in the strain of Syrian hamster which suffers from a hereditary cardiomyopathy have also shown mitochondrial abnormalities (Sordahl *et al.*, 1972). Similarly, experiments on myopathic tissue obtained from a transplant recipient have also indicated abnormalities in the properties of the mitochondria (Sordhal *et al.*, 1972; Schwartz *et al.*, 1973).

The increased levels of lactate dehydrogenase in the congestive cardiomyopathy tissue from both left and right ventricle are striking. This increased enzyme activity may be the result of the impaired mitochondrial function. Difficulty in producing sufficient ATP by aerobic glycolysis leads to enhanced anaerobic glycolysis which would be associated with increased lactate dehydrogenase levels in the tissue. This has been clearly shown in experimental animals (Yang, 1963; Williamson, 1966; Bishop and Altschuld, 1971). In patients with cardiomyopathy, lactate production, rather than lactate extraction, can be shown even in the presence of normal blood flow (Brachfeld, 1974). It would be of considerable interest to assay certain of the intermediates of carbohydrate metabolism in tissue from patients with congestive cardiomyopathy in an attempt to clarify the possible defects in mitochondrial function.

The levels of enzymes associated with the other cell organelles, viz. sarcolemma (5′ nucleotidase),

lysosomes, (acid phosphatase, N-acetyl- β-glucosaminidase, β-glucuronidase, cathepsin D), and endoplasmic reticulum (α-glucosidase) are not significantly different in the two patient groups whether left or right ventricles are studied. It is, therefore, unlikely that any major defect in the functions of these organelles occurs in the cardiomyopathies.

We are grateful to Dr. Elizabeth Welman for her helpful suggestions. We wish to thank Dr. M. Davies and Dr. E. Olsen for reviewing the histology of the biopsies. This work is supported by the British Heart Foundation and The Wellcome Trust.

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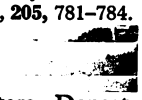

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