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GENERATION OF FREE RADICALS DURING COLD INJURY AND REWARMING

Jaisimha Iyengar, M.D., Saswati Samanta, Anna George, M.D., John C. Russell, M.D.,

and Dipak K. Das, Ph.D.

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Surgical Research Center, Department of Surgery

University of Connecticut School of Medicine

Farmington, Connecticut 06032

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Running Head: Free Radicals in Cold Injury

ABSTRACT

Cold injury is often associated with irreversible cell damage. However, to date the pathophysiology of cold injury is not known. The present study examines the mechanism of such injury. New Zealand white rabbits were anesthetized with ketamine and xylazine, and then ventilated using a Harvard ventilator. The femoral artery and vein were exposed. A flow probe was placed around the femoral artery, which in turn connected to a six-channel recorder. A continuous display of electrocardiogram was obtained on Lead II by connecting the limb leads to the same recorder. A thermocouple probe was inserted inside the leg to be cooled. After baseline measurements, one leg was cooled with a freezing mixture up to 0°C, which was followed by rewarming; the other leg served as control. During the experiment, blood samples were withdrawn from the femoral artery for the subsequent analysis of creatine kinase (CK), lactate dehydrogenase (LDH), and malonaldehyde (MDA). At the end, salicylate was injected through the femoral vein to trap any hydroxyl radical (OH·) formed. Rabbits were immediately sacrificed, and biopsies were withdrawn and frozen at liquid N₂ temperature to analyze OH·. Our results indicated that local blood flow in the cold-exposed leg was reduced significantly, suggesting that cold injury was associated with ischemic insult. CK and LDH were increased after cold exposure, then increased further during rewarming. MDA formation followed a similar pattern. OH· generated after cooling increased significantly upon rewarming. These results indicate that rewarming is associated with an episode of ischemia/reperfusion, with simultaneous generation of free radicals which, at least in part, may be responsible for cellular injury associated with rewarming. *Key words: cold injury, free radicals, ischemia/reperfusion, blood flow, electrocardiogram, thermocouple, salicylate, biopsies, liquid nitrogen, creatine kinase, lactate dehydrogenase, malonaldehyde, hydroxyl radical.*

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INTRODUCTION

Cold injury is often associated with irreversible cell damage^{1,2}. However, to date the pathophysiology of cold injury is not known. During prolonged exposure to cold, intracellular water moves to the interstitial space, and the cells become hyperosmolar and then freeze³. In addition, the hemoglobin dissociation curve is shifted to the left, thereby preventing release of O₂ to the tissue⁴. The poor perfusion of capillaries in conjunction with decreased ability to release O₂ may induce severe ischemic insult to the affected tissues despite the fact that hypothermia reduces the rate of cellular metabolism significantly.

During rewarming, with the shifting of the hemoglobin dissociation curve to the original position in concert with reinstitution of perfusion, the tissue is relieved from the ischemic insult. Although the phenomenon has never been explored in conjunction with cold injury, it is quite possible that cold-induced ischemic tissue is subjected to the so-called "reperfusion injury" during the rewarming phase. Current research has pointed to the "reperfusion injury", which presumably amplifies ischemic injury and is clearly distinct from the injury associated with ischemia⁵. A role of free radicals has been indicated in the pathogenesis of reperfusion injury in many tissues⁶⁻⁹.

This study was designed to explore the possibility of the occurrence of reperfusion injury after cold injury. Our results indicated that cold injury was associated with an ischemic episode. Oxygen-derived free radicals were formed during rewarming, resulting in further tissue damage as frequently observed in "reperfusion injury".

MATERIALS AND METHODS

Animal Preparation

New Zealand white rabbits of about 2.5 kg body weight were anesthetized with xylazine (5 mg/kg) and ketamine (30 mg/kg) and were maintained under anesthesia during the entire experiment. An electric clipper was used to remove hair from the hind limb as well as from the front of the neck area. A tracheostomy was performed, and the rabbits were ventilated by a Harvard ventilator (15 ml volume and 50 strokes/min). Femoral arteries and veins of both sides were exposed and dissected free

of tissue. A flow probe (Transonic, Ithaca, NY) was placed around the femoral artery, which in turn was connected to a six-channel simutrace chart recorder (Honeywell Inc., Pleasantville, NY). The femoral vein of the same side was cannulated with an I.V. placement catheter for withdrawal of blood samples. Another such catheter was placed in the femoral artery of the other limb. A continuous display of electrocardiogram (EKG) was obtained on Lead II by connecting the limb leads to the same recorder. The baseline values were established by measuring EKG and blood flow, as well as by estimating the arterio-venous difference of creatine kinase (CK), lactate dehydrogenase (LDH), and malonaldehyde (MDA) from the blood samples. One of the legs was then cooled down to 0°C with a freezing mixture containing ice and salt. Continuous monitoring of the interstitial temperature was achieved by inserting a thermocouple probe (Omega Engineering, Inc., Stamford, CT) into the limb. Tissue temperature was maintained for 20 min at 0°C. The ice was then removed, and the limb was allowed to rewarm to room temperature. During the experiment, blood samples were withdrawn at regular intervals of time from both the vein and artery for the subsequent assay of CK, LDH, and MDA formation. Blood flow was also continuously monitored. At the end of the experiment, salicylate (2 mM) was injected through the femoral artery to trap free radical. The rabbits were immediately sacrificed by an overdose of sodium pentobarbital. Tissue biopsies were withdrawn for assay of OH $\cdot$.

Assay for OH $\cdot$

The method used to trap and quantitate OH $\cdot$ was similar to that described by Grootveld and Halliwell¹⁰. The tissue was homogenized under liquid N₂. The ground tissue was suspended in a buffer containing a mixture of sodium citrate (0.05 M) and sodium acetate (0.03 M) (pH 4.5); 50 μ l of 70% perchloric acid was then added to the mixture. The resultant mixture was degassed and filtered through a Rainin Nylon-66 membrane filter (0.45 μ M). The sample (20 μ l) was injected onto an Altex Ultrasphere 3 μ ODs (75 x 4.6 mm) equipped with a Water Associates HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. The hydroxylated products of salicylic acid were eluted with buffer (degassed and filtered) containing 0.03 M sodium acetate and 0.05 M sodium citrate

(pH 4.5) at a flow rate of 0.8 ml/min. The detector potential was maintained at 0.6 V, employing Ag/AgCl reference electrode.

Measurement of Lipid Peroxidation

Malonaldehyde was measured as an index for lipid peroxidation. Plasma (0.5 ml) was added to 0.5 ml ice-cold perchloric acid (15%) and then treated with 0.75% thiobarbituric acid (TBA) as described previously¹¹. Samples were boiled for 20 min and centrifuged to remove the pellet. The color of the supernatant was read at 535 nm. The concentration of MDA (nmol/ml) was calculated by using a molar extinction coefficient of $156 \text{ mM}^{-1} \text{ cm}^{-1}$.

Assay for CK and LDH

CK and LDH were assayed in plasma samples obtained from the femoral artery, using an assay kit obtained from Sigma Chemical Company (St. Louis, MO) as described elsewhere¹².

Statistical Analysis

All measurements are expressed as mean values $\pm$ SEM. Student's t-test or two-tailed t-test was used for comparison of the data between two groups or within each group for each variable. For multigroup comparisons, analysis of variance was used. For those variable and time points at which the groups were not equal, multiple comparison methods were used to establish all possible pair-wise comparisons to better identify which groups differed from the others. Differences were considered significant when the *p* value was less than 0.05. Each point shown in Figures 1-7 is the mean value of at least six different experiments in each group.

RESULTS

Monitoring of Heart by EKG

The electrocardiographic pattern showed regularity in rate and rhythm of heart during the entire period of cooling the leg from 28°C to 0°C (Fig. 1). Heart rate was maintained at a steady condition even during rewarming after keeping the limb at 0°C for 20 min.

Blood Flow During Cooling and Rewarming

Hemodynamic changes in the femoral artery were continuously monitored with the flow probe. The flow pattern was also recorded during the experiment (Fig. 2). Under normal conditions, the flow rate was about 3.5 ml/min. During cooling of the limb, the flow rate continuously dropped. By the time the interstitial temperature became 0°C, it decreased to about 28% of the normal value to 1 ml/min (Fig. 3). A steady low flow rate was maintained for the rest of the cooling period. When rewarming was initiated by removing the freezing mixture from the leg, the flow rate gradually increased even when the interstitial temperature remained at 0°C. The flow rate continued to rise, and at the end of the rewarming the flow rate was completely restored.

Release of LDH and CK

The release of arterial plasma LDH is plotted against temperature during cooling and rewarming of the rabbit leg (Fig. 4). There was no change in plasma LDH levels during cooling from 30°C to 15°C, but below that temperature there was a slight increase in LDH release. A remarkable increase in plasma LDH (about 2-fold) was noted at the end of rewarming, suggesting that tissue damage occurred mostly during the rewarming period. CK, another marker for tissue necrosis, followed a similar pattern (Fig. 5). After cooling, a slight increase in CK activity was noticed, but the differences were not statistically significant. During rewarming, however, CK increased dramatically. At the end of reperfusion, these values were 2.5-fold higher compared to the baseline levels.

Estimation of Lipid Peroxidation

Malonaldehyde formation, a presumptive marker for lipid peroxidation, remained unchanged during cooling (Fig. 6). During rewarming, MDA formation increased significantly and reached a 1.4-fold higher value compared to control at the end of the rewarming phase. This suggests that lipid peroxidation occurred only during rewarming of the cooled tissue.

Estimation of Free Radical by HPLC

In some experiments, salicylate was injected through the femoral vein to trap any OH $\cdot$ radical which might be generated in the tissue. We assayed the OH $\cdot$ prior to cooling and at the onset of

rewarming, when ice was removed from the leg and blood flow began to rise. We choose this point because oxygen-derived free radicals are known to be produced at the onset of reperfusion of an ischemic organ. The results are shown in Figure 7. The $\text{OH}^\cdot$ signal increased about 3-fold in the cooled tissue (after ice was removed, when blood flow began to rise, as shown in Fig. 3) compared to baseline control values (Fig. 6B compared to 6A).

DISCUSSION

The results of our study demonstrate for the first time that free radicals are formed during rewarming of cooled tissue. The results also suggest that the cooling/rewarming episode may undergo similar pathophysiologic changes as those usually associated with ischemia/reperfusion of tissues. This gives rise to a novel and interesting hypothesis concerning the pathophysiology of cold injury. Our results clearly indicate that cellular injury mediated by cold exposure was amplified during rewarming, suggesting occurrence of a phenomenon similar to that of so-called "reperfusion injury" which is associated with reperfusion of ischemic tissues^{13,14}.

In the present study, we have clearly demonstrated that hypothermic insult resulted in reduction of blood flow, which dropped to 28% of the original value after cooling to 0°C. This value was almost restored after rewarming. We chose to hold the leg at 0°C up to a period of 20 min, because in this model, holding the temperature beyond 20 min resulted in only partial recovery of blood flow during rewarming. This suggests some irreversible injury during prolonged hypothermic insult. Indeed, it is known that poor perfusion in conjunction with anoxia and flow through cold-injured vessels can cause aggregation of red cells, platelets, and polymorphonuclear leukocytes (PMN) leading to thrombosis and tissue infarction, which ultimately may lead to gangrene formation¹⁵.

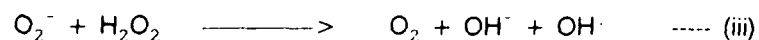
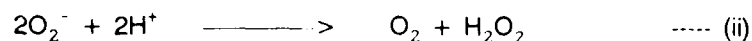
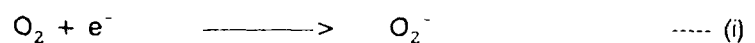
Cold injury resulted in significant damage to the cells as evidenced by the enhanced release of CK and LDH. The levels of CK and LDH were further enhanced during rewarming, suggesting further damage to the cell during this period.

Development of oxidative stress was indicated by the increased amount of MDA formation (presumptive marker for lipid peroxidation) during cooling. There is evidence in the literature regarding

the development of oxidative stress in cold-induced ischemic damage. For example, it is general practice to cool the heart to 4°C and induce cardiac arrest by hypothermia during open-heart surgery. Increased lipid peroxidation and glutathione release from heart has been reported during hypothermic arrest^{6,16}. Oxidative stress has also been cited as a probable cause for the deteriorating function in long-term perfused kidneys¹⁷. In this study, MDA formation was continuously increased in cortex samples as hypothermic storage time lengthened. Studies with liver cells demonstrated that isolated hepatocytes stored as a suspension in ice for 24 hours form more MDA compared to those from freshly isolated cells¹⁸. Our study supports these previous observations and further demonstrates the presence of OH· in cold-injured tissue.

The amount of OH· was further elevated during rewarming, suggesting development of increased oxidative stress. This result correlated with the formation of enhanced MDA during rewarming. Further cellular injury was also observed, as evidenced by increased LDH and CK release from tissues after rewarming. Taken together, these results suggest the development of "rewarming injury" similar to that observed during reperfusion of ischemic tissues. The result of our study further suggests that oxygen-derived free radicals may be responsible for such injury.

However, our results did not demonstrate the mechanism of free radical generation, nor did they indicate any potential source of free radicals in the cold-rewarmed tissue. However, several possible sources may be considered. For example, the depletion of ATP during ischemia would cause generation of hypoxanthine and xanthine, which might react with xanthine oxidase present in tissue to generate oxygen-derived free radicals¹⁹. In addition, PMN may be activated during cold injury and may cause generation of oxy radicals by well-known mechanisms²⁰. Whatever the source of injury may be, our results clearly demonstrate increased formation of OH· in the rewarmed leg. OH· may be formed during the incomplete reduction of molecular oxygen according to the iron-catalyzed Fenton reaction²¹:



The HPLC technique to trap $\text{OH}^\cdot$ (as described in this study) is a unique way to establish the presence of $\text{OH}^\cdot$. When salicylic acid is allowed to react with $\text{OH}^\cdot$, the hydroxylated products formed are extremely stable and can be detected by HPLC¹⁰. In order to demonstrate that the signal was indeed due to the formation of $\text{OH}^\cdot$, we used a specific $\text{OH}^\cdot$ scavenger such as dimethylsulfoxide (DMSO) to scavenge the $\text{OH}^\cdot$ signal. The presence of any $\text{OH}^\cdot$ signal described in this study was always confirmed by its scavenging with DMSO.

In summary, our study demonstrated for the first time the occurrence of "rewarming injury" associated with rewarming of cold tissue. This rewarming injury is similar to that observed in case of reperfusion injury. The increased presence of $\text{OH}^\cdot$ as observed in the cold/rewarmed tissue suggests a role of oxygen-derived free radicals in the "rewarming injury."

Address correspondence to:

Dipak K. Das, Ph.D.

Cardiovascular Division, Department of Surgery

University of Connecticut School of Medicine

Farmington, Connecticut 06032

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FIGURE LEGENDS

- Figure 1. Electrocardiogram of rabbit heart on lead II by connecting the limb leads to simultaneous chart recorder using a chart speed of 25 mm/sec.
- (A) Under normal conditions at room temperature.
- (B) During cooling of the leg at 0°C.
- (C) During rewarming period at 15°C.
-
- Figure 2. Changes in the pattern of blood flow waves in the femoral artery of rabbit leg using an ultrasonic flow probe.
- (A) Under normal conditions at room temperature.
- (B) During cooling of the leg at 0°C.
- (C) During rewarming period at room temperature.
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- Figure 3. Changes in the blood flow rate (ml/min) in the femoral artery of rabbit leg during cooling and rewarming. Blood flow was recorded using an ultrasonic flow probe.
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- Figure 4. Changes in the level of lactate dehydrogenase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.
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- Figure 5. Changes in the level of creatine kinase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.
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- Figure 6. Changes in the level of malonaldehyde in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.

Figure 7. Chromatograms showing generation of OH^- in tissue biopsies obtained from leg. Salicylic acid was injected through the femoral vein, and then biopsies were withdrawn and immediately frozen under liquid nitrogen as described in Methods. Chromatograms were obtained with an HPLC using an electrochemical chemical detector.

- A. Control tissue (pre-cooling).
- B. Cooled tissue at the onset of rewarming.

FIGURE 1

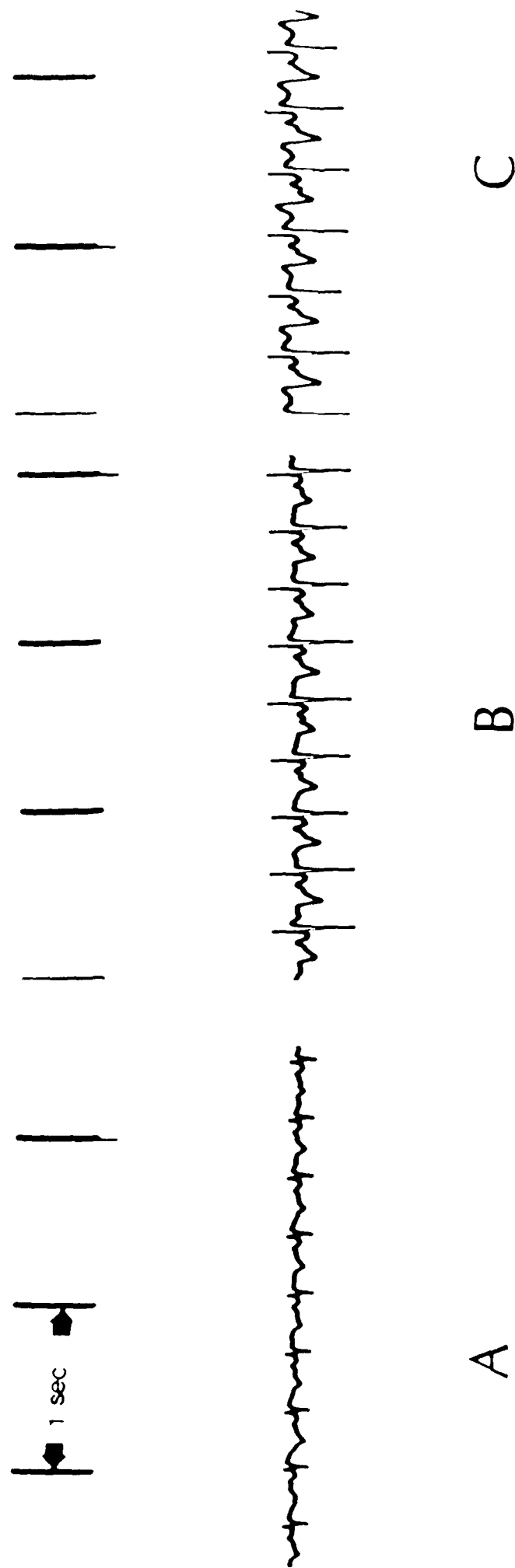
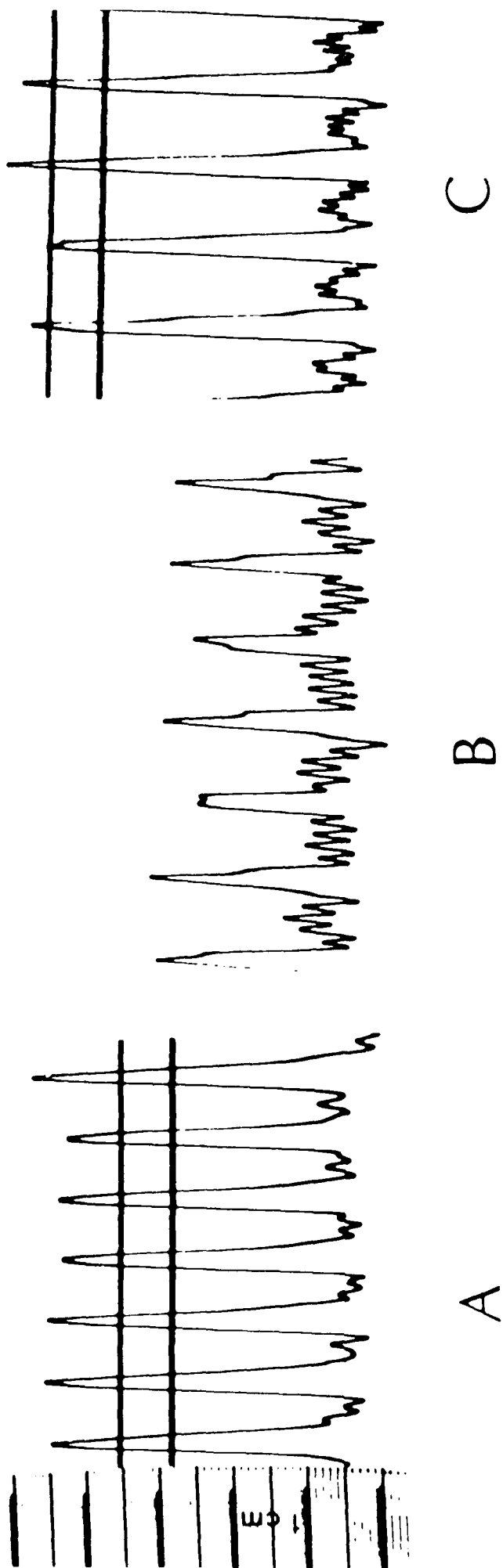


FIGURE 2



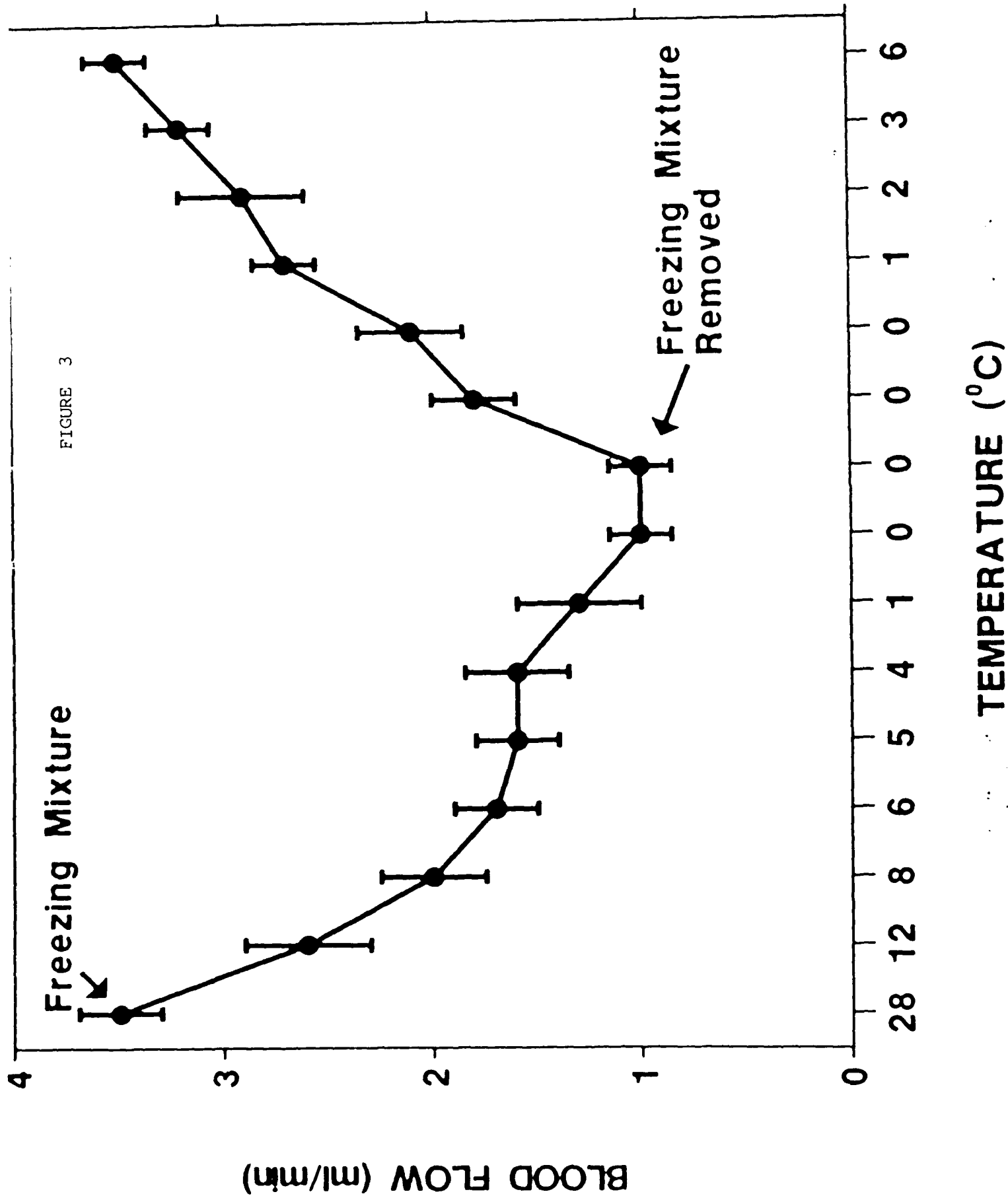


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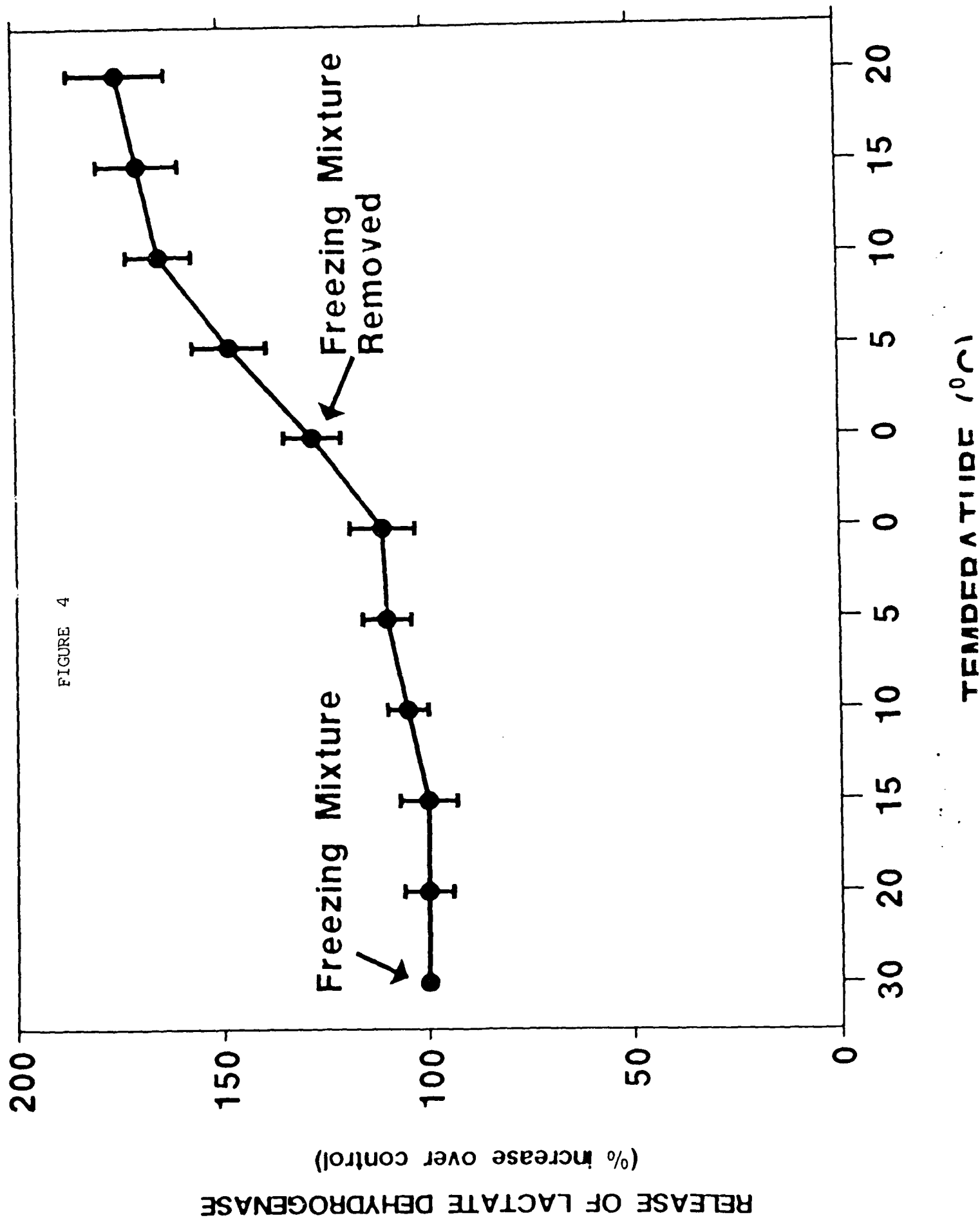
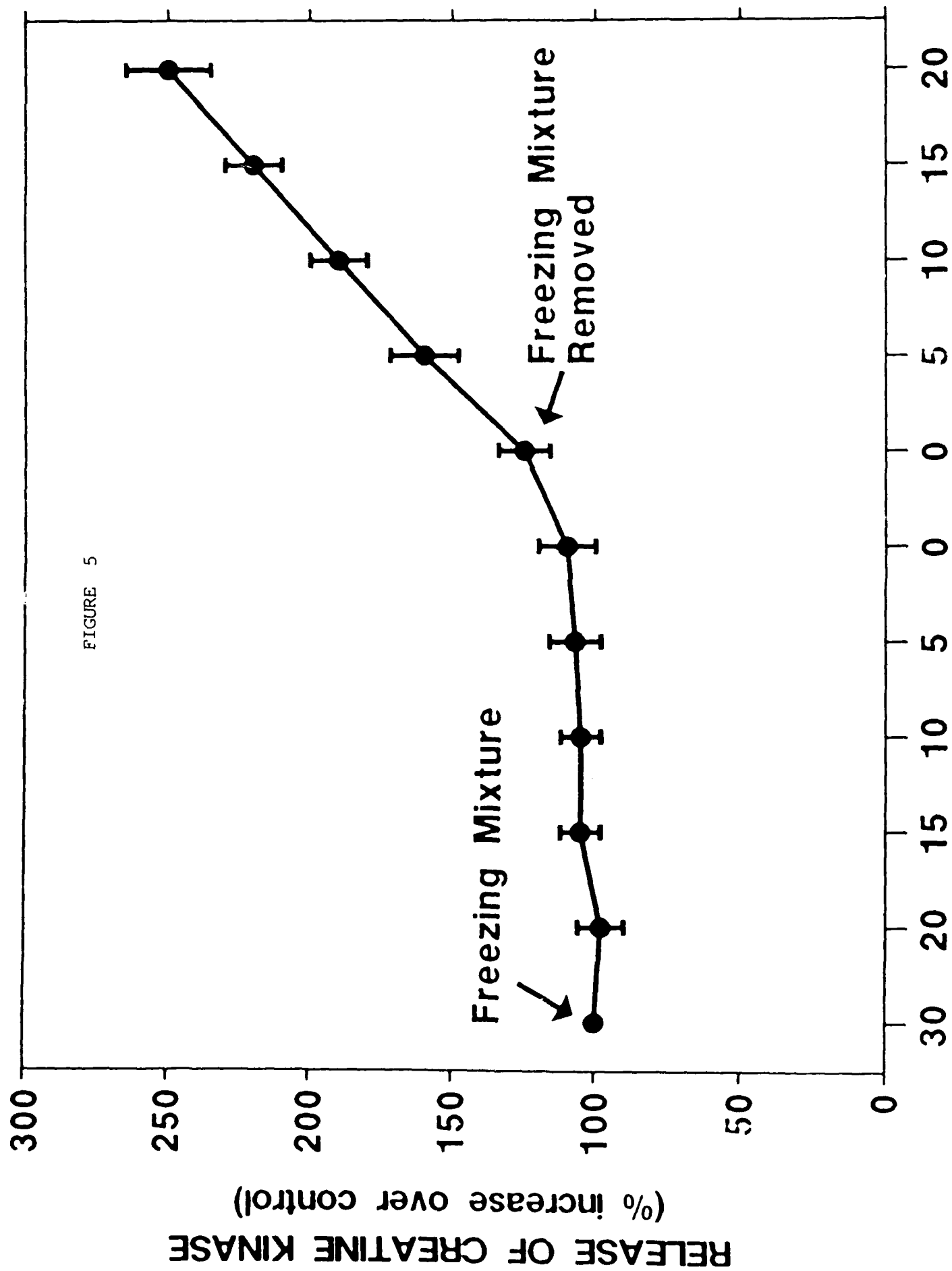


FIGURE 5



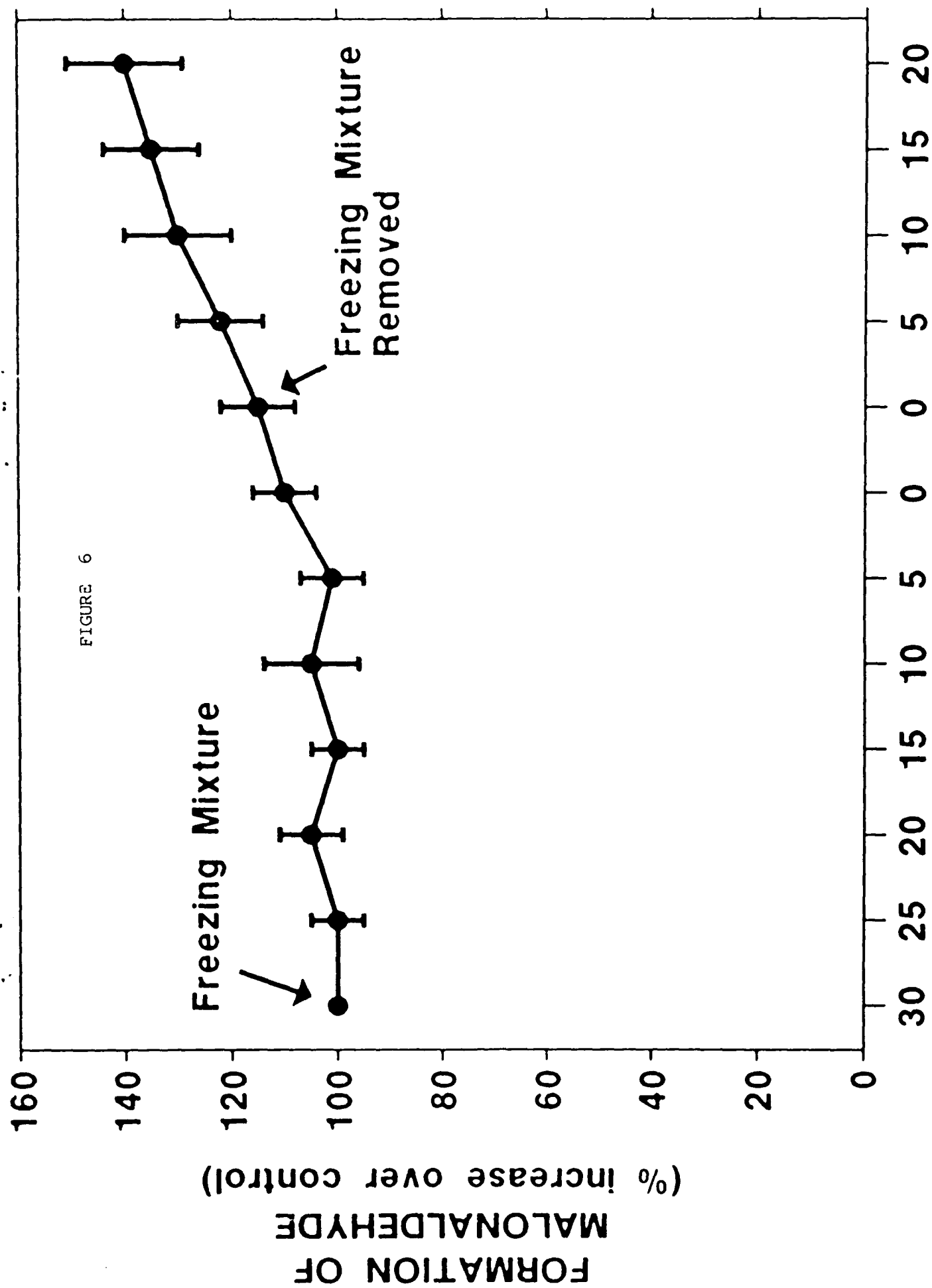
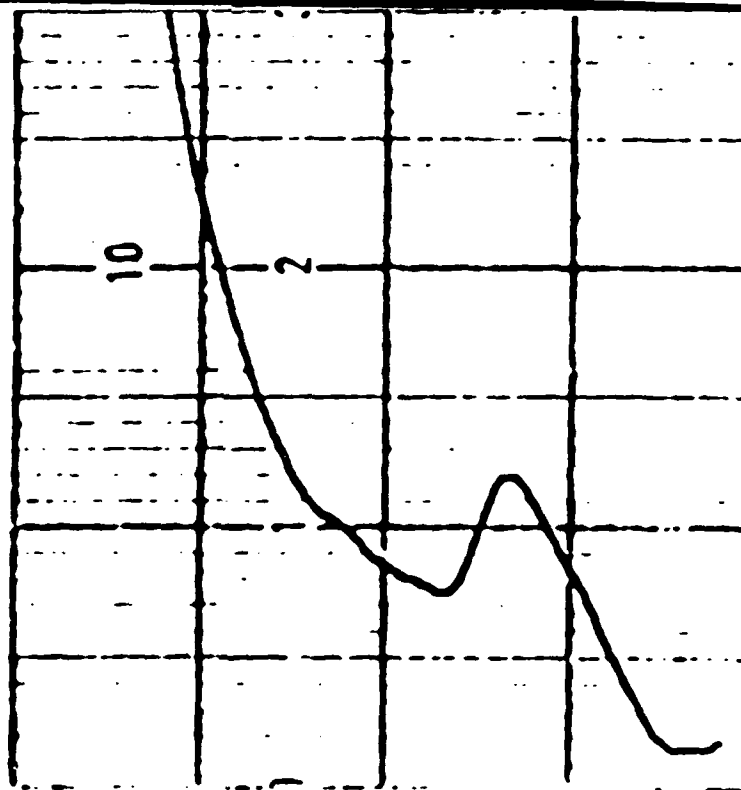
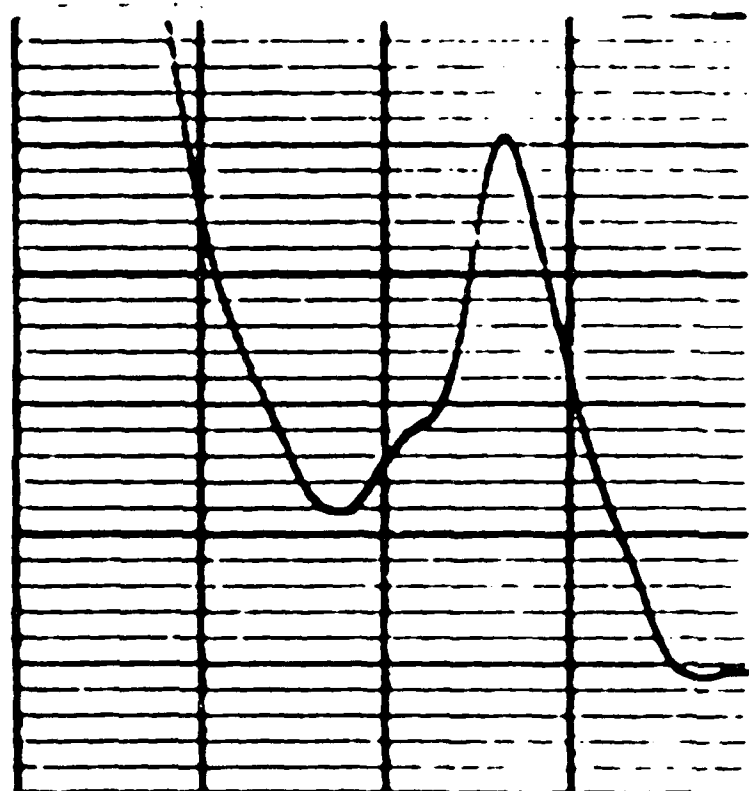


FIGURE 6

FIGURE 7



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B