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BY

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Preservation Studies on Canine Kidneys Recovered from the Deep Frozen State by Microwave Thawing

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Abstract—**Uniform heating of frozen canine kidneys (−79 to 23 ± 10°C) has been achieved using a microwave heating system at 2450 MHz. The kidneys contained the cryoprotective agent dimethyl sulfoxide; uniform cooling was obtained at 1°C/min, with fluorocarbon perfusion down to −40°C. Slow environmental cooling was used from −40 to −79°C, the temperature at which the organs were stored for periods up to several days. After microwave thawing at rates between 2 and 4°C/s, temperature differences were ≤10°C, provided the initial perfusion of the kidney had been complete and uniform. For comparative purposes, these microwave heating rates were also applied to organs immediately after perfusion cooling to +4°C. Microfil injection and histological studies of thawed and reimplanted kidneys have shown some preserved capillary blood vessels, but, as yet, frozen-thawed kidneys have not functioned. Viability tests—oxygen and para-amino-hippurate (PAH) uptake—have been performed on kidney slices recovered from −79 or −196°C by the same method. As yet PAH uptake has not survived kidney slice thawing, although light microscopic structure of these slices is well preserved.**

The work describes a complete system for the uniform deep freezing and thawing of whole organs, a system in which all the variables can be controlled, and from which viability studies can be established.

I. Introduction

THE CLINICAL need for long-term organ storage is apparent from the requirement to match all 4 HL-A antigens of both recipient and donor under circumstances where 26 different HL-A antigens are now known to be present in Caucasoid populations. Thus, for a given donor, a large pool of several hundred possible recipients is necessary to obtain full tissue matching [1]. At present, storage techniques at +4°C limit the time for tissue typing, transportation, and transplantation to about 24–48 h; even this requires elaborate perfusion techniques which have been recently reviewed by Pegg [2]. Without perfusion, 12 h at 0–5°C is the limit [3], [4], and there appears little hope of ever extending the total storage time above 0°C for kidneys or hearts beyond 72 h [5], [6]. Storage for long periods in banks will only be achieved, if at all, in the deep frozen state.

The history of man's attempts to induce latent life was reviewed in 1958 by Keilin [7]. Recovery of nucleated cells from temperatures at which the metabolic rate is sufficiently low to permit theoretically indefinite periods of storage requires, among other factors, the use of cryoprotective agents. The more successful of these, so far, are dimethyl sulfoxide (DMSO) and glycerol. These particular compounds penetrate cell membranes, depress the freezing point, and reduce the water content and thereby the damage from high concentrations of intra- and extracellular solutes that would otherwise be present at that temperature. These and other nontoxic hydrogen bonding agents have been very successful in preserving cells [8], skin [9], and corneas [10], [11]. The protective mechanisms of a great many substances are currently being investigated [12]. In a recent paper, Mazur [13] discussed the difficulty of extending these techniques to large masses of organized tissue containing many different cell types, such as the kidney. The salt concentration tolerated by the most sensitive cell is one determinant; the tolerance of the most sensitive cell to toxic concentrations of the cryoprotective agents is another. Both glycerol and DMSO have toxic levels to a mammalian heart, which increase from 1.0 M at 37°C to 2 or 3 M at 0°C [14].

Complete diffusion of a protective agent also poses problems; certainly 15–20 min is required for diffusion of DMSO from a perfusate through a complete kidney [15], [16]. Even if complete diffusion is achieved, theoretical considerations suggest that different cells require different freezing rates [13], and the same may apply to the thawing rate. Thus there are many unknowns and inevitable compromises, all of which mitigate against the recovery of whole organs from a frozen state.

However, a surprising degree of success has been achieved. Chick heart anlage have been successfully frozen for short periods and recovered from liquid nitrogen using ethylene glycol as a cryoprotective agent [38]. Studies on heart preservation have been reviewed by Luyet [17]; partial resumption of activity in all parts of adult frog hearts has recently been achieved by Rapatz [18]. Mouse embryos, containing from 2–8 cells, have been recovered from −196 and −269°C, with a high percentage subsequently becoming growing fetuses on implantation in pseudopregnant mice [39]; DMSO was found to be more effective than glycerol in cryoprotection. Slow (0.3–2°C/min) cooling rates were used. Although suspensions of kidney tubular epithelial cells have been frozen and thawed with success [19], preservation of the whole kidney has not been achieved.

Beltran and Blumenthal [20] reported experiments on whole rat kidney in 1959. Using 30-vol/vol % glycerol perfusion for 30 min, and freezing in liquid nitrogen, they achieved 70–80-percent histologic preservation when tissue was examined 16 days after subcutaneous slice reimplantation. But normal histologic appearance, by light microscopy, is not *per se* an index of tissue viability and there is a need for detailed viability tests at all levels, the methods for which have been discussed by Abbott [21], [22].

In the unfrozen state (−6°C), 15-vol/vol % DMSO is tolerated by kidneys for 8 h [23]. These authors claim that DMSO is preferable to glycerol and remains the most likely cryoprotective agent for kidneys. However, Pegg [2] has recently thrown doubt on this, as he has shown that the renal microcirculation in rabbit kidneys is better preserved by glycerol than DMSO.

Different perfusion fluids have been used. Manax *et al.*

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[24], in addition to DMSO, used low-molecular-weight dextran. The latter is also cryoprotective but nonpenetrating. These authors deduced that slow thawing (~20°C/min) was a major limitation in sustaining viability after freezing at 1°C/min to −196°C, and, in fact, proposed dielectric thawing to increase the rate. As Meryman points out [24], many different kidney perfusion and freezing systems, liquids, and rates have been used—so far, all without success.

Microwave thawing has been reported briefly by Lehr *et al.* [25]. From the limited dielectic and thermal data available on frozen organs, it seemed to us several years ago that research in this aspect of the preservation technique lagged behind research in freezing methods, and that the use of microwaves with frequencies in the range 500–5000 MHz offered the greatest promise of controlled uniform thawing.

We have verified that microwave thawing is tolerated by hamster tissue culture cells [26], and, very recently, that the electrical activity of deep-frozen whole fetal mouse hearts is retained for up to 90 days after reimplantation in over 50 percent of hearts thawed by microwaves at 200°C/min—a survival comparable to that obtained with water bath thawing. The electrical activity of these hearts was recorded at successive intervals of time after reimplantation in the ear of syngenic mice (unpublished data). Thus we have evidence that the microwave insult (of the order of 10 W/g, with electric field strengths probably reaching peak values of 1000 V/cm), is acceptable to nucleated cells and late-fetal hearts at temperatures below zero.

All these were test systems, and it is our purpose here to describe a complete freeze-thaw test system, suitable for both cells and organs, in which the rates of freezing and thawing can be controlled, while maintaining uniformity of temperature change throughout a sample.

The choice of DMSO in kidney preservation is logical. Fluorocarbon was chosen for subsequent perfusion at temperatures below +4°C, because it is biologically and physicochemically acceptable [27]–[30], and yet can be used as a perfusate through the critical tissue freezing range (−15 to −40°C) without requiring abnormally high perfusion pressures. Because DMSO and intracellular electrolytes are insoluble in fluorocarbon, perfusion with this compound could not remove these substances.

Using fluorocarbon, followed by environmental cooling, it was possible to obtain controlled freezing for whole kidneys and, without fluorocarbon perfusion, for kidney slices.

Our choice of the microwave thawing frequency is arbitrary; equipment is conveniently available in the 2400–2500-MHz range, an ISM band. For reasons to be discussed in Section IV, a lower frequency (1 GHz) might be preferable, but this can be determined only from a system that controls all the variables in the complete cycle. We have tried to establish such a system.

II. Methods

In acute experiments, whole kidneys and kidney slices were frozen, stored in dry ice or liquid nitrogen, thawed, and studied according to methods described in the following sections. In a small number of experiments, kidneys were reimplanted after various manipulations and studied chronically. The kidneys were obtained from healthy mongrel dogs.

A. Freezing

1) Whole Kidneys: A vasodilator (dibenzylene) was injected into the dog prior to nephrectomy. After removal, each

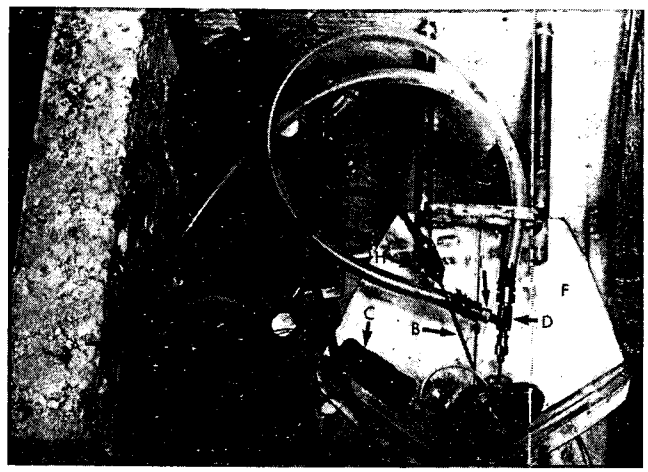

Fig. 1. Inside of the freezing chamber. A liquid nitrogen jet impinges on the cooling coil *A* which carries the perfusate (fluorocarbon) into the organ's artery *D*. The perfusate is collected in the tray *F* from the vein cannula *E* and returned through the bubble-trap *G* to the cooling coil. The liquid nitrogen valve in the lower chamber is controlled by the thermocouples *B*, *C*, and *H*. The pressure is recorded at *I*.

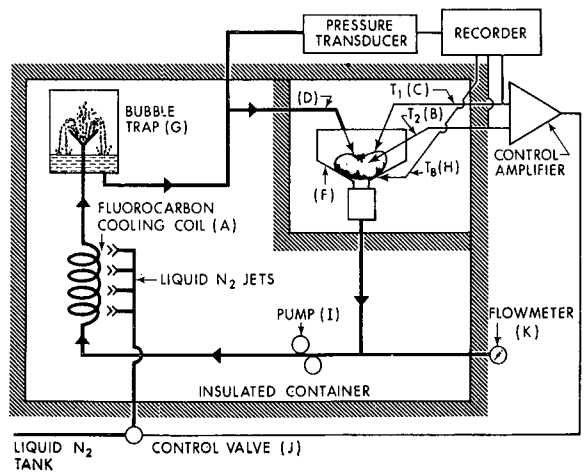

Fig. 2. Line diagram of organ cooling system. Letters (*A*, etc.) are the same as those used in Fig. 1.

kidney was rinsed for 5 min with lactated Ringers solution (Baxter) at 25°C. When whole kidneys were to be frozen, DMSO was added to the Ringers in a two-step perfusion process. First, 10-vol/vol % DMSO in Ringers was perfused for 10 min followed by 20-vol/vol % DMSO in Ringers for a further 10 min. During this period when the kidney was taking up DMSO from perfusates at either 4 or 25°C, the organ was immersed in a bath of lactated Ringers at 4°C.

The kidney was then transferred to the temperature-controlled perfusion freezer shown in Fig. 1. Cooling was achieved by continuous perfusion with fluorocarbon (FC47, 3MCo) cooled by a liquid nitrogen jet. Because DMSO is not soluble in FC47, it would not be removed from the tissue by fluorocarbon perfusion. The controlled cooling system is shown diagrammatically in Fig. 2. Thermocouples placed in the cortex (T_1), ureter (T_2), and the organ-holding chamber (T_B) were connected to a differential amplifier, which operated the control valve on the liquid nitrogen jets. Cooling proceeded only if the temperature differential across the organ, $\Delta T = |T_1 - T_2|$, ($T_B \simeq T_2$), was less than a preselected value; we chose $\Delta T \leq 5$°C and achieved an average cooling rate of approximately 1°C/min from +4 to about −40°C. About −40°C is the lowest temperature at which fluorocarbon would perfuse (10 cm³/min at 200 mmHg). No pressure was main-

tained on the venous side, the effluent draining by gravity into the reservoir. The flow rate at 4°C was 80–100 cm³/min when the pressure was 100 mmHg. The flow rate was adjusted during cooling to ensure that the pressure, monitored just proximal to the needle tied into the artery, never exceeded 200 mmHg. When the flow had effectively stopped, in the range −30 to −40°C, external cooling was used, with ΔT maintained at <5°C. Frozen kidneys were then stored in dry ice (−79°C) for periods of not less than 3 h or, in some cases, several days. The thermocouples were carefully removed before storage, because their presence during microwave exposure would lead to local areas of rapid heating.

2) Kidney Slices: Thin (0.028-in-thick) sections, weighing between 0.1 and 0.3 g, were cut from kidneys which had been rinsed with lactated Ringers solution immediately after removal from a dog. The slices were placed in Cross solution or a culture medium (specified in Table II) containing 10- or 15-vol/vol % DMSO and allowed to incubate for 20 min, with mechanical agitation and bubble oxygenation. Some slices were then transferred to an emulsion of Ringers and fluorocarbon, the emulsifying agent being pluronic F68 (polyethylene oxide glycol, Wyandotte), itself a high-molecular-weight nonpenetrating cryoprotective agent [31], [32].

From each batch of 20 slices, 5 were taken as controls and placed in a Warburg apparatus [33]. The remainder were placed in various solutions (A, B, or C) in 10-ml vials and then cooled to −100°C at rates between 0.5 and 0.7°C/min, before being transferred directly to liquid nitrogen storage. After thawing in a water bath or by microwave heating, these samples were transferred to the Warburg apparatus for 1 h where O_2 and para-amino-hippurate (PAH) uptake was measured.

B. Thawing

1) Whole Kidneys: Frozen organs were placed individually in a Teflon holder and gently clamped into position by Teflon bolts (Fig. 3). The supported organ was then immersed into a glass container filled with fluorocarbon at +4°C. The assembly was then placed on a Teflon table in the microwave heating system, a resonant multimode cavity shown in Fig. 4. After thawing, different kidneys were perfused with Ringers lactate containing either 10-percent DMSO or 10-percent Mannitol, initially, and then Ringers lactate. This perfusion step was calculated to displace the hyperosmolar fluorocarbon from the vascular tree, and remove DMSO from the hyperosmolar interstitial and intracellular fluid by diffusion without subjecting the cells to too great an osmotic gradient. Some experiments were terminated at this point, and the thawed organ was studied for temperature gradients. In experiments where DMSO was removed, Ringers lactate containing 10-percent DMSO was first perfused for 10 min, followed by a 10-min perfusion with Ringers alone. Mannitol, in decreasing concentrations, was also used to remove the DMSO, while at the same time minimizing the osmotic gradients across cell walls.

The transfer time for the liquid nitrogen to the microwave system was typically 2 min; the thawing time (see Section III) was of the order of 1 min. The *total* time that a kidney had been maintained without normal circulation above +4°C was about 1 h before subsequent manipulations (for microfil[1] injection, histology, or reimplantation).

2) Slices: The frozen slices were thawed by placing the glass vials directly into the microwave system [see Fig. 4(c)]. By reducing the input power level and loading the oven with water samples, a technique also used in the study of microwave thawing of tissue culture cells [38], the rates of controlled heating ranged between 0.1 and 10°C/s. The more uniform heating rates achieved with the whole kidneys (2–4°C/s) were later used with the slices.

The microwave cavity has cubic sides of 43.2 cm, dimensions which give rise to a large and fairly even number of modes of resonance over the bandwidth of the magnetron, when the cavity is lightly loaded [34]. The stripline antenna, coupling the transmission line B to the cavity in Fig. 4, is not shown in Fig. 4, but is one of several antennae studied for the

[1] Canton Bio-Med Products, P.O. Box 2017, Boulder, Colo. 80302.

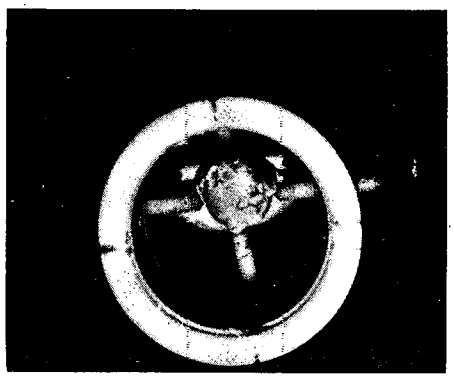

Fig. 3. Teflon holder. Frozen canine kidney clamped lightly in Teflon holder. The holder fits into a beaker of fluorocarbon which is inserted into the microwave oven.

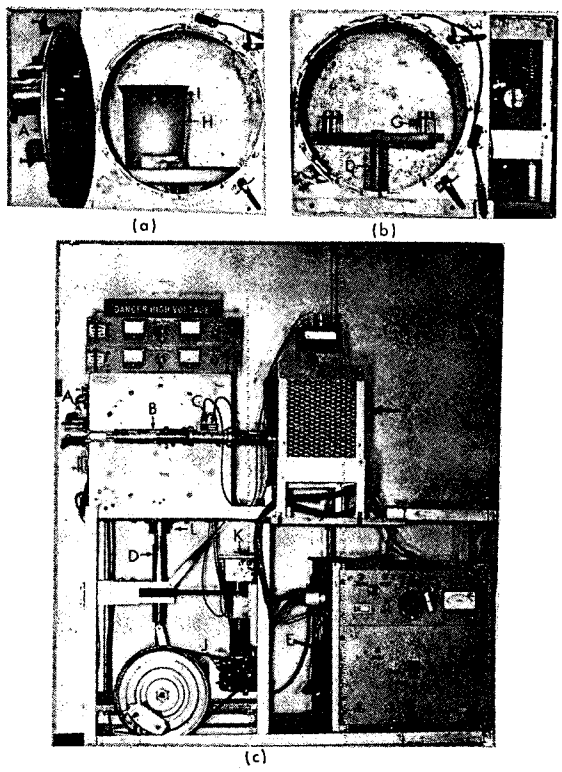

Fig. 4. Microwave thawing system: 1.6 kW at 2450 MHz. *A*—cavity door, with interlocks and tuning posts. *B*—sidewall with antenna and power coupling unit. *C*—power monitor on transmission line. *D*—Teflon shaft for oscillating turntable. *E*—magnetron power supply and control system (Amperex Corp.). *F*—2-kW (Philips) 2450-MHz magnetron encased with protection systems. *G* (inset b)—turntable rack for slice holders. *H* (inset a)—Teflon holder in polyethylene beaker. *I*—mesh facing on inside cavity wall to increase wall loss and enhance coupling. *J*, *K*—variable-speed dc driving shaft through wavetrap *L*, independently adjustable in speed.

work. The design of the antenna used is due to Johnston [35] and gave satisfactory results for a wide range of frozen loads in the cavity, provided these loads are moved through the electromagnetic fields in the cavity in a rapid but random fashion. Fig. 4 shows the method used: the rotating turntable, driven by motor K, is oscillated by motor J through a flywheel and lever. Although the method could be refined further, and an arbitrary predetermined set of path lengths and velocities were used, the method has proved surprisingly effective. (A different method, which might be advantageous if the technique were ever to become a clinical reality, is discussed at the end of the paper.) In the cavity system, the fluorocarbon and Teflon absorb a negligible amount of microwave power. The function of this large mass, we postulate, is to enclose the organ in a quasi-resonant liquid chamber coupled to the microwave oven, the coupling resonant field patterns of which are continuously stirred (or changed) by the movement. On a time average basis, 50 percent of the incident power is reflected back to the magnetron, leaving, in our system, a maximum of 800 W coupled to the cavity. Of this, about 75 percent is transferred to the organ, and the absorbed power density, at the maximum heating rate, is of the order of 10 W/g. The remainder of the power is absorbed in the cavity walls. The actual thawing rate can be changed for whole organs or slice samples by altering the input power, the coupling, or the loading of the cavity, the latter being possible by adding stationary water samples.

Perfect uniformity of heating with whole kidneys has not, however, been achieved as yet, but the variations are probably acceptable in raising the tissue to a final temperature of 20 ± 10°C. In determining heating times empirically, we assume that if all the tissue is above the freezing point and below 36°C, we are within a viable range. Hence an *average* temperature of 20°C allows variations up to ± 15°C with a factor of safety. If any large temperature differences were to occur during thawing, the heating would become unstable.

C. Unfrozen Kidneys

To study the microwave insult and the effects of the cryoprotective agent, some kidneys were not frozen. They were perfused and cooled to +4°C by the method described above and immediately rewarmed in the microwave system.

D. Microfil Injection

This injection technique was used to outline the microcirculation of the kidney. Microfil is a catalyzed silastic rubber, which will pass through the glomeruli and postglomerular capillary circulation into the venous system. The kidney was sliced, after the microfil had set, dehydrated with increasing concentrations of alcohol, and then made transparent with methyl salicylate. Photographic records were taken at a magnification of $\times 40$ to $\times 65$.

E. Histology

In some instances, hematoxylin and eosin preparations were prepared by the usual staining procedures after microfil injection of whole kidneys. In assessing these stained preparations, a 1–4 grading system was used: 1 being normal and 4 being complete loss of normal structure. The glomeruli, tubules, and vessels were graded separately. Arbitrary criteria of damage had to be devised, because the tissue had been subjected to various insults under circumstances which precluded the normal inflammatory response to tissue injury, responses which are normally mediated by biochemical substances and cellular elements in circulating blood. These arbitrary criteria included swelling of cells, disruption of the glomerular capillary wall or the walls of renal tubules, loss of glomerular or tubule cell nuclei, separation of tubules to give wide interstitial spaces, loss of tubule cell cytoplasm, roughening of arterial and arteriolar lining endothelium, and disruption of the elastic membrane in arterial and arteriolar walls. Finally, each slide was assigned a general overall grading: v—viable, pv—possibly viable, pnv—probably not viable, nv—not viable, dnv—definitely not viable. Although the word "viable" is used in this general assessment, its use is also quite arbitrary, because it could not be correlated with experience with reimplantation. However, these criteria were deemed to be the best that could be devised for assessment of tissue damage in our experiments to date.

F. Reimplantation

One kidney was reimplanted for 5 days and then removed for histologic and microfil examination. Other kidneys were reconnected externally to the femoral artery and vein of the same dog, and allowed to perfuse for 3–4 h. Assessment of this tissue, after blood recirculation, was in accord with usual pathologic criteria. As might be expected, after recirculation, evidence of tissue damage was much greater than could be detected by histology of thawed tissue prior to reperfusion with normal blood.

G. Temperature Profiles

Some kidneys, at different stages, were used to study temperature profiles. For all temperature measurements, 25-gauge copper-constantan thermocouples were used with a Leeds–Northrop potentiometer equipped with a standard cell (Model 8693-2). Profiles, as opposed to isolated temperature measurements within an organ, were taken by cutting a kidney in the medial plane and probing beneath the surface of the different area.

H. O_2 and PAH Uptake Tests (Warburg Apparatus) on Kidney Slices

Two biochemical parameters were used to assess viability: oxygen uptake in a Warburg apparatus (with carbon dioxide absorption) and the uptake of I^{131} PAH as judged by slice/medium (s/m) concentration ratios [21], [22], [26] and the end of a period of slice incubation. Respiration is a crude index for intact cell metabolism, since isolated mitachondria can respire [37]; I^{131} PAH uptake, a function of proximal tubule cells, is dependent on complex mechanisms, including the membrane sodium and potassium dependent ATP-ase, and is much more sensitive an index of intact cell viability.

III. Results

A. Whole Kidney Experiments

The first series of experiments were on the relationship of the adequacy of perfusion of whole kidneys to the uniformity of organ temperatures after microwave rewarming.

This question was researched by using the perfusion system, already described: perfusion with a cryophylactic agent (such as DMSO) and then cooling to −40°C with FC47 perfusion, surface freezing to −79°C, and microwave rewarming. Measurement of temperature gradients was then made by thermocouple probing of hemisected kidneys and comparison of these temperature measurements a) with visible evidence of adequate blood removal by prior perfusion, b) with histol-

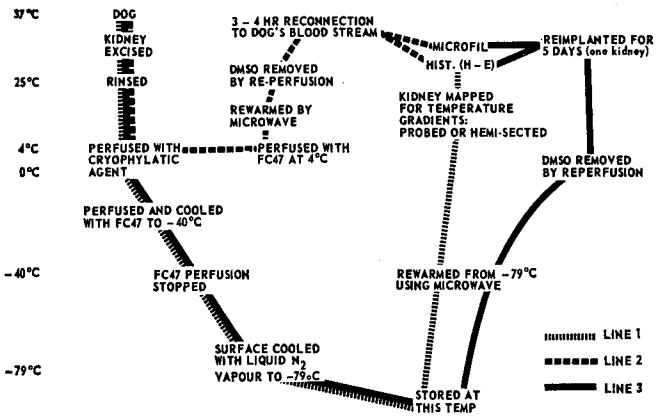

Fig. 5. Line 1—Kidney for temperature map. Line 2—Kidney perfused at 4°C with FC47, then rewarmed by microwave. Line 3—Kidney reimplanted for 5 days.

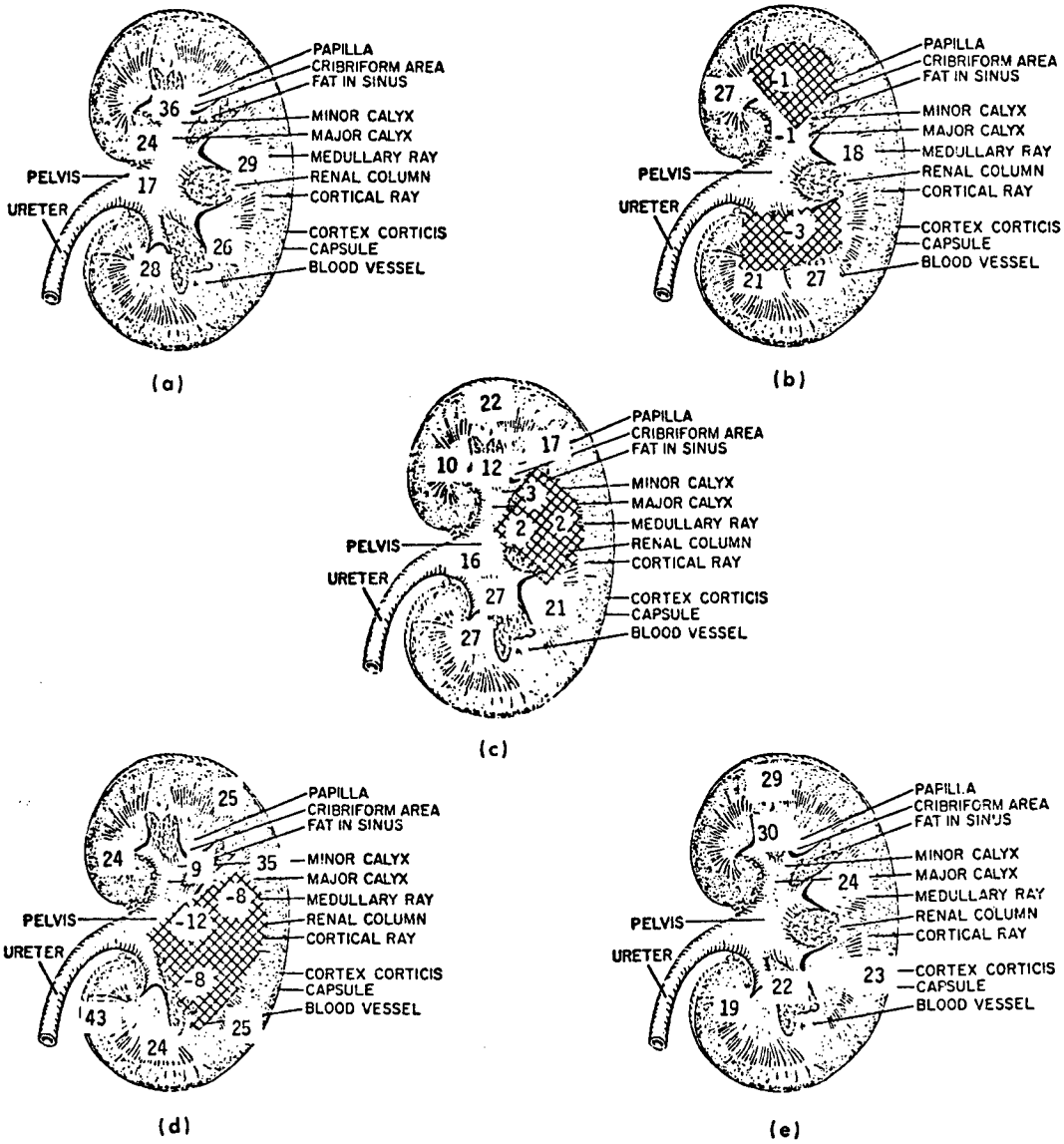

Fig. 6. Temperature profiles (°C) of hemisected kidneys. (a) Well-perfused section of kidney with a ΔT of ± 10°C. (b) Two nonperfused areas (hatched) which resulted in a lower temperature compared to the well-perfused cortex and medulla. (c) and (d) show similar results. (e) Well-perfused kidney section with a ΔT of ± 10°C.

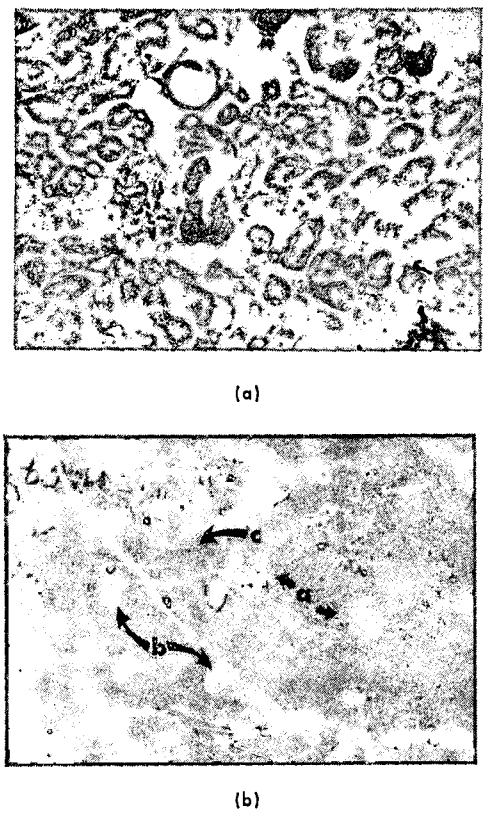

Fig. 7. Kidney frozen and thawed by the protocol indicated in Fig. 5 (line 1) and examined for tissue histology [Fig. 7(a)] and by microfil injection before excision of the microvasculature. (a) Tissue damage assessment: glomeruli 3, tubules 2.5, interstitium loose. General comment: "not viable." (b) There are some well-preserved glomeruli *a*, though others show leakage of injection fluid into Bowman's space *b*. Some well-preserved areas of the peritubular capillary plexus are seen *c*, but not as many as in the normal (see Fig. 8).

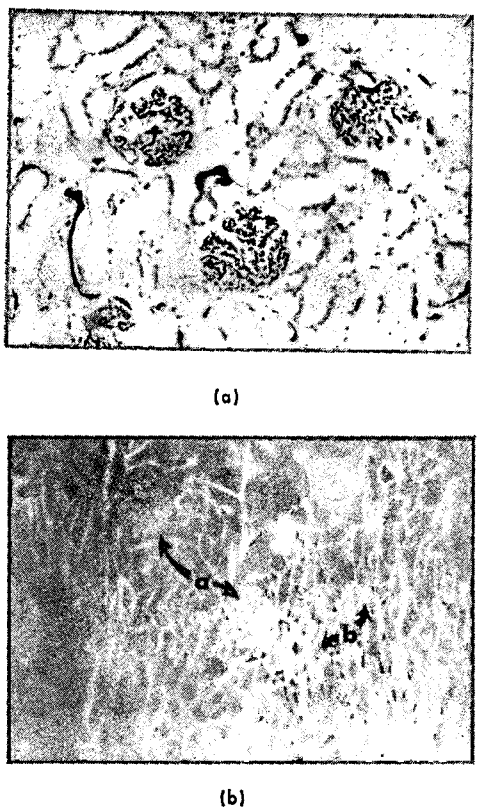

Fig. 8. Unfrozen kidney that has been perfused with cryophylactic agent and fluorocarbon at 4°C and warmed by microwaves to 37°C, and then reimplanted into the dog for 3 h. After excision, microfil was injected and portions examined for histologic structure [Fig. 8(a)] and the microcirculation [Fig. 8(b)]. (a) Stained by hematoxylin and eosin. Interpretation: glomeruli 2, tubules 2, interstitium 1, vessels 1. General comment: "viable" (microfil seen in glomeruli). (b) Some well-preserved glomeruli *a*; areas of well-preserved peritubular capillary plexus *b*.

ogy, and c) with preservation of the microcirculation after microfil injections of intact whole kidneys. The data from the experiments are presented as follows:

1) The experimental procedure is summarized and coded by different lines, diagrammatically, in Fig. 5.
2) Temperature gradients in hemisected kidneys, probed with thermocouples after thawing by microwave exposure, as illustrated for some kidneys in Fig. 6 and tabulated for several kidneys in Table I.
3) Histology by light microscopy of hemotoxylin and eosin stained sections of thawed whole kidneys [Fig. 7(a)] compared with nonfrozen kidneys perfused with FC47 at +4°C and then warmed to 37°C by microwaves [Fig. 8(a)].
4) Microfil injection preparations of thawed whole kidneys [Fig. 6(b)] compared with similar preparations of nonfrozen kidney perfused with FC47 at 4°C and then warmed to 37°C by microwave [Fig. 8(b)], for analysis of preservation of the kidney's microcirculation in glomeruli and the postglomerular peritubular capillary plexus.

1) Analysis of Data: A group of kidneys which were not frozen but had been permeated with a protective agent, followed by perfusion at 4°C with FC47 and warming with microwave, showed what was judged to be near-normal histology and assessed as "viable" after being anastomosed via the femoral artery and vein of the same dog for a number of hours. The histology and microfil injection indicate a normal-looking kidney (Fig. 8). They were not left in the dog long enough for the ultimate test of viability—renal function after kidney reimplantation.

Heating rates of 100–200°C/min have been obtained in thawing fluorocarbon-loaded kidneys from −79°C. In a group of kidneys which were hemisected for the temperature profiles in Table I, it was found that the poorly perfused renal medulla shows a much greater temperature gradient with the cortex than when the medulla was well perfused—except for one area, marked by an asterisk in Table I. The poorly perfused medulla was also colder than the fluid in the renal pelvis when the latter had been previously filled with FC47. In the one example where urine was left in the renal pelvis (assuming that FC47 did not reach this area), it remained frozen, as did the medulla when the cortex had thawed.

In another series, biopsies were taken from whole kidneys removed after 3 h of reimplantation. These kidneys had been processed through the same experimental procedure but not hemisected. Some of these series were also injected with microfil after excision. These biopsies indicated that some tissue integrity has been preserved [Fig. 7(a)]. As seen in Fig. 7(b), microfil injection of the microcirculation shows good preservation of some glomeruli and in some parts of the peritubular capillary plexus, but other areas show damage to the glomeruli with microfil leakage into Bowman's space.

A single kidney reimplanted for 5 days, after freezing: Following the experimental procedure outlined in Fig. 5, a kidney was implanted into the groin of the same dog for 5 days.

TABLE I

TEMPERATURE PROFILE OF HEMISECTED KIDNEYS FOLLOWING THAWING FROM −79°C BY MICROWAVE

(Cases selected demonstrate uniformity obtained when perfusion is complete; see also Fig. 6)[a]

	Temperature (°C) in Various Parts of Kidney				
		Medulla Areas		Pelvic Areas	
Sample No.	Cortex	Well Perfused	Poorly Perfused	Urine in Pelvis	FC47 in Pelvis
13	0	−12		−9	
	2	−10		−14	
	2	−11		−14	
	5	−10		—	
14		26			17
		28			17
	27		−1		17
	27		−1		17
17	25		−8		—
	27	30			—
	23	22			—
15	22	12			16
	17		3		16
	17	21			27
18	36		24*		—
	15		−1		—
	23		7		—
	4		1		

[a] Table I gives data from kidneys shown in Fig. 6. The well-perfused hemisected kidneys [in Fig. 6(a) and 6(e)] have a ΔT of $< \pm 10$°C. The other hemisected kidneys in Fig. 6 show nonperfused (hatched) cold areas as compared to the well-perfused cortex and medulla.

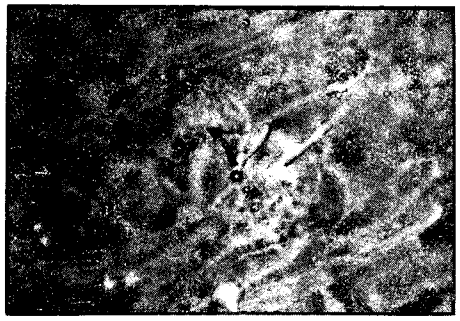

Fig. 9. Microfil injection of 5-day implanted kidney. There is still some vascular integrity in some areas with preserved glomeruli *a* and peritubular capillary plexus. Pattern of the vessels is less uniform and neither glomeruli nor capillaries are as normal as those seen in Figs. 7(b) or 8(b). However, this degree of integrity after a 5-day implantation period indicates that the glomeruli and associated tissue are "viable" but not necessarily functioning.

Upon removal, it was injected with microfil and examined histologically by H and E section and light microscopy.

2) Analysis of Data: The 5-day reimplanted frozen-thawed kidney shows damage to the microcirculation (Fig. 9). Some isolated areas look quite good with the glomeruli and capillary plexus well preserved, indicating that blood was being perfused through the kidney. However, at no time was there evidence that the kidney was capable of forming urine. Also, in other parts of the kidney, the microcirculation was grossly damaged and, in fact, could not be properly injected.

B. Kidney Slice Experiments

Kidney slices were used to analyze further the effects of freeze-thaw injury on renal tissue, in contrast to effects on the microvasculature.

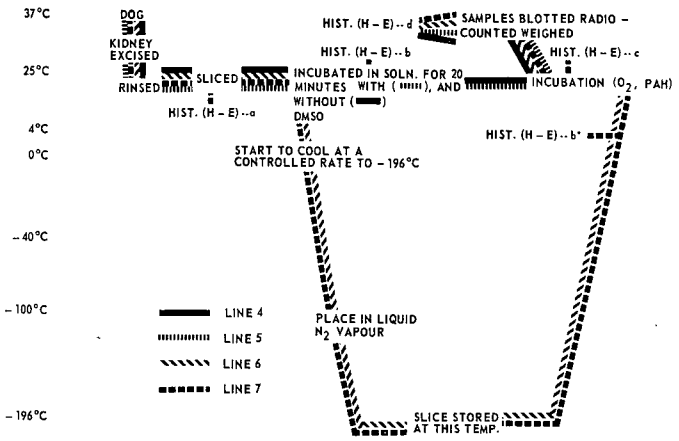

Fig. 10. Line 4—Kidney slice not frozen and incubated in Cross solution. Line 5—Kidney slices not frozen and incubated in protective agent prior to Warburg. Line 6—Kidney slices incubated in protective solution and then frozen and thawed. Line 7—Kidney slices incubated in protective solution and histology samples taken along experimental procedure.

The experimental procedures are shown diagrammatically in Fig. 10. The biochemical results are presented in Table II for both unfrozen and freeze-thawed slices. Certain histologic aspects are tabulated in Table III and illustrated in Fig. 11.

Unfrozen kidney slices: Following the experimental procedure indicated by lines 4 and 5 in Fig. 10, experiments were carried out to compare the effect of Cross solution [33], 10- and 15-percent DMSO in Cross solution, and Culture Medium 199 (Bio-Cult Labs, Glasgow) +15-percent DMSO +10-percent fetal calf serum (fcs) and Hepes buffer. Unfrozen kidney slices were incubated for 20 min at room temperature prior to Warburg incubation for measurement of O_2 and PAH uptake. These experiments were designed to detect any deleterious effect of DMSO at room temperature when added to Cross solution, and to study DMSO and fcs in Culture Medium 199.

Freeze-thawed slices: Using the protocol line 6 in Fig. 10, the uptake of oxygen and I^{131} PAH by frozen-thawed kidney slices was measured in order to compare freezing solutions, concentrations of the protective agents, and thawing by microwave or by water bath. Tissue was also studied by routine hematoxylin and eosin staining. Table II indicates which solution was used for the 20-min slice incubations prior to freezing in 5 ml of the same solution.

1) Analysis of Data of Oxygen Utilization and I^{131} PAH Uptake: Using O_2 and PAH uptake in the Warburg as an index of viability, it would seem from Table II that incubation for 20 min at room temperature in 10- and 15-percent DMSO in Cross solution, prior to the Warburg incubation, has little effect on PAH or O_2 uptake, because there is no statistical difference for groups 2 and 3 compared with group 1. The s/m ratio for PAH uptake is significantly lower for slices preincubated in Culture Medium 199 with 15-percent DMSO, fcs, and Hepes buffer. However, it should be noted that there are very few samples in group 4.

Kidney slices which were frozen in different solutions, and warmed by a water bath or microwave at different heating rates, also had good O_2 uptake in the Warburg following thawing. However, radio-labeled PAH uptake, using s/m as the index of viability, was poor. This poor uptake was independ-

TABLE II

COMPARISON OF O_2 AND s/m IN THE UNFROZEN AND FROZEN SLICES WHEN INCUBATED AND FROZEN IN DIFFERENT SOLUTIONS

Group No.	Incubation Solution Prior to Warburg	No. of Samples	Mean O_2 ±SD mm/mg	Mean s/m ±SD[a]	Comparison of s/m and O_2 Values for Groups 2, 3, and 4 Against Group 1 (Control); p value
Nonfrozen Slices					
1	Cross soln.	8	0.837 ±0.28	—	
		14	—	6.2 ±1.05	
2	10 vol/vol % DMSO in Cross soln.	15	1.01 ±0.47	—	O_2 – ns
		25	—	8.4 ±2.95	s/m PAH: $p<0.05$
3	15 vol/vol % DMSO in Cross soln.	9	1.0 ±0.39	—	O_2 – ns
		12	—	7.1 ±0.89	s/m PAH: $p<0.05$
4	15 vol/vol % DMSO in Culture Medium 199+10% fcs (with Hepes)	3	1.14 ±0.31	—	O_2 – ns
		4	—	4.0 ±1.14	s/m PAH: $p<0.05$
Frozen Slices to −196°C and Warmed with MW or WB					
Group No.	Incubation Soln. Prior to Freezing to −196°C, Stored and Rewarmed with MW or WB and then Incubated in Warburg	No. of Samples	Mean O_2 ±SD mm/mg	Mean s/m ±SD	No. of Samples Warmed by MW or WB
5	10 vol/vol % DMSO in Cross soln.	38	0.87 ±0.32	—	20 WB
		53	—	1.3 ±0.328	33 MW
6	15 vol/vol % DMSO in Cross soln.	12	0.82 ±0.409	—	6 WB
		20	—	1.4 ±0.28	14 MW
7	Mem. +10 vol/vol % DMSO+10% fcs (with Hepes)	14	0.968 ±0.39	—	5 WB
		14	—	1.33 ±0.24	9 MW
8	Mem. +10 vol/vol % DMSO+10 vol/vol % fcs+1% pleuronic+20% FC47	6	1.22 ±0.596	—	3 WB
		6	—	0.92 ±0.083	3 MW
9	Culture Med. 199+15 vol/vol % DSMO+10% fcs (with Hepes)	5	1.09 ±0.48	—	5 MW
		5	—	1.138 ±0.078	

ns: Not significant; MW: Microwave; WB: Water bath; SD: Standard deviation; $p<0.05$: 95-percent probability that the result is significant.
[a] Typical sample(s) values were in the range of a few thousand counts/mg. The background radiation was at least two orders of magnitude smaller than the lowest value measured for a medium (*m*).

TABLE III

RESULTS OF HISTOLOGY SAMPLING AT DIFFERENT POINTS ALONG THE EXPERIMENTAL PROCEDURE AS INDICATED IN FIG. 10 (v—"VIABLE," pv—"PROBABLY VIABLE," nv—"NOT VIABLE," pnv—"PROBABLY NOT VIABLE")

Sample No.	Glom.				Tube				Vessel				Gen. Com.			
	a	b	b*	c	a	b	b*	c	a	b	b*	c	a	b	b*	c
Not frozen																
114	1	1.5		2.5	1	1.5		2.5	1	1		2	v	v		pnv
115	1	1.5		2.5	1	1.5		1.5	1	1		2	v	v		pnv
116	1	2		2.5	1	2		2.5	1	1		2	v	v		pnv
117	1	2		3	1	2		2	1	2		2	v	v		pnv
Frozen-thawed																
118	1.5	2	2.5	2.5	1	2	2	2.5	1	2	2	2	v	pv	pv	nv
119	1.5	2	2.5	2.5	1.5	2	2.5	3	1	2	2	2	v	pv	pv	pnv
120	1	2	2.5	3	1.5	2	2.5	3	1	2	2	3	v	pv	pnv	nv
121	2	1.5	2	3.5	1.5	2	2.5	2.5	2	1	2	3	v	v	pv	nv
122	2.5	1.5	2	3	2	2	2.5	3	1	1	2	2	pv	pv	pv	pnv

ent of the type of freezing solution used and method of thawing. Abbott [21] reported a similar finding in 1969.

2) Analysis of Renal Slice Histology: Table III shows an analysis of slice histology at various times in the experimental handling of both unfrozen and freeze-thawed slices. The unfrozen tissue is normal, histologically, when examined immediately after slicing, as expected (point *a* in Fig. 10, column a in Table III), and after incubation in different solutions for 20 min at 25°C (point *b* in Fig. 10, column b in Table III). However, this tissue was damaged by a subsequent 20-min period of incubation in the Warburg apparatus (point *c* in Fig. 10, column c in Table III) and was judged to be "probably not viable" (using the arbitrary criteria described in Section II).

This deleterious effect of Warburg incubation on histologic appearance is very evident in the frozen-thawed slices (lower part of Table III). Only minor changes occurred with freeze-thawing (comparing histology at point *b* with *b** in Fig. 10, and column b and b* in Table III). Only one slice was judged "probably not viable," with four slices "probably viable." After subsequent incubation in the Warburg, none of these slices was deemed "viable."

Thus histologic examination of thawed slices can be falsely interpreted as normal, and histology after incubation of un-

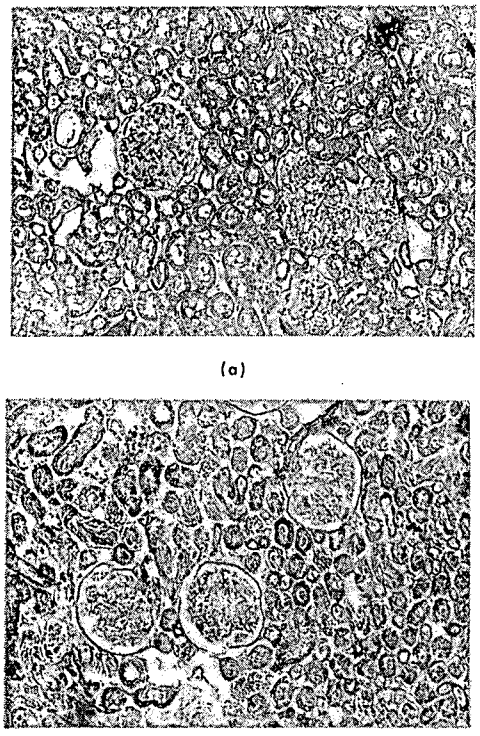

Fig. 11. Deterioration of histologic appearance of freeze-thawed renal slices examined immediately after thawing [Fig. 11(a) represents point *b** in Fig. 10 and column b* in Table III] compared with appearance after 20 min of incubation in a Warburg apparatus after thawing [Fig. 11(b) represents point *c* in Fig. 10 and column c in Table III]. (a) Glomeruli 1, tubules 2.5, interstitium loose. General comment: "probably viable." (b) Glomeruli 3.5, tubules 3.5, interstitium loose. General comment: "definitely not viable."

frozen slices can be falsely interpreted as abnormal. Histology is clearly a very difficult parameter to use in this type of research. The uptake of PAH is still the most sensitive index of tubular cell viability, but these experiments, with freezing, failed to show preservation of viability by this parameter.

The histologic conclusions presented in Table III are illustrated by Fig. 11, where marked deterioration is seen between point *b**, after thawing but prior to incubation, and point *c*, after Warburg incubation.

IV. Discussion

In the search to establish possible methods for preservation of mammalian organs in a deep frozen state and their subsequent recovery, it is concluded that electromagnetic energy in a limited frequency range offers real promise for both the uniformity of thawing and the control of the thawing rate. From a consideration of the penetration depth of the wave, frequencies in the range from 500 to 5000 MHz (5 to 50×10^8 Hz) are of interest. In this paper, a convenient frequency in the middle of this range, 2450 MHz in the ISM band, has been chosen. With this system, it has been possible to thaw kidneys from -197°C to above zero at a heating rate of 100–200°C /min, while still maintaining uniformity to within ±10°C. In the process of testing out the uniformity of thawing, it became apparent that a uniformly thawed kidney resulted only when prefreezing perfusion had been uniform and complete. When the kidney was hemisected, following thawing, it was clearly seen that areas which still had traces of blood, indicating incomplete perfusion, were those that remained frozen. Kidneys which had good perfusion during the initial rinse, during the addition of the protective agent, and during the cooling with FC47 were those which thawed uniformly with a ΔT of less than ±10°C, when measured after thawing.

The difficulty in assaying the damage due to the freeze-thaw insult, as previously pointed out by Abbott [21], is clearly verified in these experiments.

Prolonged reimplantation following the insult was tried on one canine kidney. The result indicated that some vascular integrity was maintained, even though the functional capacity of the kidney did not return. Clearly, much more research is needed before kidneys can be made to survive the freezing insult, but thawing by microwave, after adequate perfusion, may be accepted as a partial solution to one aspect of this highly complex problem.

The kidney slice experiments were designed to study another aspect of the overall problem, namely, protection of renal epithelial cells from freeze-thaw injury. In these experiments, it was decided to assess viability by three parameters: oxygen uptake, uptake of radio-labeled PAH after slice incubation in a Warburg apparatus, and the microscopic state of the tissue by hematoxylin and eosin staining of suitably fixed tissue sections. In the control experiments, unfrozen slices were shown to tolerate 10- and 15-percent DMSO in Cross solution as indicated by O_2 and PAH uptake.

With the frozen-thawed slices, using freezing rates between 0.5 and 1°C/min, thawing rates between 200 and 300°C/min, and different protective agents, O_2 uptake was comparable to normal, but there was no PAH uptake. Experiments showed that microwaves, when used to warm from 4°C up to 30°C, did not affect the PAH uptake capability, and there is no reason to believe the microwaves *per se* would specifically affect this function when used on frozen tissue. Some support for this hypothesis, that microwaves would not cause intrinsic damage to frozen tissue, was also obtained when tissue culture cells were used as a method of assay [26].

A useful conclusion from the work on kidney slices was the definition of the value of routine light microscopic sections, stained by hematoxylin and eosin. It was found that the Warburg incubation, even when PAH uptake was good, caused marked deterioration of the appearance of the tissue when stained after incubation. But it was also found that relatively well-preserved histologic appearance of frozen-thawed tissue (when stained immediately after thawing) was also misleading, because even histologically well-preserved tissue did not take up PAH when subsequently incubated. Reliance cannot be placed on routine light microscopic appearance as indicating preserved viability, although tissue that appears damaged histologically will certainly prove to be "nonviable" by the more sensitive parameters that must also be employed.

So far, we have no reason to reject microwave thawing. Looking ahead, with the obvious need to control thawing parameters for organs of different sizes, the resonant cavity has severe limitations. A particular thawing rate would be more easily achieved in a traveling-wave applicator. Recent experiments performed here by Walker (unpublished data) on the specific heat and microwave absorption of frozen biological solutions and kidney slices in a waveguide indicate that the 0°C point can be detected both optically and electromagnetically. Either method would provide an endpoint, or near-endpoint, control system. Human organs could be rotated in a container held in *L*-band waveguide but not in *S*-band waveguide, if the dominant mode were used. Thus a frequency

around 1 GHz may be more practical than 2.45 GHz. It may also be possible, at either frequency, to heat a stationary organ suspended in the waveguide by the intermittent (pulsed) use of two magnetrons coupled through circulators to each end of the waveguide "applicator." Dielectric and thermal data on frozen tissue can be obtained far more easily in traveling-wave systems.

V. Conclusion

A uniformly frozen canine kidney with controlled freezing rates can be produced with the system used in these experiments. Uniformly and rapidly thawed kidneys were obtained only when initial perfusion had been complete and uniform, but function did not occur in one subjected to prolonged reimplantation.

Unfrozen kidney slices tolerated 10- and 15-percent DMSO. PAH uptake never returned to normal following the freeze-thaw insult, and this remains a barrier to further research with kidney slices. As tissue culture cells tolerated microwave [26], there is reason to suppose that the failure of kidney slice preservation is not due to the use of microwave thawing, but to other unknown problems.

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