Scanning Microscopy

Volume 5 | Number 1 Article 22

10-29-1990

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Scanning Microscopy, Vol. 5, No. 1, 1991 (Pages 239-245)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/91$3.00+.00

CHANGES IN SODIUM CONCENTRATION IN CARDIAC MYOCYTES FROM DIABETIC RATS

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(Received for publication May 5, 1990, and in revised form October 29, 1990)

Abstract

The effects of streptozotocin induced diabetes on rats were studied. The animals showed an increase in blood glucose concentration and a loss of weight from both the body and the heart. Loss of weight from the heart was less severe leading to an increased heart to body weight ratio. Study of element concentrations by X-ray microanalysis showed that there was an increase in intracellular Na concentration in cardiac myocytes from the diabetic animals, but no change in Mg. These results agree with studies which show changes in Na/K ATPase after the onset of diabetes.

Key words: X-ray microanalysis, cardiac tissue, sodium, potassium, calcium, diabetes.

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Introduction

X-ray microanalysis is being widely applied by a number of groups to the study of heart tissue. This interest in the heart is not surprising, as heart disease remains a major killer in western society; in Britain diseases of the heart and circulatory system account for some 50% of deaths (Morgan 1989). Interest in elemental analysis of cardiac muscle centres on the element Ca. Normal functioning of cardiac muscle depends on controlled release of Ca from intracellular stores, with the intracellular concentration of Ca being maintained within narrow limits, whereas loss of function and cell death are accompanied by increases in the intracellular concentration of Ca (Katz and Reuter 1979). At present it is not clear whether this increase is the cause or effect of cell dysfunction in disease (Bond 1988).

A number of different methods are used for the study of Ca (and other element) concentrations; intracellular electrodes and fluorescent dyes being the most popular. X-ray microanalysis offers different capabilities from these more standard techniques and provides complimentary results. Undoubtedly, a major contribution of X-ray microanalysis to elucidating the role of ions in cell function is the ability to undertake analyses of subcellular sites without resort to fractionation procedures (Somlyo 1984). However the importance of being able to analyse individual cells within a tissue, and to estimate the concentrations of a number of elements simultaneously should not be overlooked. In heart, as in many other tissues, the intracellular concentration of Ca is regulated by the complex interplay of a number of mechanisms (Chapman and Tunstall 1987) including exchange for other elements such as Na. Thus the intracellular concentrations of other cations may be equally important.

The potential of X-ray microanalysis for the study of cardiac muscle is being realised (see the section on heart in LeFurgey et al. 1988a), and a summary of different applications is shown in Table 1. Different methods can be used for preparing heart tissue for X-ray microanalysis, the method chosen depends on the aims of the study. The preparation most frequently used is a working physiological preparation such as

Table 1. Applications of X-ray microanalysis to the study of cardiac myocytes.

Preparation	Intervention	Reference
papillary muscle	control	Wendt-Gallitelli and Wolburg 1981,1984 Tormey 1983 Cantino et al. 1986 Jorgensen et al. 1988 Hagler and Buja 1986
	ouabain	Wendt-Gallitelli and Jacob 1982,1984
	low Na	Wheeler-Clarke and Tormey 1985,1987
muscle strip	ischaemia	Walsh and Tormey 1988a,b
Cultured neonatal myocytes	IAA ouabain	Buja et al. 1985,1988 LeFurgey et al. 1986,1988b
Isolated adult myocytes	voltage clamp	Wendt-Gallitelli and Isenberg 1989
	control	Ward and Warley 1989 Chiesi et al. 1981
In situ fixed	control	von Zglinicki et al. 1986 Bond and Jaraki 1989 Warley 1989
	ageing	von Zglinicki and Bimmler1987
cardiomyopathy		Bond et al. 1989

isolated papillary muscle, which can be fixed in known functional states (Wendt-Gallitelli and Wolburg 1981, 1984; Wendt-Gallitelli and Jacob 1982, 1984; Wheeler-Clark and Tormey 1985,1987; Cantino et al. 1986; Hagler and Buja 1986; Walsh and Tormey 1988 a,b; Jorgensen et al. 1988). Other preparations are neonatal myocytes in culture (Buja et al. 1985,1988; LeFurgey et al. 1986, 1988b) and isolated adult myocytes (Chiesi et al. 1981; Wendt-Gallitelli and Isenberg 1989; Ward and Warley 1989). For the study of element concentrations after the onset of disease there has been a move towards the use of heart tissue cryofixed 'in situ' (von Zglinicki et al. 1986; von Zglinicki and Bimmler 1987; Bond and Jaraki 1989; Bond et al. 1989; Warley 1989). Although such preparations represent a specimen which is inherently more complex, they may be more suitable in the study of disease tissue.

Cardiomyopathy may develop as a long term complication of diabetes, and drug-induced diabetes in different animals has been used as a model. Hearts from diabetic animals, untreated with insulin show depressed ventricular function (Regan et al. 1974; Hearse et al. 1978; Garber and Neely 1983), altered contractile properties of isolated papillary muscles (Fein et al. 1980) and increased susceptibility to anoxia and ischaemic damage (Hearse et al. 1978). Biochemical studies show lowered activity of the Ca activated myosin ATPase (Garber and Neely 1983), altered permeability of the sarcolemma to Ca (Heyliger et al. 1987) and defective transport of Ca by the sarcoplasmic reticulum (Ganguly et al. 1983; Lopaschuk et al. 1983). The biochemical findings are supported by ultrastructural studies which show deterioration of the myocardium in diabetic animals (Seager et al. 1984; Jackson et al. 1985). These various findings led Pierce et al. (1988) to propose that diabetes mellitus leads to an alteration of Ca movements in the heart and that this is the causative factor in the development of cardiac pump insufficiency. Some bulk analytical studies have been carried out on ventricular tissue from diabetic animals, but the results from these are contradictory. No significant changes in Na K or Ca but a decrease in Mg were reported for tissue from rabbits (Bhimji et al. 1985), whereas increases in tissue concentrations of both Na and K were reported for rodents and dogs. This paper reports preliminary results from X-ray microanalysis of cardiac tissue from diabetic rats.

Materials and Methods

Diabetes was induced in male CSE Wistar rats (weight 300 g) by intraperitoneal injection of the drug streptozotocin (55mg/kg), untreated animals of the same weight were used as controls. After the induction of diabetes the weight of the animals was measured daily and the presence of glucose in the urine was monitored.

Operative procedures were the same for control and diabetic animals, for the X-ray microanalysis studies diabetic animals were used 8 weeks after induction of the disease. The animals were anaesthetised by subcutaneous injection of sodium pentobarbitone. For studies of blood glucose and organ weights, the femoral artery was cannulated, heparin was injected into the animal and a blood sample was withdrawn one hour after the injection of anaesthetic. The animal was killed and organ weights were measured. For the study of elemental concentrations the heart was frozen 'in situ' using cooled copper pliers which had been exposed to liquefied propane (Warley 1989). The animals were anaesthetised and one hour later a ventral incision was made and the thorax opened to expose the beating heart. The heart was gently lifted away from the thorax, the inferior vena cava was cut, and the heart cryofixed immediately. The tissue was freed from the jaws of the pliers under liquid nitrogen, and pieces of the ventricle were selected and stored under liquid nitrogen. For sectioning a small piece of tissue was broken away from the large block and clamped in the jaws of a vice type specimen chuck. Sections 200nm thick were cut from the external edge of the frozen tissue at a temperature of -130°C to -125°C. The frozen sections were transferred to Pioloform coated Ni grids (100 mesh) and the sections were pressed onto the grids using a cooled polished copper rod. The grids were transferred to a cooled brass block within the chamber

Table 2. Heart weight, body weight and blood glucose in diabetic rats.

Days diabetic	0	4	25	60
Start weight (g)	296±5	297±5	311±7	296±6
Weight loss (g)		22±6	78±12	106±28
Heart weight(g)	0.82±0.4	0.84±.02	0.76±.03	0.7±0.07
Heart/Body	.0027	0.003	0.0033	0.0037
Glucose mM/l	3.7±0.2	16.8±2.5	23.4±2.9	22.4±4.9

Table 3. Element concentrations in cardiac myocytes from control (C) and diabetic (D) rats (mmoles/kg dry weight ± S.E)

myofibrils

	n	Na	Mg	P	S	Cl	K	Ca
C	4	71±7	61±4	349±14	126±19	98±16	478±18	1±2
D	4	141±13	72±8	358±31	251±40	89±8	463±40	3±2

mitochondria

	n	Na	Mg	P	S	Cl	K	Ca
C	4	39±4	36±2	443±8	125±18	40±9	269±5	2±2
D	4	66±7	43±4	436±22	204±35	45±8	288±28	-1±1

of the microtome, the block was transferred to a freeze drier and the sections freeze dried overnight (vacuum < 10^{-3} Torr). The sections were allowed to warm to room temperature under vacuum and were coated with a thin layer of carbon before viewing in the electron microscope.

Analysis was carried out using a JEOL 100CX STEM electron microscope at 100 kV and a beam current of 1.5nA (measured using a Faraday cage). The myofibril region of the cells was analysed using a reduced area raster of 1 um and mitochondria were analysed using a raster size of 0.5 μm. Spectra were collected for 100s live time and data was processed with a Link analytical 860 series 2 EDS detection system and Quantem software. The system was calibrated against frozen sections of gelatin containing known amounts of salts. Details of the freezing and analytical procedures have been published previously (Warley 1989).

Cells were analysed from four control and four diabetic animals. From each animal 15 to 20 cells were analysed. The mean values from the individual cells from each animal were averaged to give the two groups of control and diabetic animals. The significance of differences between the two groups was tested using Student's t-test.

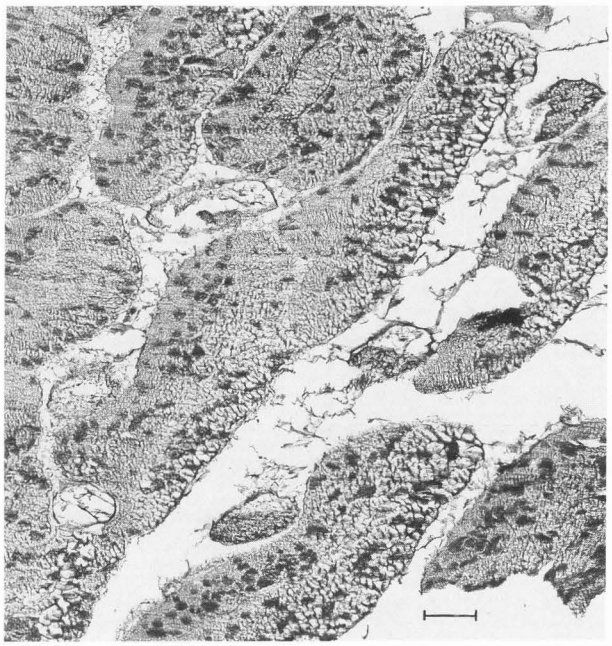

Figure 1. Electron micrograph showing a freeze dried frozen section of heart tissue from a rat 8 weeks after the onset of diabetes. Mitochondria and myofibrils are clearly seen. Only regions with little ice damage were analysed . Bar = 3 μm.

Results

Induction of diabetes with streptozotocin resulted in a decrease in body weight and increase in concentration of glucose in the blood. Weight loss from the heart is not as great as that from the body resulting in an increased heart to body weight ratio. Results for weight loss and blood glucose measurements at varying times after the onset of diabetes are shown in Table 2. The control animals used for X-ray microanalysis had a mean body weight of 294 ± 9.6 (SD) g. For X-ray microanalysis animals were studied 8 weeks after the onset of diabetes. These animals had a mean starting weight of 290 ± 4 g and the mean weight loss from these animals during the time of diabetes was 100 ± 6 g.

An electron micrograph of a freeze dried frozen section of ventricular tissue is shown in Fig 1. The epicardium, and individual myocytes can be clearly seen. The morphology is sufficiently good to be able to determine myofibrils and mitochondria. Only the superficial layers of cells which were largely ice crystal free, were used for analysis.

Concentrations of the elements Na, Mg, P, S, Cl, and K in ventricular myocytes from control and diabetic animals are shown in Table 3. Comparison of the two groups of animals shows that there is a significant increase in the concentrations of Na and S in the myofibril region of myocytes from the diabetic animals

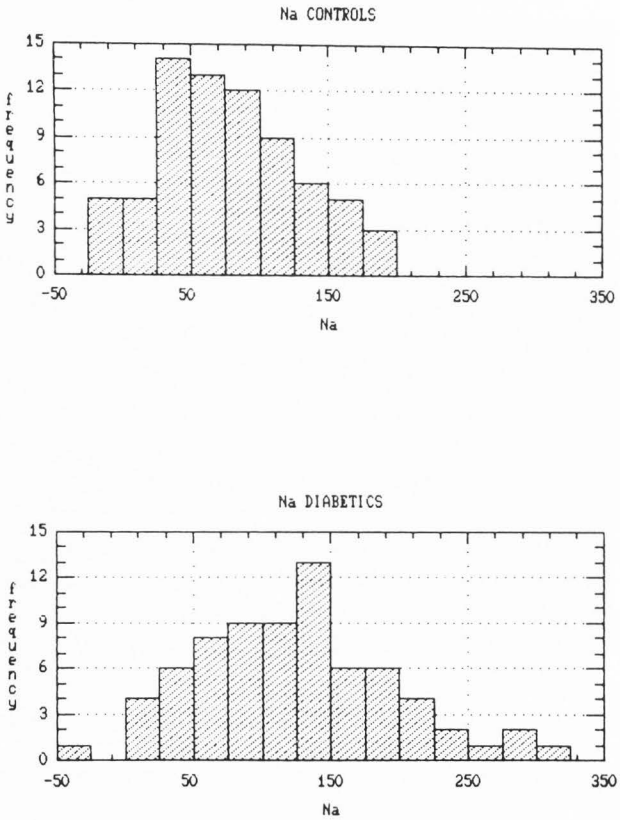

Fig 2. Distribution of Na concentrations in individual myocytes (myofibril region) from control and diabetic rats.

and a significant increase in the concentration of Na in the mitochondria. There were no significant changes in the concentrations of the other elements. The increased concentration of Na seen in myocytes from diabetic animals could be due to either an overall increase in Na in the majority of cells, or the presence of a number of cells containing very high concentrations of Na. Analysis of Na concentrations in individual myocytes (Fig. 2) shows that both are occurring. In control animals the majority of values for Na concentrations is in the range 0-100 mmoles/kg and no values over 200 mmoles were recorded. In the diabetic animals there is a shift of concentrations to higher values (over 100 mmoles/kg) and some cells were recorded with values greater than 200 mmoles/kg. In all of these latter cells the high values for Na were accompanied by lower values for K. In some animals cells with low K/Na ratios were found adjacent to cells with high K/Na (Fig. 3) suggesting that localised cell death is occurring.

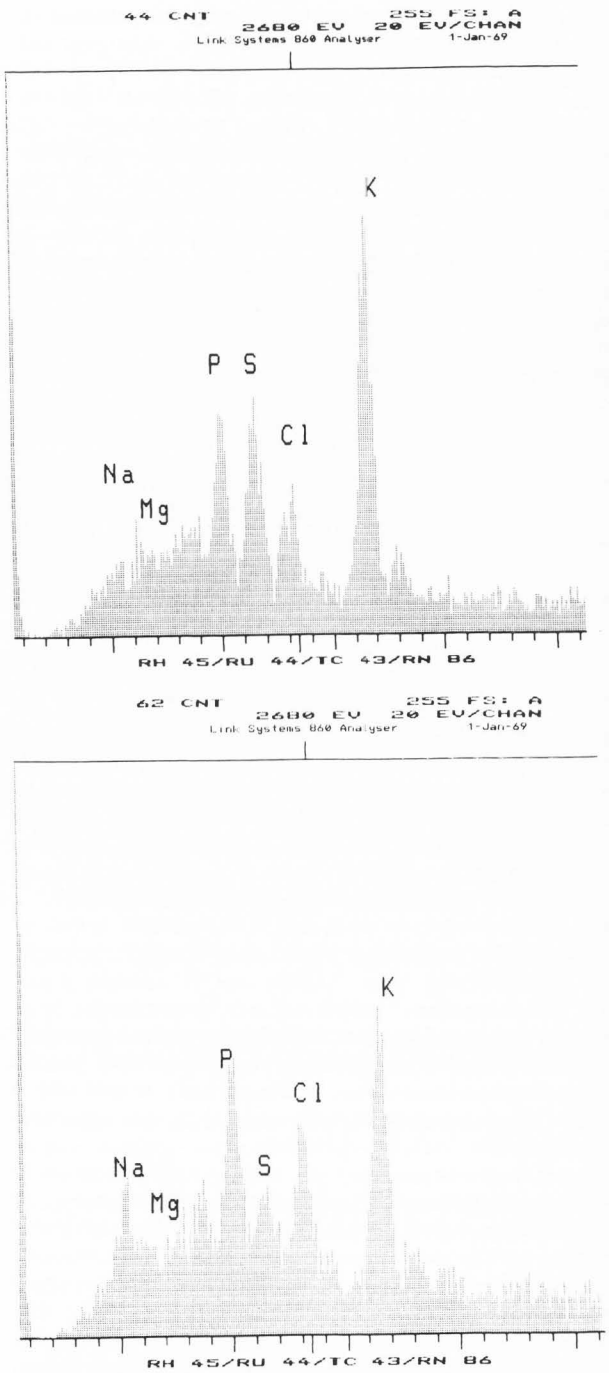

Figure 3. Individual spectra from adjacent myocytes in a diabetic animal (myofibril region). One cell (a) shows high K and low Na typical of cells from control animals. The other cell (b) shows increased Na and lowered K.

Discussion

This study reports elemental concentrations in cardiac myocytes from rats eight weeks after induction of diabetes. The progress of the disease was similar to that reported by other authors for the rat and other animals (Ganguly et al. 1983; Jackson et al. 1985; Kjeldsen et al. 1987).

The heart tissue was cryofixed 'in situ' and should most closely resemble the physiological state of the animal. In situ cryofixation was shown by Somlyo et al. (1985 a,b) to be suitable for studies on liver, and since then has been applied successfully to heart (von Zglinicki et al. 1986; Bond et al. 1989; Warley 1989), despite the relative bulk of this tissue. Element concentrations in myofibrils and in mitochondria obtained for rat heart cryofixed 'in situ' from two different laboratories show comparable values (von Zglinicki et al. 1986; Warley 1989). The results from these two laboratories are also in line with those obtained by X-ray microanalysis of isolated rat papillary muscle (Jorgensen et al. 1988) and with results obtained from other techniques (discussed in von Zglinicki et al. 1986). This agreement of results from different sources enhances confidence in the techniques used for this study.

The increased Na concentrations in cardiac muscle from the diabetic animals agree with the report of Kjeldsen et al. (1987) who found an increased Na content, without change in K in ventricular muscle from diabetic rats. However, the results reported here are in contrast with the results of Bhimji et al. (1985) on diabetic rabbits which showed no changes in Na and K but altered concentrations of Mg. The differences could be due to either the different species studied, or to differences in the techniques used for estimating elemental concentrations. In the present study Ca was present at very low concentrations or was not detected. Long counting times and a large number of analyses are required in order to detect statistically significant changes of this element at very low levels, so it is not possible to conclude from this study that intracellular concentrations of this element were not changed at a low level. However if large increases in intracellular Ca had occurred these would have been detected.

The increase in intracellular Na concentration could be attributed to low levels of circulating insulin in the diabetic animals. Insulin is known to stimulate the activity of the membrane bound Na/K ATPase. The activity of this enzyme is decreased in cardiac tissue from diabetic rats (Ku and Sellers 1982) and the concentration of pump sites has also been shown to be decreased (Kjeldsen et al. 1987). The results for cardiac muscle reported here differ from skeletal muscle in which the increase in Na is coupled with a decrease in intracellular K (Moore et al. 1983). Alterations in intracellular Na concentration could affect contractility of cardiac muscle either directly, or by affecting the intracellular concentration of Ca through Na-Ca exchange. Thus the preliminary results reported here show that more extensive studies of cardiac tissue from diabetic animals will be justified.

Acknowledgements

This work was supported by the British Heart Foundation. The author would like to thank Dr. M. Osborne, Department of Physiology, The Medical School, University of Birmingham, U.K. for use of their analytical electron microscope.

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Discussion with Reviewers

R. Wroblewski: In the discussion you state that increases in intracellular Ca could be detected. Do you exclude the possibility of lowered Ca concentrations in experimental animals? Na influx might cause the efflux of Ca and thus might cause lowered contractile capacity.

Author: That is an interesting point. The reason for

looking for an increase was that increased Ca had been predicted from indirect studies.

R. Wroblewski: Non contractile tissue (a.o fat and connective tissue) or water in the heart might be the cause of almost unchanged heart weight in experimental animals. If the higher water was taken into account it will of course give significant elemental changes of other elements if data were given on a wet weight basis. Do you have data on the water content of diabetic hearts from your experiment or from literature?

Author: My own studies (using CrEDTA as a marker for the extracellular medium) suggest that there is no change in water content in the diabetic heart.

L.M. Buja: Since cryopreservation of cardiac cells has generally been obtained from isolated cell preparations or thin pieces of tissue, such as papillary muscles rather than whole hearts, what criteria were used to establish that cryopreservation by the author's in situ freezing technique of the heart was adequate enough to allow for in situ preservation of electrolytes in morphologically defined regions of interest?

Author: With the method of freezing used, only muscle close to the epicardium can be studied. I think the freezing is adequate both on morphological grounds (see included micrograph) and because the elemental concentrations are in agreement with those presented by other authors for this tissue.

L.M. Buja: Although the author postulates impaired Na/K ATPase activity as the cause of the increased Na in cardiac myocytes of the diabetic animals one would expect to have decreased K as well as increased Na as a result. How does the author explain the increase in Na without a change in K? What proportion of cells showed decreased K and K/Na ratio as shown in Fig 2b.

T. von Zglinicki: Your Fig. 2 suggests an inverse correlation between Na and K. Even if mean K is not decreased in diabetic rats this correlation appears possible due to the high SE for K in the diabetic group. A correlation analysis would be of help to decide whether the Na/K ATPase is influenced under diabetes or not.

Author: It seems most likely that any fall in K may be masked by presenting the results as mean values. I calculated the ratio K/Na in the myofibril region for individual cells and found it to be 10.2 ± 9 (SD) for control animals and 5.6 ± 5 (SD) for the diabetic animals. This difference is statistically significant ($p < 0.001$ Student's t-test). Similarly analysis of the correlation between K and Na in the diabetic animals shows a significant negative correlation.

T. von Zglinicki: You present S analysis although your measurements were done at room temperature. It would be interesting to know whether diabetes could lead to chemical changes in S-containing proteins or lipids rendering them more susceptible to radiation damage.

G.M. Roomans: Although the paper according to its title deals only with changes in sodium in cardiac myocytes, could you speculate briefly on the significant changes in sulfur that you observe? Is there a relationship with ultrastructural changes?

J.M. Tormey: The doubled S concentration in both myofibrils and mitochondria is intriguing. Please discuss any possible biological significance to this finding. Alternatively, how likely is it that this difference is an analytical artifact? It is well known that sulfur is lost easily from tissue sections under electron bombardment, especially if analyses are carried out at room temperature. Are there any differences in analytical conditions that might explain this difference in your case?

Author: There were no differences in analytical conditions, such as time of exposure to the electron beam or the use of a cold stage, which could account for the observed differences in sulfur. Therefore, with the proviso that the analyses were performed at room temperature, it appears that this change is associated with the diseased state. Sulfur is largely associated with proteins so the increase in this element could reflect a change in protein constituents, particularly enzymes (in diabetes there is a shift to the utilisation of lipids and proteins as fuels therefore the enzyme profile of the cell must change). The utilisation of lipids also leads to an increased formation of acetyl CoA, a sulfur containing molecule, which is localised in both cytoplasm and mitochondria. So an increase in S could be expected. Ultrastructural changes which have been reported include an increased thickness of basement membrane which includes sulphated polysaccharides (though why this should lead to increased intracellular S I'm not sure). Therefore the increased S could well reflect the complex changes in metabolism which occur as the tissue adapts to the diabetic state.

J.M. Tormey: From the altered Na gradient alone it might be expected that the contractility of the diabetic hearts would be increased. Is this the case after 8 weeks of diabetes? Would you speculate how the altered gradient may be causally related to fully developed cardiac myopathy?

Author: The reported effect of diabetes is in fact a decrease in contractility. I think that one has to balance any increase which might occur from the increased Na with the reported increased amount of stiff connective tissue. I favour the idea that localised cell death is occurring which decreases the effective muscle mass.

J.M. Tormey: Can you estimate how much of the variation in Na concentrations are related to analytical error (such as poor counting statistics) and how much might be due to biological variation? Would you conclude that intracellular concentrations are significantly more variable in the diabetic heart?

Author: I have not looked at lowest detectable levels of Na in this analytical system so my answer must be a guess. I would have thought that there would be less uncertainty associated with the detection of the higher levels of Na in the diabetic animals than with the lower values found in the control cells, and, looking at the coefficient of variation for the analyses this is so.