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EFFECT OF GLYCEROL ON ICE FORMATION OF ISOLATED MITOCHONDRIA AT DIFFERENT FREEZING RATES

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Kuo-Chun Liu (1979) Effect of glycerol on ice formation in freezing isolated mitochondria. *Bull. Inst. Zool., Academia Sinica* **18**(2): 71-78. The mitochondria isolated from renal cortical tissue of mouse is used as a model organelles to study the effect of glycerol on ice formation and to understand the cryoinjury and cryoprotection nature of the two different freezing rates used.

Samples with and without glycerol pretreatment were cooled either at −38° C/sec in liquid nitrogen to −196°C or at −3.8°C/min on dry ice to −78°C, and freeze-substitution was used to preserve the ice spaces after frozen. After rapid freezing samples showed both intra and extramitochondrial ice spaces in the absence of glycerol. The shapes of the mitochondria were changed due to extramitochondrial ice formation, and descreased in size. In the presence of glycerol, there were 30-40% of mitochondria with, and 60-70% of them without intramitochondrial ice spaces. The 60-70% of mitochondria without intramitochondrial ice space were severe dehydrated and shrunken in size.

Slow freezing in the absence of glycerol, only extramitochondrial ice spaces were formed. All of the mitochondria were dehydrated and shrunken in size, due to the extramitochondrial ice formation. Freezing injury of mitochondria of rapidly freezing samples were caused both by intramitochondrial and extramitochondrial ice formation. Freezing injury of slowly freezing samples was only due to salt concentration caused by extramitochondrial ice formation.

In rapid freezing, the presence of glycerol reduced the number of mitochondria having intramitochondrial ice from 100% to 30%. In slow freezing, the presence of glycerol greatly reduced the degree of mitochondrial dehydration. Isolated mitochondria also proved to be a convenient system for cryobiological study.

To reduce the degree of injury and to obtained higher percentage and better quality of thawed cells, tissues and eventually organs[9] are the major aim of cryobiological research. This aim can only be realized through the understanding of the events happened during freezing; freezing storage; the thawing process; the action of cryoprotective substances; optimal rates of freezing and thawing of different cells and tissues. The importance of cryobiological research on cellular organelles as the structural and functional units have been proposed[8,9] but it still is a rather neglected area[18].

The study of freezing-thawing injury and the protective effects of glycerol and dimethyl sulfoxide on the *in situ* and isolated mitochondria of renal cortical tissue of mouse revealed that greater damage occurred after rapid freezing and slow thawing than after slow freezing with rapid thawing both in the presence and absence of protective substance[19]. Similar results were obtained in the study of

rough endoplasmic reticulum of rat pancreatic cells[21]. As has been proposed the knowledge of cellular organelles independent or interdependent reactions in the freezing-thawing processes are essential to our understanding of the mechanism in cryoinjury and cryoprotection[12,24].

To a frozen biological system the after thawing structural integrity and functional survival are the final approval and disapproval of certain freezing procedure. Yet the after thawing result represents a colligative effects of at least two different steps, freezing and thawing. The study of the frozen state will provide the information related to rate of freezing, effects of cryoprotective substances on ice formation which is the primary cause of freezing injury.

This report is to present observations made on isolated mitochondria from renal cortical tissue of mouse at the frozen state to study the freezing injury before the thawing effects were added. Hopefully though this and similar studies better freezing techniques can be developed to circumvent the injurious factors of freezing toward the direction of more protective for different cells and tissues.

MATERIALS AND METHODS

Isolation of mitochondria

Kidney cortical tissue of two-month old female mice of Webster strain were collected in 0.25 M ice cold sucrose solution. Slices of the cortical tissue were rinsed and homogenized in sucrose solution, in the propotion of one gram of tissue in 10 ml of sucrose solution. The homogenate was centrifuged in RC2-B Sorvall centrifuge, rotor SS-34, at 2,000 rpm for 12 minutes at 4°C. The supernatant was collected and centrifuged at 8,500 rpm for 10 minutes at the same condition. The process was repeated three times until the mitochondrial pellets were creamy in appearance[24].

Preparation of mitochondria for freezing and freezing-substitation

Pretreatment of mitochondria for freezing was done by resuspending the pellets in 0.25 M of sucrose solution with 15% of glycerol (V/V) for 15 minutes at room temperature. The suspension was centrifuged for 10 minutes at room temperature at 8,500 rpm. Samples were taken for electron microscopic study as pre-freezing control[18].

Freezing of mitochondria was done by placing glycerol treated mitochondrial samples in the dimension of about 1 mm^3 on a small aluminum carrier. For rapid freezing the aluminum carrier was submerged in liquid nitrogen (−196°C) for two minutes. This will give an overall freezing rate of −38°C/sec to the final temperature of liquid nitrogen. For slow rate of freezing the aluminum carriers were placed in glass stendor dishes which were covered with glass cover and put on the surface of solid carbon dioxide within an insolated box. This arrangement will give a overall freezing rate of −3.8°C/min to −78°C[18]. The frozen state was preserved by the technique of freeze-substitution. Frozen samples were placed in the substitution fluid (1% osmic acid in a solvent of equal part of absolute acetone and absolute ethanol) for a period of three weeks at −85°C[3,9,21].

Electron microscopy

At the end of three weeks, the freeze-substitution fluid were replaced with precooled acetone[3,9,21]. Then the sample were left at room temperature to warm up. After a 30 minutes wash in absolute ethanol, the samples were treated with propylene oxide and then infliltrated and embedded in Epon-Araldite mixture for electron microscopic study. Thin sections were cut on a Poter-Blum microtome with a diamond knife. The sections were stained with uranyl acetate and lead citrate. Observation and photographs were made with a Siemens 1A Elmiskop.

RESULTS

Observations on the ultrastructure of isolated mitochondria of unfrozen control and of the frozen state are represented in five figures.

Control samples (Fig. 1) showed that about 60 to 70% of the mitochondria were in con-

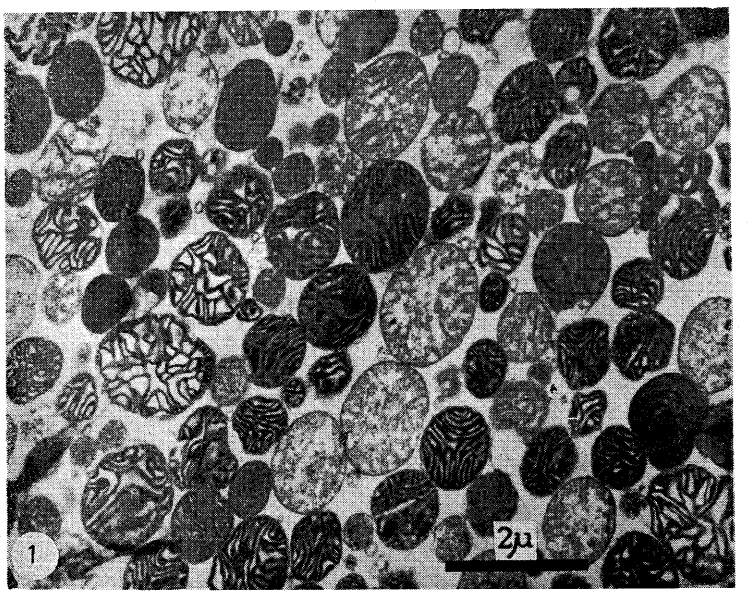

Fig. 1. Mtiochondria, isolated from renal contical tissue of mouse, consist of about 30 to 40% in orthodox and 60 to 70% in condensed conformation, ×15,000.

densed and 30 to 40% were in orthodox conformation[6]. They were circular to oval in shape and their diameters were ranged from 0.5 to 1.5 micrometers. Dense granule was very seldom being observed. There was a slight loss of critae and mitrix density among some of the mitochondria. This is believed to be caused during the isolation process[18].

After rapid freezing in the absence of glycerol, samples showed both extra and intramitochondrial ice spaces (Fig. 2). The intramitochondrial ice spaces of most of the mitochondria were very small and evenly distributed throughout the whole mitochondria. There were some mitochondria with slightly large intramitochondrial ice spaces. It was also evident that due to extramitochondrial ice formation there was alteration of mitochondrial configuration from circular to polygonal (Fig. 2, arrow).

In the presence of glycerol, about 30 to 40% of the mitochondria showed intramitochondrial ice spaces (Fig. 3), and the rest of the 60 to 70% of the mitochondria did not show any intramitochondrial ice space. These mitochondria devoid of intramitochondrial ice space were severely dehydrated, reduced size and altered in shape, due to extramitochondrial ice formation. These with intramitochondrial ice spaces were still more or less maintained their regular shape. Disruptures of mitochondrial membrane due to intramitochondrial ice formation were also observed (Fig. 3 arrow).

Slow freezing without the presence of glycerol, samples showed extramitochondrial ice spaces only. The mitochondria were dehydrated, reduced in size, showed a shrunken appearance. They aggregated in small groups and these groups were interconnected. The shape of these mitochondria were altered from original circular to irregular due to the dehydration and physical expension of ice formation (Fig. 4).

At the presence of glycerol, slow freezing resulted slightly larger extramitochondrial ice spaces than in the samples without glycerol freezing at the same rate (Fig. 5). The general appearance of the mitochondria were very similar, but were less dehydrated and deformed than these from the samples without glycerol. Some of the mitochondria were even still cir-

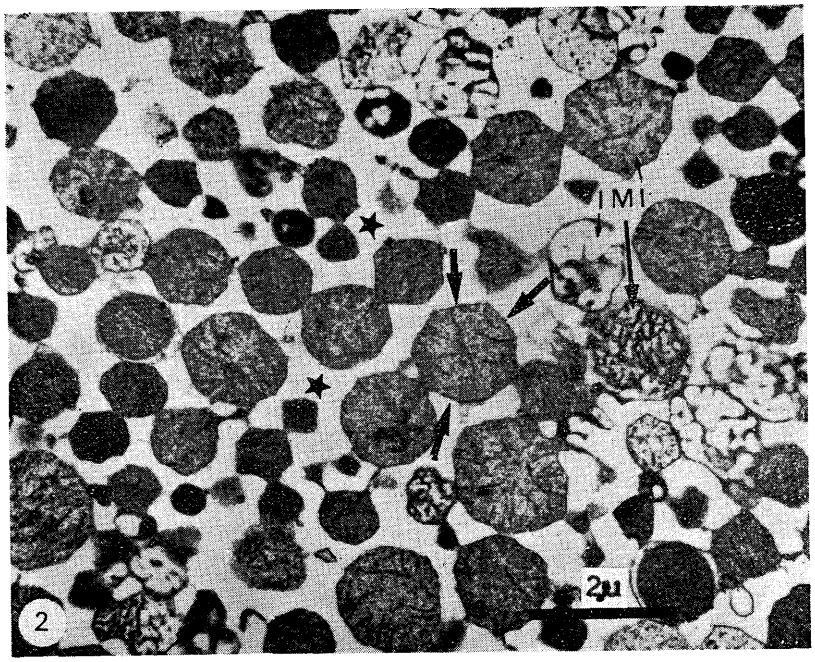

Fig. 2. Mitochondria after rapidly froze to −196°C at the rate of −38°C/sec. The stars indicate the locations where the extramitochondrial ice used to be. The sides of a polygonal mitochondrion were pointed out by the arrows, and the intramitochondrial ice spaces (IMI) were indicated (arrows) ×15,000.

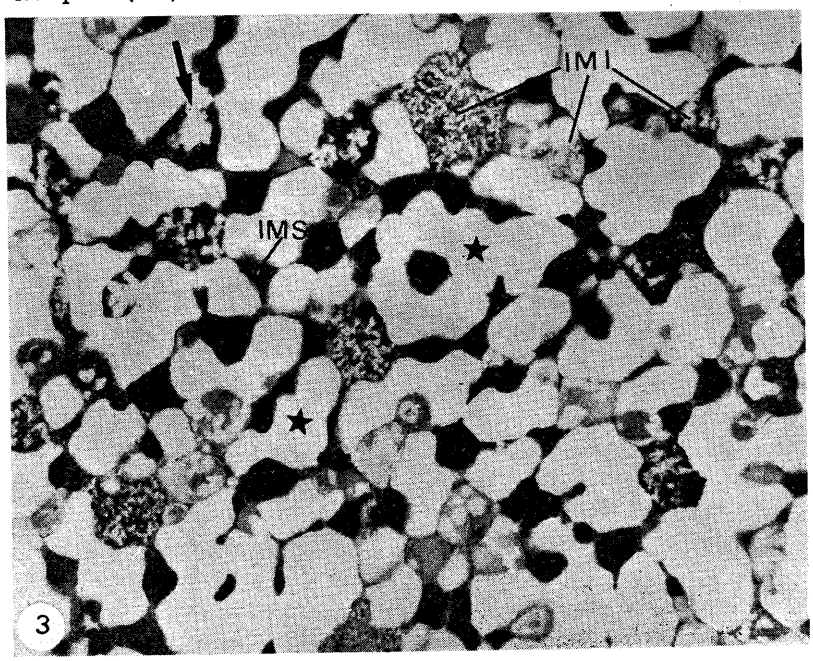

Fig. 3. Mitochondria were cooled at the same rate as those in Fig. 2, except glycerol was added. The intramitochondrial ice spaces (IMI) and the intramitochondrial spaces (IMS) between cristae were clearly shown. The stars indicated the spaces used to be occupied by extramitochondrial ice. ×15,000.

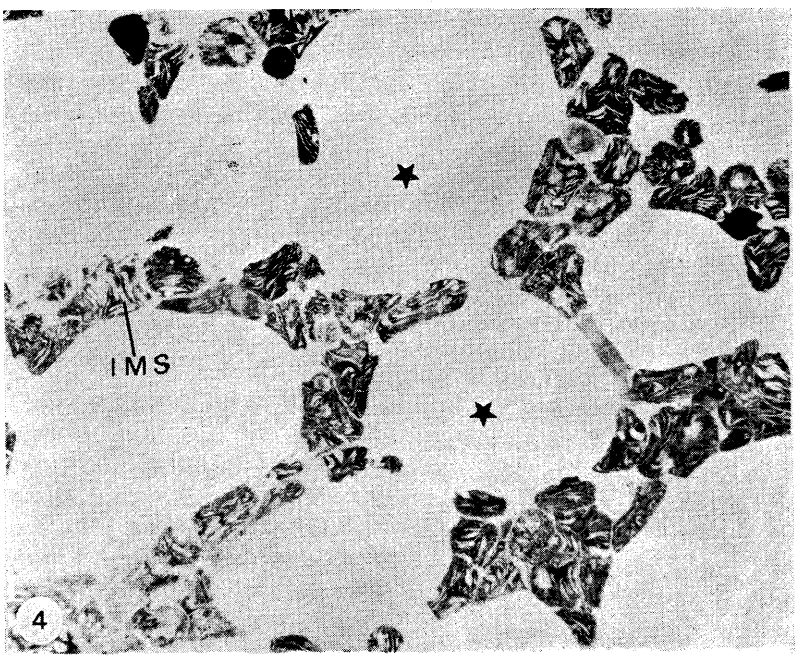

Fig. 4. Mitochondria were frozen slowly at the rate of −3.8°C/min to dry ice temperature (−78°C). The extramitochondrial ice spaces (stars) and the extramitochondrial spaces (IMS) were clearly shown. ×15,000.

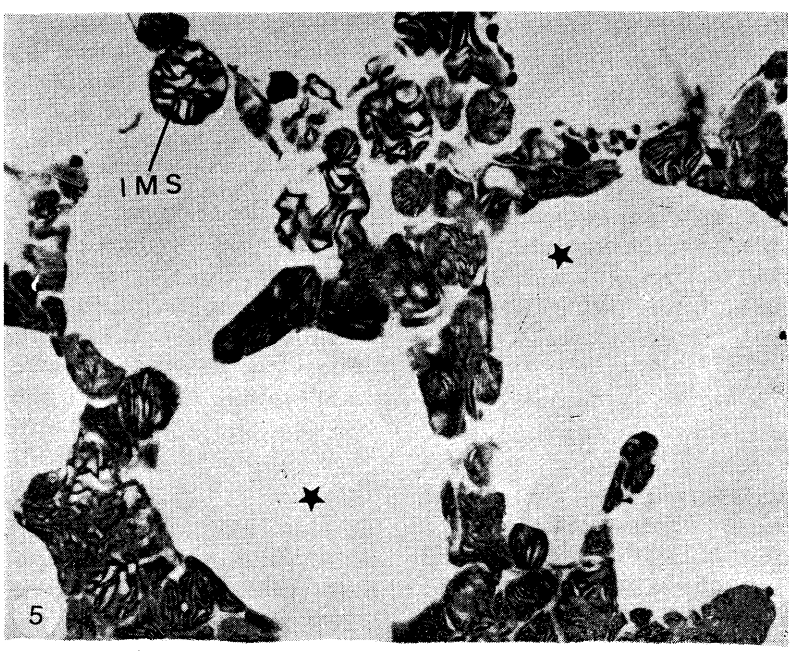

Fig. 5. Mitochondria were frozen at the same condition as in Fig. 4 except that glycerol was added to the samples before freezing. Stars indicated the extramitochondrial ice spaces and the intramitochondrial spaces (IMS) were clearly shown. ×15,000.

cular in shape with evident spaces between cristae similar to those of condensed conformation of control samples. (Fig. 1).

DISCUSSION

The dual effects of freezing on killing and preserving living cells and the protective effects of various substances such as glycerol and dimethyl sulfoxide on freezing cellular organelles [4,5,18], cells[1,2,10,11,14,15,17,19,22,23] tissues[5,7,8,11,20,21] have been interested by many researchers. The causes of freezing injury as explained by the two factors hypothesis[15] were mainly mechanic disruption and salt concentration, both were caused by ice formation. The protective effects of various substances during the freezing processes were discribed as: lowering the eutectic point of freezing; facilitating supercooling; and acting as salt buffer to reducing the extent of extracellular freezing[17]. Freezing injury of isolated mitochondria after rapid freezing without the presence of glycerol, as observed from the frozen state electron micrograph (Fig. 2) should include both mechanic injury due to intramitochondrial ice formation, and the effect of salt concentration, due to both intra and extramitochondrial ice formation. In the presence of glycerol, cryoinjury of 30 to 40% of the mitochondria was due to both mechanic disruption and salt concentration since there were only 30 to 40% of the mitochondria showed both intra and extramitochondrial ice. And rest of the 60 to 70% of the mitochondria should reflect the salt concentration effect alone due to dehydration of extramitochondrial ice formation (Fig. 3).

The drastic change of the pattern and the location of ice formation from without glycerol (Fig. 2) to with glycerol (Fig. 3) suggested that during the freezing process glycerol either altered the permeability of the membrane system[13] or reduced the velocity of crystallization[14] facilitated extramitochondrial ice formation. Thus 60 to 70% of the mitochondria devoid intramitochondrial ice and reduced one factor of freezing injury. The differentiation between the 30 to 40% and 60 to 70% mitochondria by different in location of ice formation under the same freezing rate may also represent the heterogenity and difference in cryosensitivity of the mitochondrial population[4,18].

Cryoinjury of slow freezing mitochondria was mainly by salt concentration which was caused by dehydration of extramitochondrial ice formation since there was no intramitochondrial ice formed (Fig. 4, 5). From the alteration of the size and shape of the mitochondria from both samples without (Fig. 4) and with (Fig. 5) glycerol indicated that the degree of dehydration was greatly reduced at the presence of glycerol. Less injury from salt concentration should be expected from the samples with glycerol.

The intramitochondrial spaces observed in those samples without intramitochondrial ice spaces were occupied presumably by the unfrozen phase of glycerol and solutes. They were the largest in the mitochondria of slow freezing with glycerol (Fig. 5), smaller in the samples of slow freezing without glycerol (Fig. 4) and undistinguishable in the samples of rapid freezing with glycerol (Fig. 3). The variations in size of these intramitochondrial spaces between cristae suggested that the buffering action of glycerol varied according to the rate of freezing might also associate with the final temperature reached.

The after thawing injury of isolated mitochondria of slowly freezing samples should be less severe than that of the rapidly freezing samples as reported[18], because there was no intramitochondrial ice formation which was considered more fatal especially added their growth during thawing than their initial formation[25]. This was also to be true in the situation of rough endoplasmic reticulum as detected at the frozen state and after thawing observation[20,21].

Mitochondria isolated from their natural, *in situ* condition without the protection of cytoplasm, cellular membrane and neighboring cells of the tissue reacted less as complicated as highly organized cells. For it simplicity in structure and small in size should provided an unique system for cryobiological study.

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在不同冷凍速率下甘油對於分離出之粒腺體結冰之影響

劉 國 鈞

這報告是以小白鼠腎器管皮質部分離出來的粒腺體做材料，在使用和不使用冷凍保護劑—甘油處理的情況下，在高速和低速冷凍後，用冷凍取代脫水處理，在電子顯微鏡下觀察，冰粒所留下的究隙，來了解冷凍結冰的情形和粒腺體結構上的變化，用以分析冷凍傷害的原因，及冷凍保護劑的作用。

高速冷凍是分別將經過與未經過甘油處理的樣品 (1 mm^3) 直接浸入液態氨中，平均溫度降低速度是 $-38°C/sec$ 到 $-196°C$ 低速冷凍是將相同樣品放於四周隔溫良好固體二氧化碳上，平均溫度下降是 $-3.8°C/min$ 到 $-78°C$，結果明確顯示出，高速冷凍除了內外結冰造成脫水的傷害外，更因內部結冰造成結構上的機械傷害，所以高速冷凍的傷害要比低速冷凍結冰時，單受脫水的傷害要大，在高速冷凍時使用甘油，可以減少粒腺體內部結冰而低速冷凍時使用甘油，可以減輕因外部結冰造成脫水的狀況，結果並表明粒腺體以其簡單的結構和微小的體積適於用來做一般低溫生物學上的研究。