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# CRYOFIXATION OF HEART TISSUE FOR X-RAY MICROANALYSIS

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## Abstract

Cryofixation of tissues is necessary to be able to study the concentrations of elements by X-ray microanalysis. Simple dissection of heart tissue fragments of the size needed for optimum cryofixation by the conventional methods of plunge or slam freezing leads to the development of ischaemia in the tissue fragments and a consequent redistribution of the diffusible elements. Heart tissue can be frozen in vivo using liquid nitrogen cooled Cu clad pliers, but the morphological detail is preserved better if the cooled pliers are exposed to liquefied propane immediately before freezing. Concentrations of Na are lower and concentrations of K are higher in the tissue which has been frozen in vivo compared to tissue frozen after dissection.

KEYWORDS: Cryofixation, heart tissue, X-ray microanalysis, rats, sodium, potassium.

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## Introduction

Changes in the concentrations of the inorganic elements Na, Mg, K and Ca are thought to control the functions of many different cells. Although much is known about total elemental concentrations especially of normal tissues, more remains to be learned particularly about elemental concentrations in individual cells, subcellular organelles, and whether alterations occur after the onset of disease. The technique of X-ray microanalysis offers a means of unequivocally measuring elements at the cellular and subcellular levels and, for the study of the diffusible elements, cryofixation and cryopreparation are necessary, to arrest physiological processes and to retain elements in their in vivo situation. Although there has been an increasing amount of interest in the cryofixation of biological material and several reviews and books on the subject have been published (see, e.g., Plattner and Bachmann, 1982; Robards and Sleytr, 1985; Steinbrecht and Zierold, 1987; Elder and Bovell, 1988), in the majority of instances emphasis has been placed on the development of cryotechniques to produce specimens suitable for morphological studies. However, if the intention is to study the specimen by X-ray microanalysis, then the aim must be to preserve the distribution of elements within the specimen. It is vital that movement of elements does not occur at any stage during specimen preparation and morphological considerations may have to take second place. During the development of X-ray microanalytical techniques attention has tended to focus on the later stages of specimen preparation, sectioning (discussed in Roomans et al., 1982) and transfer to the electron microscope (see, e.g., Frederik and Busing, 1981; Hagler and Buja, 1986) as points at which movement of elements could occur. These possibilities have now been well discussed (Hagler and Buja, 1984; Hagler, 1986; von Zglinicki and Uhrik, 1988; von Zglinicki et al., 1988). However it is essential to bear in mind that preparation of the specimen for freezing is the most likely step at which alteration in the distribution of elements may occur; this is often overlooked.

The various methods which may used for preparing tissues for freezing are: dissection of pieces small

enough to give rapid freezing; tissue preparations (e.g., papillary muscle) isolated under well defined experimental conditions; and isolation of single cells from the tissue. Each of these methods has its own advantages and drawbacks. For dissected fragments of tissue immersion in a liquid cryogen or impact freezing against a cooled metal block are the techniques which have been used for cryopreservation. There is no doubt that excellent morphological detail can be obtained using either method of cryofixation (see e.g. Sitte et al., 1987; Roos and Barnard, 1985) but the effects of preparing the tissue for freezing need to be considered. Dissection of a tissue leads to damage at the cut surface and loss of elements from the outermost layer of cells (Somlyo et al., 1977; Hagler et al., 1981; Zierold and Schafer, 1988). In addition if a tissue remains metabolically active after dissection, changes due to anoxia can develop rapidly (von Zglinicki et al., 1986) leading to a loss of the ionic gradient across the plasma membrane. Isolated preparations of papillary muscle (Wendt-Gallitelli and Wolburg, 1984; Wendt-Gallitelli and Jacob, 1984; Wheeler-Clarke and Tormey, 1987) or epithelium (Gupta, 1989; Rick and Schratt, 1989) have proved useful for study by X-ray microanalysis. The physiology of such isolated preparations has been well studied, the preparations are of low bulk, and often suitable apparatus has been designed so that optimum cryofixation is obtained with the tissue in a known functional state. Similarly isolated cells provide a specimen of low volume, present in a well defined medium, and it possible to control such preparations right up to the moment of freezing. However, several X-ray microanalytical studies have now shown that intracellular elemental concentrations can be altered in freshly isolated cells (Warley, 1986; Zierold and Schafer, 1988; Warley, 1989) and, unless single cells of known viability can be selected (Wendt-Gallitelli and Isenberg, 1989), the effects of isolation need to be taken into account when interpreting results from these preparations. The use of physiological preparations or isolated cells might seem the best approach for preparing specimens for X-ray microanalysis. However these methods may not be appropriate for studying changes in element concentrations due to disease, as it is possible that any alterations *in vivo* could be destroyed during the isolation process. This paper reports preliminary results obtained by freeze clamping heart tissue *in vivo* using cooled copper clad pliers which have been exposed to liquefied propane immediately before freezing.

## Materials and Methods

A pair of Cu clad pliers were made after the designs in Hagler and Buja (1984) and Ingram and Ingram (1984). The pliers were cooled initially by plunging into liquid nitrogen; the cooled pliers were placed in the cooled chamber of a Reichert KF80 plunge freezing device to allow the pliers to remain cold and any excess liquid nitrogen to evaporate. The cool dry pliers were then placed into the coolant vessel which contained liquefied propane until needed for freeze clamping of tissues. Male CSE Wistar rats weighing 300 g were used as control animals. The animals were anaesthetised heavily with sodium pentabarbitone (35 mg/kg). The thorax was opened, the vena cava cut, and the beating heart rapidly clamped in the jaws of the cooled copper clad pliers. The flattened piece of tissue obtained was stored under liquid nitrogen until sectioning. In one experiment small pieces of heart tissue were dissected placed on silver pins and frozen by plunging into liquefied propane using a Reichert KF80 freezing device.

The method used for sectioning was similar to that described by Hagler et al. (1983), but the wafer of tissue is somewhat thicker (approx. 1 mm), because of this the block needs to be trimmed to an angle before sections can be cut. Sectioning was carried out using a Reichert FC4D cryoultramicrotome. The flattened tissue was fractured under liquid nitrogen using a cooled razor blade. A small piece of tissue was placed in the jaws of a vice type chuck (obtainable from Reichert-Jung). The knife was set at as large an angle as possible and the block trimmed. Sections 200 nm thick were cut from the point of well frozen tissue at a temperature of -125°C to -130°C. The sections were pressed between two Pioloform coated grids. The grids were separated and placed into a precooled brass block for transfer to a freeze drier. The sections were allowed to freeze dry overnight and were coated with a thin layer of carbon before analysis.

Analysis was carried out using a JEOL 100CX electron microscope in STEM mode. The accelerating voltage was 100 keV and the beam current (1.3 nA to 1.8 nA) was measured with a Faraday cage. The area of analysis was 0.25 μm x 0.25 μm for myofilaments and 0.125 μm x 0.125 μm for mitochondria. For analysis the specimen was tilted to 35. Spectra were collected and quantitative data were obtained by use of a Link Analytical 860 series 2 EDS detection system and Quantem software. This program is based on the continuum normalisation procedure of Hall and has full facilities for gain calibration and background subtraction. The energy region used for determining the continuum was 6 keV to 16 keV. For further details of this analysis system see Spencer et al. 1988.

## Results

### Morphology.

When liquid nitrogen cooled Cu clad pliers were used to freeze clamp intact heart tissue, the results were disappointing (Fig. 1), good preservation was not achieved to any great depth in the tissue. Invariably a gradient of ice crystal damage could be seen even in the first layer of myocytes. The presence of ice crystals in the remainder of the block made sectioning difficult, and the sections were prone to breaking and difficult to pick up.

Exposure of the cooled pliers to liquefied propane

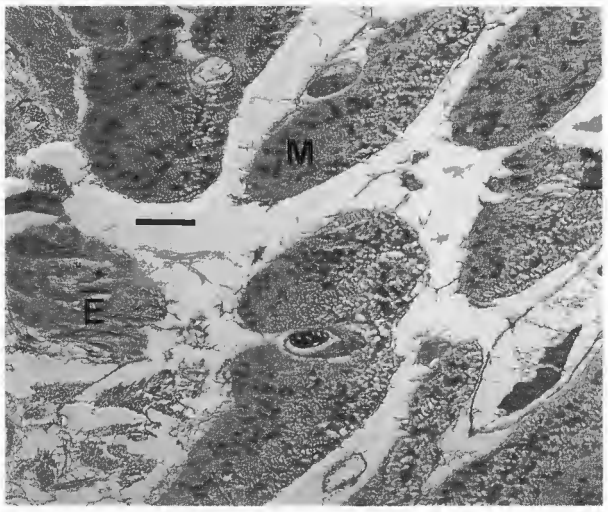


Fig. 1. Cardiac tissue frozen using liquid nitrogen cooled Cu clad pliers. E - epicardium, M - myocyte. Bar = 5 μm.

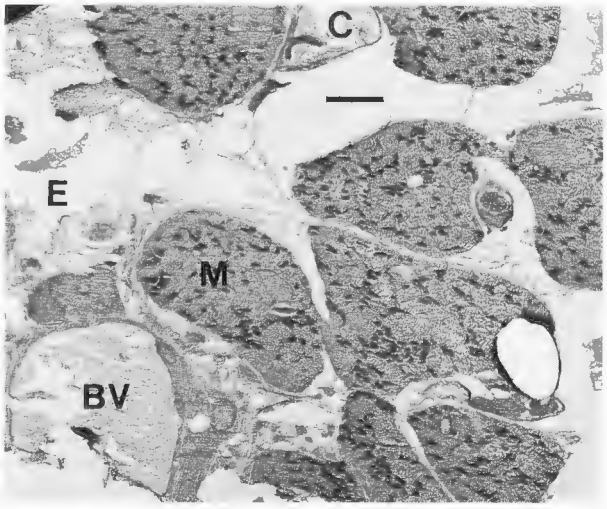


Fig. 2a. Cardiac tissue frozen using cooled pliers exposed to liquefied propane before freezing. E - epicardium, M - myocyte, BV - large blood vessel, C - capillary. Bar = 5 μm.

before freezing resulted in a greater depth of preservation of the heart tissue (Figs. 2a,b). The separation of the myocytes from each other seen in Fig. 2 is due to shrinkage of the section during freeze drying. Because of this it is difficult to estimate accurately, from the freeze dried sections, the depth of good cryofixation obtained. Several points stand out. As would be expected the structure of cells in the epicardium, which is closest to the pliers, is well preserved. The myocytes show good freezing and are suitable for analysis 3-4 cells in depth away from the

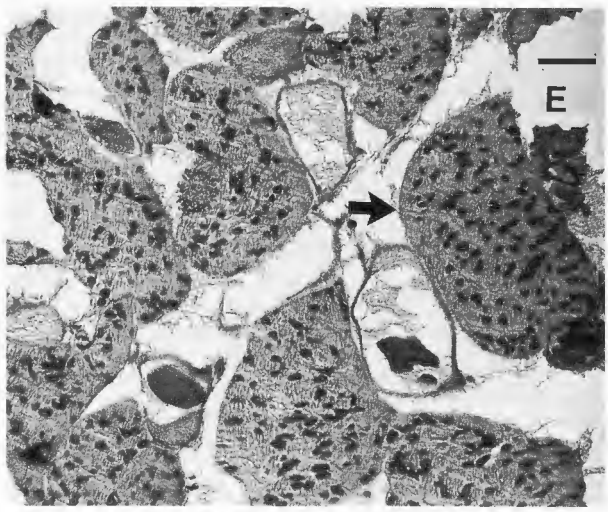


Fig. 2b. Cardiac tissue frozen using cooled pliers exposed to liquefied propane. Z bands (arrow) can be seen in this preparation. Bar = 3 μm.

epicardium. Subcellular details are recognisable in the individual myocytes, the mitochondria and myofibrils are easily distinguished and, depending on the angle at which the myofibrils are cut, the Z bands can be seen (Fig. 2b). The capillary vessels are well preserved, even at a considerable depth in the tissue, and the lack of distortion of these vessels indicates that the outer layers of tissue are not being crushed during the clamping process.

Analysis.

The concentrations of elements in myofibrils (myo) and mitochondria (mit) of myocytes prepared either by dissection or by freezing in vivo are shown in table 1.

Table 1. Concentrations of elements in myocytes and mitochondria of rat cardiac myocytes (mmol/kg dry weight).

| | n | Na | Mg | P | S | Cl | K |
|---|---|---|---|---|---|---|---|
| dissection | | | | | | | |
| Myo | 10 | 154±20 | 31±9 | 196±26 | 95±5 | 89±11 | 277±14 |
| Mit | 10 | 104±14 | 22±7 | 373±17 | 105±11 | 63±10 | 223±45 |
| in vivo | | | | | | | |
| Myo | 79 | 73±6 | 61±4 | 353±10 | 121±5 | 100±4 | 473±10 |
| Mit | 60 | 42±4 | 37±3 | 438±7 | 107±4 | 47±3 | 270±6 |

Results shown as mean ± SEM, n=number of observations.

The results for dissected material are from one animal and are typical of results found for dissected tissue. The results for in vivo freezing are pooled results of analyses of myofibrils from four animals. Intracellular concentrations of Na are low and those of K high in the tissue frozen in vivo. The results are in good agreement with microanalysis results for rat myocardium

reported by von Zglinicki et al. (1986) who also used an in vivo technique for freezing heart tissue. Higher concentrations of Na and lower concentrations of K are found in the myocytes from tissue which was dissected before freezing. The results for the dissected tissue are similar to those reported by other workers (Dykes et al., 1979) who also used dissection.

## Discussion

The results presented in this paper confirm the observations of von Zglinicki et al. (1986), that dissection of heart tissue before freezing can lead to redistribution of elements. Nevertheless dissection followed by cryofixation should not be condemned outright as a method for freezing all tissues, as the results obtained may depend not only on the speed of dissection but also on the level of metabolic activity of the tissue. Heart tissue with its high metabolic rate may be particularly vulnerable to the changes which are caused by the development of ischaemia. Earlier studies on the thymus gland (Warley, 1988) using dissected fragments of tissue the elemental concentrations in the control cells were similar to those obtained by other workers using different methods of analysis.

Cryofixation of tissues using liquid nitrogen cooled aluminium clad pliers was introduced by Wollenberger et al. (1960) for fixation of tissues for biochemical studies. Pliers coated with Cu rather than Al have been successfully used for fixation of specimens of small volume such as papillary muscle (Hagler and Buja, 1984), isolated skeletal muscle (Ingram and Ingram, 1984), biopsy specimens (Tvedt et al., 1987) or cells cultured as monolayers (Buja et al., 1985; Tvedt et al., 1988). However the results presented here show that the liquid nitrogen cooled pliers did not give sufficiently good morphological preparation of a bulkier tissue, the whole rat heart. Morphological preservation was much improved by exposure of the pliers to liquefied propane immediately before freezing.

In vivo freezing has been proposed by Somlyo et al. (1985) who constructed a device using a clamp containing solid Freon (Freon popsicles) and by von Zglinicki et al. (1986) who used a chilled cryobiopsy needle. On the whole, an in vivo freezing technique might be expected to produce poor results morphologically, as the bulk of the tissue should lead to low freezing rates. However, Barnard (1987) has commented on the remarkable quality of freezing obtained by Somlyo et al. (1985) with the Freon popsicles and attributes the improvement to absorption of heat from the tissue by the latent heat of the solid/melting Freon. In their studies von Zglinicki et al. (1986) obtained better cooling rates with a biopsy needle cooled in liquefied propane rather than liquid nitrogen. These latter authors attribute the better cooling to a thin layer of propane remaining on the surface of the needle and conclude that freezing is achieved by a combination of metal mirror and liquid cryogen fixation. It is probable that the improvement in freezing reported in this paper when the cooled pliers were exposed to liquefied propane before freezing is due to a similar effect. The use of pliers for in vivo freezing does offer several advantages. The pliers are easy to construct. In addition, a large area of specimen is frozen, thus it is possible to perform different investigations on material from the same animal, or to reinvestigate the same tissue if necessary.

The uncertainties about the effect of manipulation of tissues on element concentrations are removed when in vivo freezing is used making this an attractive method of specimen preparation for X-ray microanalysis particularly for studies of pathological changes. Despite this the technique is not routinely used outside the laboratories of Somlyo and von Zglinicki. The reason for this is not clear, but could possibly be due to the poorer preservation achieved compared to other techniques. The method presented here provides a simple means of achieving in vivo cryofixation providing a specimen with minimal ice crystal damage, in which the elemental composition is preserved and which is suitable for study by X-ray microanalysis.

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## Discussion with Reviewers

S.H. Ashrafi: What was the percentage of shrinkage during freeze drying and up to what depth the cryofixation was obtained?
Author: I have not measured shrinkage of tissue during freeze drying. Gross ice crystal damage was found after a depth of about six myocytes.

S.H. Ashrafi: Did the author evaluate the relocation of free ions during freeze drying of tissue following the withdrawing ice crystal interface towards the center of the specimen?
Author: I have measured myofibril element concentration (i.e. cross-section of the myocyte) up to 5 myocytes in from the epicardium. No gradient of element concentration was detected.

S.H. Ashrafi: Where was the background subtracted for elements in the spectrum?
Author: The quantitation program uses a filtered least squares fitting routine for estimation of peak areas. The continuum reading was taken in the interval 6-16 keV.

S.H. Ashrafi: Did warming of the freeze drying section at room temperature during transfer of sections to the microscope affect the redistribution of elements and their concentrations in the specimen?
Author: I do not have access to a cold stage to be able to undertake the experiments to assess whether there was any redistribution of elements due to transfer at room temperature. I do take care at this step, and the fact that the concentrations of Na and K are similar to those obtained with other techniques suggests that this is not a serious problem.

S.H. Ashrafi: Please comment about the high concentrations of Na and low concentrations of Mg, P, S, Cl and K in the dissected tissues than in vivo.
Author: The alterations in concentrations in dissected tissue are probably due to both damage and anoxia.

G.M. Roomans: The reason why the "cryoplier" technique has not gained more widespread popularity seems to be that many groups who tried the method noted a very variable success rate. Could you give an indication of the percentage of well frozen specimens that you have obtained with your technique.
Author: I have now fixed material from ten animals and have achieved successful freezing each time. I also would like to point out that while I was undertaking this work Bond et al. published work on heart tissue which had been cryofixed using the Freon popsicles method, so it appears that snap freezing of heart tissue is becoming a recognised procedure.

T. von Zglinicki: I would expect that dry pliers should give good results consistently, provided that they are sufficiently polished. Please, state clearly whether you compared dried or "wet" pliers.
Author: I have not tried deliberately to compare dry and wet pliers. I think that it is difficult to ensure that the liquid nitrogen cooled pliers remain dry as even the briefest exposure to the atmosphere results in condensation.

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