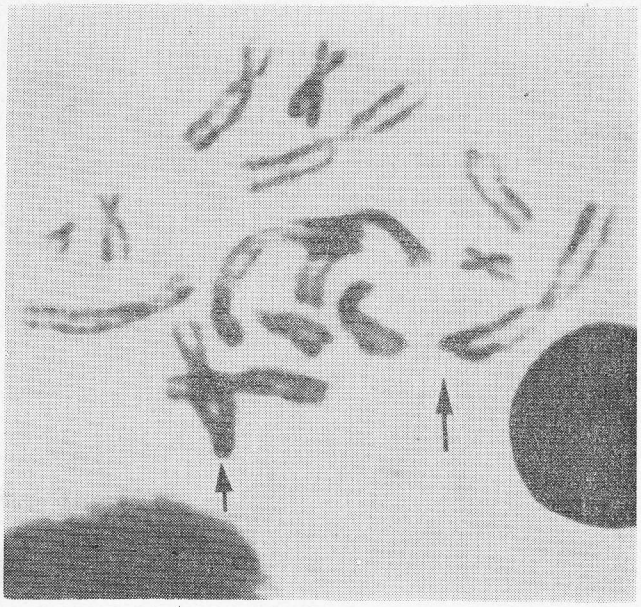

Fig. 1. Stickiness of chromosome arms.

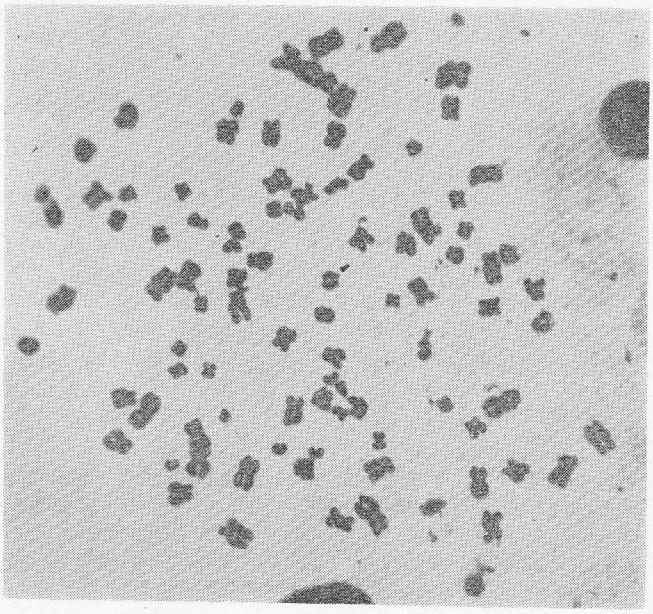

Fig. 2. A hyperploidal cell in a microwave-irradiated culture.

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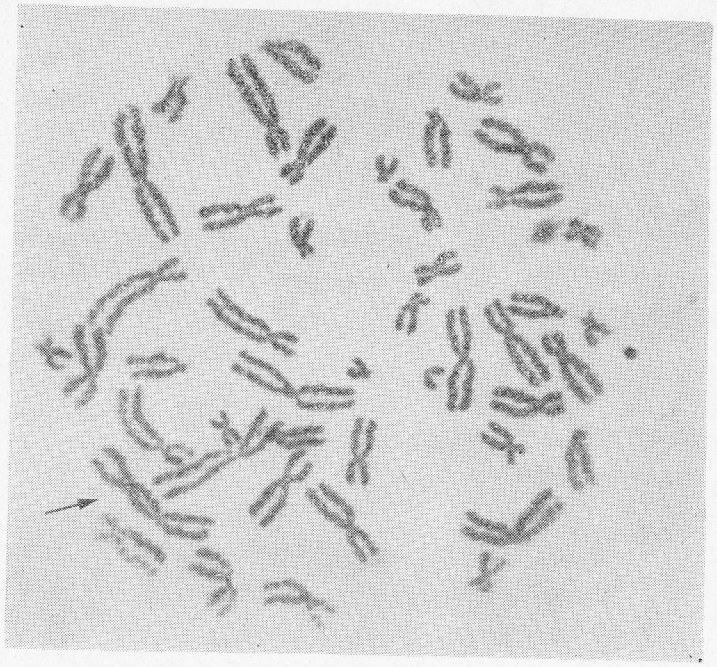

Fig. 3. A dicentric chromosome.

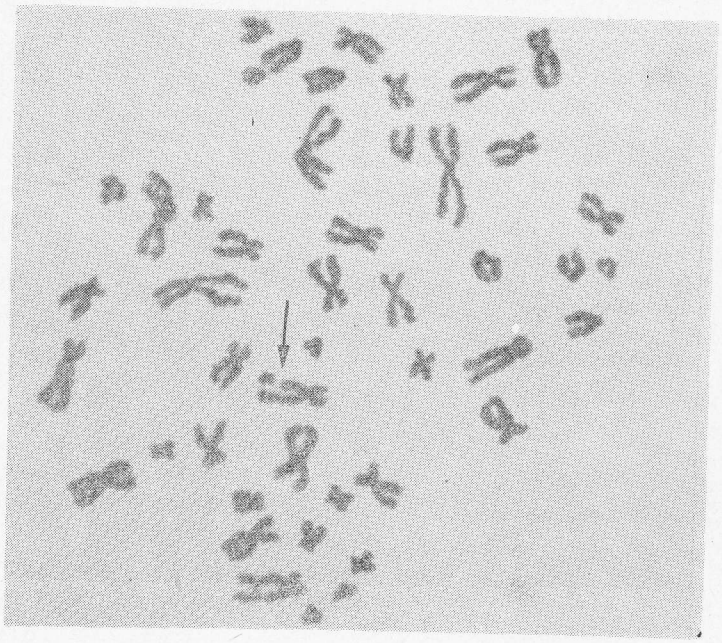

Fig. 4. Chromatid break.

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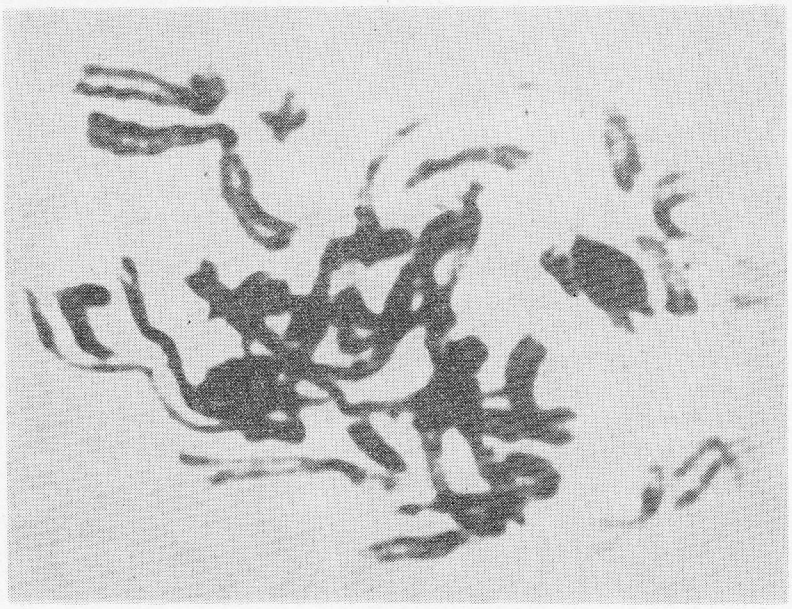

Fig. 5. Changes in chromosome spiralization.

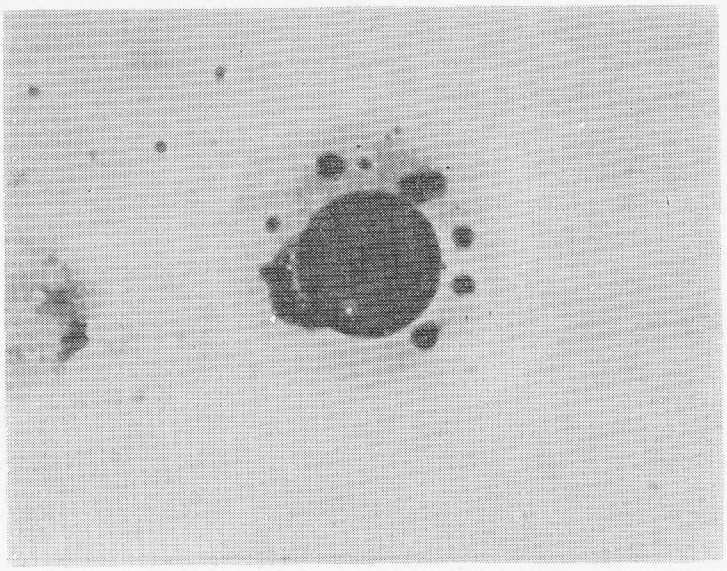

Fig. 6. A macrophage-like cell.

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DISCUSSION

The presented above results suggest that microwaves may have mutagenic effects. Reports on point mutations in *Drosophila melanogaster* (10) strengthen this suspicion. The mechanism of blastic transformation and mitotic stimulation of lymphocytes by microwaves is unclear and should be investigated further. It seems worth mentioning that an increase in absolute lymphocyte counts in peripheral blood and stimulation of lymphocytopoiesis has been observed *in vivo* in animals following long-term, low-dose exposure (2, 3, 6, 7). Microwave-induced mitotic and chromosomal aberrations have been described in other types of cells in tissue culture (6, 10) and in plants.

The results obtained indicate also that microwave exposure may induce changes in interphasic nuclei. Irregular outlines of the nucleus in several instances suggested that fragments may be split off. It should be stressed that as far as we know microwaves are the only physical agent capable of inducing lymphoblastoid transformation (1). This seems to be interesting from a theoretical point of view. It is the author's feeling that this phenomenon could be used in investigations on the mechanism operative in blastic transformation of lymphocytes.

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MICROWAVE THAWING OF CELLS AND ORGANS

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INTRODUCTION

The use of energy in the microwave spectrum provides a method of controlling the rate and uniformity of heating of deep-frozen materials. If human organs are ever to be recovered from temperatures at which storage banks are a realistic possibility, microwave thawing techniques have to be considered. A study of the biologic effects of microwave heating on nucleated cells in the range from —196°C to +35°C is an essential step in the search for methods of viable organ preservation.

High survival rates for several cell types in tissue culture thawed by short wave diathermy (27 MHz) have been reported (8). Microwave and short wave (2450 and 27 MHz) thawing of canine kidneys have been briefly reported (3), but, so far, there have been no reports of the recovery of viability and function of deep-frozen kidneys, by any of the freezing and thawing regimes used.

Thawing of biological materials is conventionally achieved in a water bath. A high degree of success has been obtained with cells of many types and certain multicellular structures. Skin and cornea are preserved clinically in the frozen state (6, 9); in fact, an encouraging number of advances have recently been made in the preservation of other multicellular structures using water bath thawing. The cryobiological methods used have been described in a number of books (6, 9) and an excellent review of freezing phenomena has been given recently by Mazur (5). Whittingham, Leibo and Mazur (11) froze 2—8 cell mouse embryos to —196°C and —269°C at slow cooling rates (0.3 to 20°C/min) and then thawed slowly at rates of 4° to 25°C/min in a water bath, with subsequent success. The cryoprotective agent dimethyl sulfoxide (DMSO), at 1 M concentration, was about twice as effective as an equal concentration of glycerol. These particular compounds penetrate all membranes, reduce the free water content and thereby the damage from high concentrations of intra- and extracellular solutes that would otherwise be present at that temperature. Optimum cooling rates have been established in many cases.

Supercooled adult hearts have resumed beating but attempts to freeze adult mammalian hearts have met with only limited success. A detailed review of the subject has been given by Luyet (4). Other organs have withstood supercooling.

The small, fully differentiated heart also provides a convenient model for studying the effects of microwave thawing. Not only is it possible to determine the effects of the insult at different power levels, the method of controlled thawing may have immediate applications in, for example, the preservation of adult heart valves, the selective preservation of certain cells, improved preservation techniques for embryos in animal husbandry and eventually, help to determine whether or not the kidney,

the organ in greatest transplant need, can be preserved indefinitely. The latter is probably the greatest challenge to preservation engineering today. It is not possible to describe the histologic, microfil, oxygen and para-amino-hippurate studies on frozen-thawed canine kidneys in detail in this paper, but the raw data and photographic information are available.

MICROWAVE HEATING SYSTEM DESIGN

In microwave biology studies, waveguide systems are preferable as the fields are known. Multimode cavities are unsatisfactory in this respect but in thawing applications, where the geometry of the material is often irregular, their use is indicated in many cases.

The two resonant microwave systems were cubic structures, the basic design following that of the conventional microwave oven. The smaller unit, a 10-inch cube, coupled to a 1 kW, 2450 MHz magnetron, was used for thawing tissue culture cells. A larger volume oven was required for thawing organs by our method: a 17-inch cube coupled to a 2 kW magnetron at the same frequency.

Both systems were equipped with rotating turntables on which the samples were placed. In the larger cavity, the Teflon turntable was oscillated as well as rotated, through a Teflon shaft powered from two independent external electric motors. This system is shown in Figure 1. The turntables and drive shafts were made of Teflon, a durable material which does not absorb microwave energy. When 2 ml or 5 ml samples (tissue

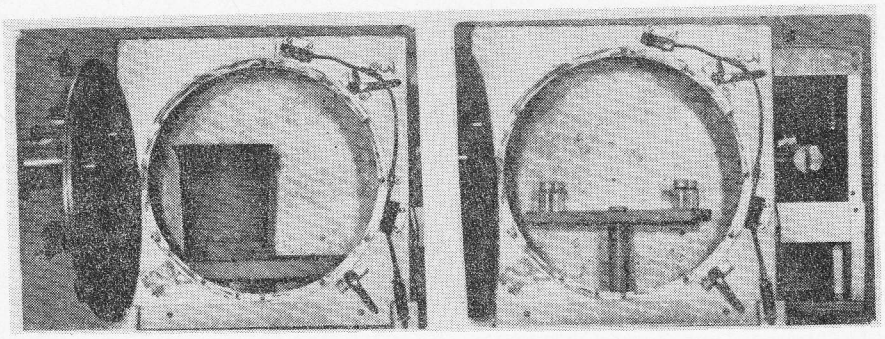

Fig. 1. Microwave thawing system. The two turntables are shown at the top through the open cavity door.

culture cells and fetal hearts in different solutions) were heated *in vitro* a thermal insulating styrofoam cover was placed over the samples. Heating times were determined experimentally in advance; samples that were not recovered in the range 0 to 40°C were rejected. Heating rates were selected to be similar to those obtained in a water bath with 2 ml to 5 ml samples. Perfused, frozen kidneys were immersed in a 1 litre Teflon container filled with fluorocarbon, a material which was also used for the organ perfusate. Organs were frozen at 1°C/min by the method shown in Figure 2.

The 17-inch cubic cavity was used or thawing canine kidneys and fetal hearts, conditions representing very different degrees of loading. For thawing tissue culture cells, we were interested in applying very high field strengths, to determine the effect on their survival. The smaller cavity was used for this; in the absence of a load, corona discharge was detected around the Teflon shaft at 1 kW input power. Rotating two 2 ml samples in the oven, with a 50 ml water load in one corner of the cavity gave rise to a significant

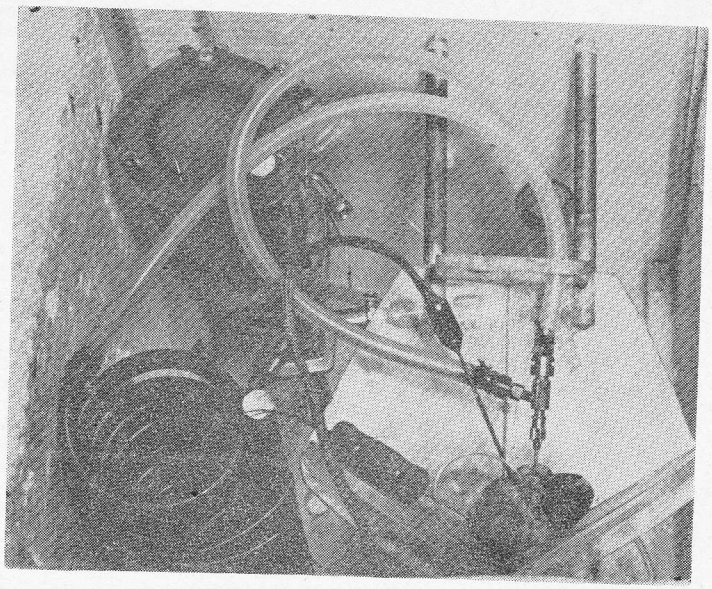

Fig. 2. Inside view of the freezing chamber. Thermocouples control the cooling rate through the liquid nitrogen jet on the perfusate cooling coil shown at the lower left.

preferential heating of these samples, at the same time reducing the reflected power back to the magnetron to about 50%. The electric field strengths in the cavity have not been measured but it is assumed that peak values are in the range 1000 to 10 000 volts/cm.

These systems lack refinement and the approach is entirely experimental. For our initial purposes the systems have worked, providing rapid uniform thawing and some basic data. In the work reported here using cavities we have operated at a mean heating rate which approximately corresponds to that obtained with 2 ml samples thawed in a 35°C water bath. However, the initial and final rates are quite different owing to the changing dielectric properties of the liquids and tissue. The mean absorbed power densities were of the order of 10 W/cm³ in the majority of cases.

MICROWAVE THAWING OF TISSUE CULTURE CELLS

The results have been reported (30) and demonstrate very clearly that no disadvantages to microwave thawing procedures, with a cell system, are apparent if the final temperature is below about 10°C. Thus, the absorption of energy resulted in rapid and uniform thawing. Survival figures as good as, or better than, those realized with standard thawing procedures can be achieved for Chinese hamster cells. Twice the conventional thawing rate is acceptable and it is to be noted that, theoretically, this is relatively independent of sample shape and size. However, this statement must be qualified as the heating rates in different temperature ranges are quite different.

Microwave thawing of fetal mouse hearts

The fully differentiated fetal heart of the mouse can be reimplanted in the ear of an adult syngeneic mouse and studied electrically over a long period of time. Im-

munologically, this is equivalent to transplantation between identical twins in man. Microwave energy (at 2450 MHz) was used as one method of thawing and compared to thawing in a water bath.

Materials and methods

Heart transplant. Hearts were removed from Balb-c fetuses obtained at 16—18 days gestation. The hearts at this time measure approximately 1 mm in diameter and beat rhythmically. Following thawing, the embryonic hearts were implanted directly into the ear of adult syngeneic mice anesthetized by Nembutal. The anterior aspect of the ear was injected subcutaneously with 0.1 ml of saline. The injection causes the two layers of skin to separate and thus form a pocket for the fetal heart. A small incision was made to introduce the fetal heart into the subcutaneous space. Fetal hearts implanted in this manner are nourished by the surrounding tissue fluid until an abundant capillary supply is formed some days after grafting (2).

Freezing. The fetal hearts were placed in prechilled solutions: Eagle's minimal essential medium (MEM) containing Hepes buffer, 10% (v/v) fetal calf serum and 10% (v/v) dimethyl sulfoxide (DMSO) or McCoy's 5a medium containing Hepes buffer, 10% (v/v) fetal calf serum and 10% (v/v) DMSO were successful. The DMSO was added slowly. The freezing rate was maintained between 0.5 and 0.7°C/min, by a thermocouple control system, down to —100°C. The samples were then placed in liquid nitrogen vapor and cooled at 5—10°C/min down to —196°C. They were stored for 72—216 h at —196°C before being rewarmed.

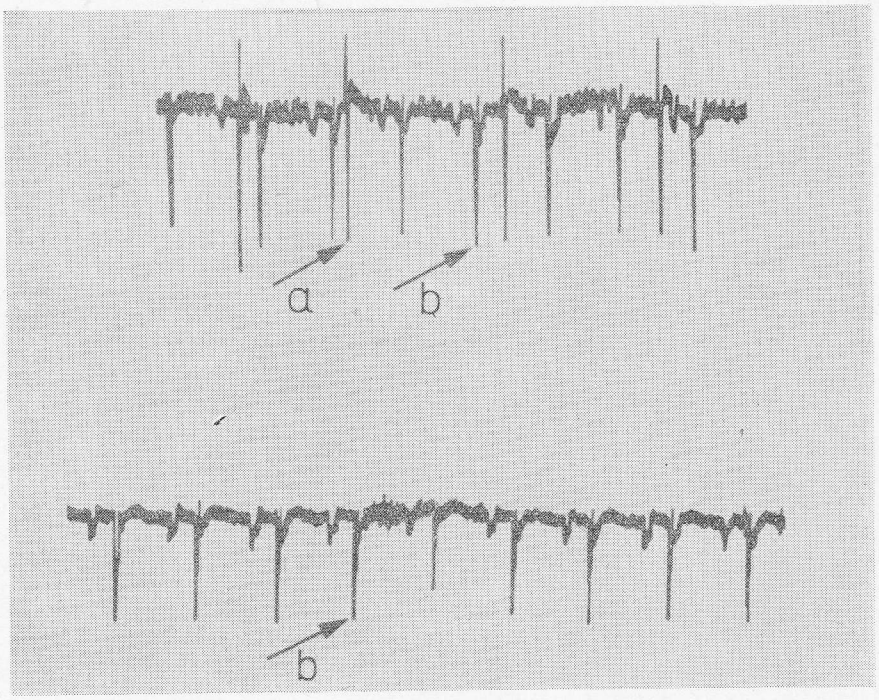

Fig. 3. A typical ECG showing normal mouse complexes (arrow b) at approximately 250 beats/min with the slower superimposed fetal heart rhythm (arrow a).

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Thawing process. Frozen hearts were rewarmed either by placing the 5 ml vials in a 35°C water bath, thereby raising the temperature at a rate of ~ 150°C/min or in the microwave system described. In these experiments, mean heating rates of 200°C/ /min were used to thaw the hearts from —196°C to 20°C ± 10°C.

Assay of graft function. It is not possible to assess the pump action of the graft; however, the electrical property was evaluated by electrocardiography and contractility could be seen with a magnifying glass through the skin of the external ear. The adult heart function (b) and that of the fetal heart (a) can be recorded simultaneously as two distinct sets of rhythmic electrical activities, Figure 3.

Results

As controls, Balb-c mice received direct syngeneic ear transplants of unfrozen fetal hearts. 95% of these control transplants had electrical activity by the 5th day and continued to function for periods in excess of 90 days. 18 out of 30 hearts thawed by microwaves and 14 out of 20 thawed in the water bath showed similar overall biphase electrical activity after 35 days.

From these results, it is concluded (a) that both DMSO and fetal calf serum may encourages the hope that it may be effective with larger tissues where water bath thaw-injury, (b) that microwave thawing is as effective as thawing in a water bath, which encourages the hope that it may be effective with larger tissues where water bath thawing would certainly be ineffective, and (c) that this model can be used, in future work, to assess different cryoprotective agents and freeze-thawing techniques on survival of a multicellular organ which is at the upper limit of size for nutritional survival by diffusion and neo-capillary ingrowth, i.e. the upper limit of size for survival without the need for direct vascular anastomosis at the time of graft implantation.

MICROWAVE THAWING OF KIDNEYS

In attempting to design a complete freeze-thaw system, our objective has been to use the most promising perfusates in a system which allows for the controlled variation of the basic parameters, uniform freezing and thawing rates. If controlled, uniform freezing and thawing cannot be obtained, there seems little purpose in other experiments on whole organ preservation.

The freezing system, shown in Figure 2 is rate controlled by the thermocouples to ± 5°C variation. Fluorocarbon was chosen for subsequent perfusion at temperatures below +4°C as it is biologically and physicochemically acceptable, and will perfuse through the critical tissue freezing range (—15 to —40°C) without requiring abnormally high perfusion pressures. At about —40°C, environmental cooling was then used down to —79°C at the same rate. For thawing, the frozen kidneys were placed individually in a Teflon holder. After thawing, kidneys were perfused with Ringer's lactate containing either 10% DMSO or 10% mannitol initially and then Ringer's lactate by itself.

Results

A group of kidneys which were not frozen but had been permeated with the protective agent, followed by perfusion at 4°C with FC47 and warmed in the microwave system, showed what was judged to be near normal histology and assessed as 'viable' after anastomosis via the femoral artery in the vein of the same dog for a number of hours. The histology of microfil injection indicated a normal kidney.

Some kidneys thawed from —79°C at rates between 100 and 200°C/min showed satisfactory temperature profiles if the perfusion was complete. Overheating of the

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external ureter was seen in several kidneys. A kidney reimplanted in the groin of the same dog after a complete freeze-thaw cycle, was examined after 5 days. Although the external appearance was completely normal, damage to the microcirculation was subsequently apparent. In isolated areas glomeruli and capillaries were well preserved indicating that the blood was being perfused through the organ. Other parts of the kidney showed gross damage, and, in fact, could not be properly injected with microfil. At no time was there evidence that the kidney was capable of forming urine.

Our histological studies, with microfil sections and thawing profiles, have clearly demonstrated the difficulty in assaying damage due to the freeze-thaw insults, in line with the experiences of Abbott (1). So far however we have no definite reason to reject microwave thawing at the organ level. The fact that some nucleated cells and one very small multicellular organ will survive a series of gross insults of this type is encouraging.

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