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LOW TEMPERATURE PRESERVATION STUDIES ON ORGANIZED MAMMALIAN TISSUES

by

RAYMOND VICTOR RAJOTTE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Low Temperature Preservation Studies on Organized Mammalian Tissues" submitted by Raymond Victor Rajotte in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Electrical Engineering, (Biomedical Engineering).

This text is dedicated to my wife, Gloria,

and three: Brian, Michael and Monique.

ABSTRACT

This thesis studies methods of preserving, in the deep frozen state, canine heart valves, fetal and neonatal mouse hearts and the islets of Langerhans.

A histological examination of adult canine heart valves recovered from storage (-196°C) shows viability with some loss of stromal fibrocytes and reversible damage to the endothelial lining.

A more complex tissue is then considered. Hearts removed from 17 - 19 day fetal and 1 - 9 day neonatal mice are frozen in liquid nitrogen, stored and tested for electrical activity after thawing. Various cryoprotective agents are used. It is shown that dimethyl sulfoxide, ethylene glycol and glycerol are all protective to the fetal hearts provided glycerol is added at 37°C. Controlled microwave thawing at 2450 MHz is comparable to the water bath thawing technique. Diffusion of both protective agent and nutrients is shown to be the limiting factor in the recovery from the deep frozen state of neonatal hearts between 6 - 9 days of age.

The methods developed are used to preserve, *in vitro* and *in vivo*, rat islets of Langerhans. Islets are taken from adult rats, frozen in 7.5 and 10% dimethyl sulfoxide to -196°C, thawed and in some cases cultured for two days prior to perifusion. Culture appears to allow for metabolic recovery, as these groups of cells responded similarily to the control groups. Electron microscopic examination

shows that some damage occurs in the frozen group, but the damage is not irreversible. Islets are harvested from several rats, frozen and stored. After thawing and culture, a transplant is made into a syngeneic rat with chemically induced diabetes. Compared to a control, the transplanted rat returns from a hyperglycemic state to normal. Some of the transplanted islets are recovered from the liver after sacrifice and further assayed.

ACKNOWLEDGEMENTS

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To Dr. George Chmura for his assistance in all experimental procedures.

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INTRODUCTION

INTRODUCTION

"In a living active organism the state of many of its constituents is the result of a dynamic equilibrium between the reactions involved in their constant degradations and regenerations. The organism must constantly provide the energy for the upkeep of its complex structure, which has a tendency to collapse. The stability of such an organism is of a dynamic nature.

During anabiosis no energy can be supplied by the organism for the upkeep of its complex structure which, nevertheless, remains intact . . . . . Only when the structure is damaged or destroyed does the organism pass from the state of anabiosis or latent life to that of death".

D. Keilin, F.R.S. (1)

"It is true that by introducing certain protective agents, e.g. dimethyl sulphoxide, into biological material, freezing damage can be prevented in special circumstances, but the freezing, without lethal damage, of whole organs - let alone of a body in its entirety has not been achieved so far.

It cannot be emphasized too strongly that if injury is to be prevented, the protective action must be carried out before freezing. Once the drastic changes caused by freezing have occurred, repair and recovery are impossible".

An excerpt from a letter entitled "Freezing of a Corpse" by N. Kurti, F.R.S. in the Times of London, Aug 10, 1968 (2).

"Nevertheless these results do provide some grounds for optimism, and when it is remembered that basic knowledge in low temperature biology is increasing all the time, and that this new information is providing ideas which can be applied to organ storage, then it is reasonable to hope that it may not be too long before the transplanter's pipe dream of an "organ bank" becomes a practical possibility".

D.E. Pegg, M.D. (3)

The clinical need for long term organ storage is apparent from the requirements to match all 4HL-A antigens of both recipient and donor under circumstances where at least 26 different HL-A antigens

are now known to be present in Caucasoid populations. In addition to the HL-A antigen it has recently become clear that matching for the LD antigen system may be very important (4) by *in vitro* procedures (the mixed leukocyte culture reaction) which presently requires 6 days. Thus, for a given donor a large pool of several hundred possible recipients is necessary to obtain full tissue matching (5). At present, storage techniques at +4°C limit the time for tissue typing, transportation, and transplantation to about 24 - 48 hr; and even this requires elaborate perfusion techniques which have been reviewed by Pegg (3). Without perfusion, 24 - 48 hr at 0 - 5°C is the limit (6)(7)(8)(9) and there appears little hope of ever extending the total storage time at 0°C for kidneys or hearts beyond 72 hr (10)(11). Storage for long periods in banks will only be achieved, if at all, in the deep frozen state.

Many anomalies exist in cryobiology. Whereas certain insects (e.g. rotifers, nematodes) and most seeds will survive freezing due to dehydration, as well as some types of organized tissue, e.g. the cornea (12) and possibly the ileum (13), with the correct use of cryoprotective agents, the unnucleated human platelets, as well as the single celled granulocyte, have eluded clinically successful preservation in the frozen state.

The history of man's attempts to induce "latent life" has been reviewed in brilliant perspective and detail by Keilin (1). This classic work chronicles all the significant studies on the natural processes of anabiosis, now referred to as cryobiosis, and the laboratory attempts at inducing latent life, from the observation of

Henry Power, M.D., in 1661 (14), and Antony van Leeuwenhoek in 1702 (15), through the intensive investigation period of the 1930's by the late Father Luyet and others (16), who studied the effects of dehydration and vitrification, followed by the work of Parkes (17), Smith (18), Lovelock (19) and others, at the end of the 1940's. In recent decades, detailed reports on the preservation of cells, spermatozoa, cornea, etc., have appeared in the Journal of Cryobiology (1964 - present). In 1966 the state of the art of cryobiology was reviewed in a book by Meryman (20). Subsequent books (21) (2) have described work at the cellular level (22) (9) and at the organ level, as well as current clinical techniques of banking up to 1974.

Recovery of both nucleated and non-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 specific cryoprotective agents at non-toxic levels. The more successful of these are so far dimethyl sulphoxide (DMSO), glycerol and ethylene glycol (EG). These particular compounds will penetrate cell membranes, depress the freezing point, and thereby reduce the damage from high concentrations of intra and extra cellular solutes that would otherwise be present at that temperature. These and other non-toxic hydrogen bonding agents have been very successful in preserving cells (3), skin (23) and corneas (13)(24). Aqueous solutions of glycerol and DMSO become viscous and glassy at low temperatures rather than forming crystaline eutectic. This is because of the H-bonds between the water molecule and the

cryoprotectants. At - 20°C, one mole of glycerol binds 2 moles of water and one mole of DMSO binds 3 moles of water. In a recent paper, Mazur (25) has discussed the difficulty of extending these techniques to large masses of organized tissue containing many different cell types such as the heart and islets of Langerhans. Salt concentration, toxicity of the cryoprotective agent to different cells, freezing and thawing rates are all factors that have to be considered. Both glycerol and DMSO can be toxic to mammalian heart; toxicity occurs at 1.0 M at 37°C but not until 2 or 3 M concentration at 0°C (26).

Unicellular or dispersed cell systems: The erythrocyte has been studied more than any other cell at low temperatures (27)(28)(29)(30)(31). Since 1950, when Smith (32)(33) first showed that small amounts of blood mixed with glycerol could be frozen to -70°C, many processes have been developed to freeze RBC: 1) The Tullis Process, 2) The American Red Cross Process, 3) The Huggens Process (9).

Interest in the preservation of leukocytes has derived largely from the studies involving preservation of bone marrow. Ashwood-Smith (34)(35)(36) and Leibo (37) using bone marrow stem cells investigated cooling and warming rates in the presence of some non-penetrating protective agents. A major problem in the study of leukocytes has been the lack of reliable and acceptable tests of function and viability. Rowe et al. (38)(39)(40) have shown that rabbit and human granulocytes frozen in 10 and 15% DMSO retain their capacity to phagocytize polystryene latex particules. Fabian et al. (41) have been able to get 25% recovery of granulocytes which have

been frozen and thawed; this is not an adequate result. Ashwood-Smith (42) has demonstrated preservation of mouse lymphocytes with DMSO using three parameters of viability: phase contrast microscope, protein synthesizing activity and the ability of the lymphocyte to produce splenomegaly when injected with F1 hybrids. Farrant et al. (43) have used different cooling rates during freezing to separate populations of human peripheral blood lymphocytes.

A fragment of another type of bone marrow cell, the megakaryocyte, is the platelet. There is a great demand for platelet transfusions in patients receiving chemotherapy for leukemia with concomitant thrombocytopenia. Preservation of platelets has posed a formidable problem due to membrane fragility. Reviews of the problems in preserving blood platelets have been given by Morrison (44), Strumia (45) and Chanutin (46). Problems encountered include the choice of a suitable container, the appropriate anticoagulants, and the best technique for separation and prefreeze storage of platelets. These difficulties are compounded by the complex function of the platelet. It has been shown by Murphy et al. (47) that preservation at 22°C gives longer survival than at 4°C; this is a significant departure from the standard thinking,that cooling favors maintenance of cell viability. The difficulty in preservation of fresh platelets suggests that freezing may be the only means to achieve long term preservation of this clinically important blood component. Several investigators (48)(49)(50)(51)(52)(53)(54)(55) have shown that glycerol gave 25% recovery of human platelets. DMSO has also been used,but better recovery has been obtained with 5% DMSO + 5% dextrose (56)(57)

(58) on rat platelets than with DMSO alone. Clearly much remains to be discovered about the metabolism of platelets before full preservation of platelet viability may be expected.

In multicellular systems, complete diffusion of a protective agent poses special problems in multicellular systems. Certainly 15 - 20 min is required for diffusion of DMSO from a perfusate through a complete kidney or heart (59)(60). Even if complete diffusion is achieved, theoretical considerations suggest that different cells require different freezing rates (25), and the same may apply to the thawing rate. Thus, there are many unknowns all of which mitigate against the recovery of whole organs from a frozen state. However, a degree of success has already surpassed the expectations of Kurti (2). Certain multicellular structures have already been successfully preserved after freezing. Thus, chick embryo heart anlage has survived freezing for short periods in liquid nitrogen (61) using ethylene glycol (EG) as a cryoprotective agent. Whittingham et al. (62) have frozen 2 - 8 cell whole mouse embryos to -196°C and -269°C at slow cooling rates (0.3° to 20°C/min) and then thawed them slowly at rates of 4° to 25°C/min, with subsequent development of 50 - 70% of the embryos into blastocyst on culture. When these were placed in pseudo-pregnant mothers, 65% became implanted as pregnancies and went to term. Dimethylsulfoxide (DMSO), at 1 M concentration, was about twice as effective as an equal concentration of glycerol. Frozen embryos are known to be crossing the world privately for animal husbandry. There is an optimum cooling rate and a probable need for slow as well as controlled thawing rates in the studies reported (62).

Supercooled adult hearts have resumed beating (63)(64)(65) but prior to the work of Offerijns and Krijnen in 1972 (66) and Rapatz in 1970 (67), attempts to freeze adult mammalian hearts had met with little success. A detailed review of the subject has been given by Luyet (68) who has tabulated the historical developments of this aspect of heart research. Rapatz (67) obtained partial resumption of activity in all parts of adult frog hearts after freezing to temperatures "below -55°C", using ethlyene glycol as a cryoprotective agent. Offerijns and Krijnen (66) added DMSO to the perfusates of isolated adult rat hearts. With supercooling to temperatures between -18°C to -30°C, young rat hearts (10 - 16 days old) recovered, but older hearts did not. Canine kidneys frozen to -196°C and micro-wave thawed have also shown some vascular integrity following re-implantation (69).

In the last decade there has been considerable work done in the fresh transplantation of isolated islets of Langerhans for the treatment of diabetes mellitus. Since 1964 when Hellerstrom (70) first microdissected islets from the mammalian pancreas, harvesting sufficient numbers of islets for transplant, has been the major problem. In 1965 Moskatewski provided the basis for current harvesting techniques, by using enzymatic digestion with collagenase to separate the acinar tissue from the intact islets (71). Since then Lacy and Kostiovavsky have improved the technique by mechanically disrupting the acinar tissue by injecting a balanced salt solution via common bile duct and, following collagenase digestion, to separate the islet

from the acinar tissue by centrifugation in a discontinuous density gradient (72).

In the last 10 years much experience has been gained in the site of transplantation of the isolated islet. Initially the islets were injected intramuscularly or interperitonealy but of late the most efficient site for transplanting the islet has been in the liver via the portal vein (73). These islets lodge in the terminal portal venule where they become embedded and revascularized. With the above technique hyperglycemic rats have been returned to normal glycemia as measured by weight gain, blood sugar, urine volume and urine glucose. One of the major problems in the harvesting of islets is getting a large enough number for transplantation. To date it takes 3 - 4 donor rats to cure one diabetic rat. If this technique of harvesting islets is ever to be used clinically, low temperature banking will have to be established. Once the islets are frozen, there exists the problem of being able to thaw the samples. Some of the methods that could be used, are water bath thawing or microwave. Microwave thawing has been reported briefly by Lehr et al. (74). From the limited dielectric and thermal data available on frozen organs, it would appear that research in this aspect of the preservation technique lagged behind research in freezing methods, and that the use of microwaves with the frequencies in the range of 500 - 5000MHz offered the greatest promise of controlled uniform thawing. It has been verified that microwave thawing is tolerated by hamster tissue culture cells (75). The microwave insult (of the order of 10W/g, with electric field strengths probably reaching peak values of 30,000v/cm),

is acceptable to nucleated cells at temperatures below zero. A system such as microwave will definitely have to be used when trying to thaw large organs from sub-zero temperature.

The following chapters describe and illustrate the techniques and models used in assessing viability after freezing and thawing of dispersed cell systems or solid organs. Different multicellular organized tissues such as the fetal and neonatal heart, heart valves and the islets of Langerhans were used to try and extend the range of organized tissue that can be preserved. Heart valve tissue, due to its simplicity, was looked at first. This tissue is similar to the cornea which is now routinely preserved at sub-zero temperatures. Going to a more complex organized tissue, the fetal and neonatal hearts were used as a model to test microwave and water bath thawing and to test different protective agents on cardiac tissue. The third and most complex of the organized tissue investigated was the adult islets of Langerhans. This is a small organ that performs a very complex function; namely that of supplying insulin to the body thereby regulating the blood glucose level.

CHAPTER II

CANINE HEART VALVES

INTRODUCTION

In the hope of extending the range of organized tissue that could be cryopreserved, it was decided that canine heart valves would be a suitable tissue to freeze and thaw. Even though valvular tissue is very simple, it is still an organized tissue composed of endothelial lining (1 to 2 cells thick) which covers the adjacent layers of collagen. The two layers of collagen sandwich the layer of connective tissue (Figure 1).

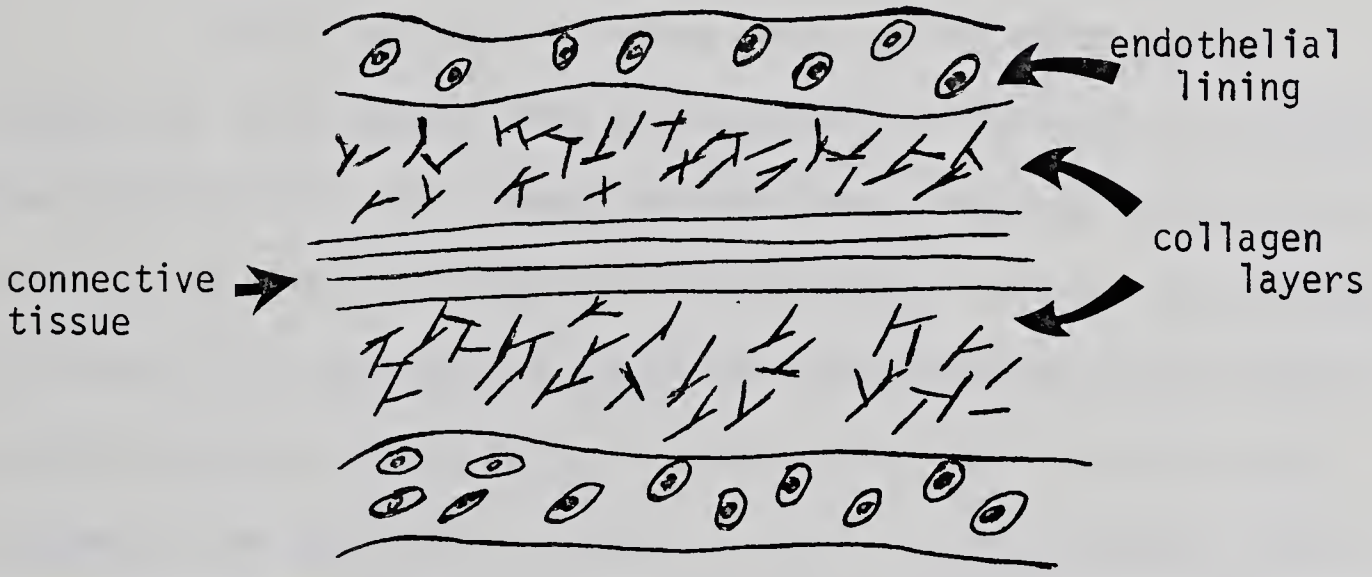

Figure 1. *Cross section of heart valve.*

Heart valve tissue is similar to cornea, a tissue which has been cryopreserved for many years and is in clinical use (76)(77). Mermet et al. (78)(79) in a brief report (1971) claimed successful implantation of adult canine valves. There is no evidence that DMSO, at 10% (v/v), is damaging to fetal heart tissue (80). In a recent book (81)

some writers have claimed success with banking human heart valves, whereas others have stated that insufficient data exists to justify use of the method at present. It is, in fact, difficult to know if a transplanted valve is alive or not, as even non-viable valves, due to their high content of collagen fibers, will have valve-like function for quite long periods of time.

Prosthetic valves are undoubtedly of greater clinical utility, but it was felt that there was still sufficient justification to pursue the concept of preserved valves in higher mammals if only to study the tissue damage.

METHOD

Mitral and aortic valves were removed under sterile conditions from mongrel dogs anaesthetized with dibutal (0.5cc/kg). The pleural cavity was opened and the hearts removed from the pericardium and placed in sterile chilled saline. The heart was opened and the valve leaflets (or cusps) were dissected away and placed in chilled Eagle's Minimum Essential Medium (MEM) to limit the warm ischemic time and to wash adherent blood from the surface of the valves. The valve tissue was then transferred to a solution of MEM, together with 10% (v/v) Fetal Calf Serum (FCS), 1% (v/v) glutamic acid, penicillin (100 units/cc) and streptomycin (100 µg/cc). DMSO was added slowly to this solution over a 20 min period to a final concentration of 10% (v/v) and an additional 10 min allowed for equilibrium to occur. Osmotic stress was thereby minimized. The addition of the DMSO was carried out at 4°C, at a rate of 0.5%/min.

Following this equilibrium period one leaflet of each valve, along with 5 ml of its solution was transferred to a small sterile freezing vial, placed in a Linde cooling chamber (described in Chapter 2), and frozen to -100°C at a rate between 0.5 and 0.7°C/min. Samples were then placed in liquid nitrogen vapor and cooled to -196°C at 20°C/min, at which temperature they were stored for periods always in excess of 6 days. After storage, samples were thawed in an agitating 37°C water bath. The mean thawing rate from -196°C to +4°C was approximately 150°C/min. Once thawed, the valves were transferred to culture flasks containing 2 ml of MEM with 10% FCS. Each valve was first placed on the bottom of a flask and left to stick for a 2 hr period without any solution, after which time the culture media was allowed to cover the tissue. This procedure was used to ensure that the valve adhered to the flask, did not subsequently float off. After 3 days, when the valve had adhered firmly to the flask, an additional 3 ml of MEM + 10% FCS were added to each flask. After a five day culture time (i.e. at the end of 8 days) the incubation medium was replaced with 5 ml of fresh MEM + 10% FCS. After a total culture period between 9 and 12 days, valves were examined for endothelial and fibrobalstic growth. If there was an outgrowth of cells, the valves were then removed from the culture flask and fixed in 4% formaldehyde for histologic examination. The interpretation of the histology slides was done by T.S.* on a blind basis. Multiple step sections were prepared from some of the

* Dr. T.K. Shnitka, Department of Pathology, University of Alberta.

valves to determine the extent of tissue survival. Valves which were devoid of fibroblast or epithelial cells outgrowth were also examined histologically. Both fibroblast or epithelial cell growth were used to assess tissue (valve) viability.

RESULTS

The results are given in Table I. By taking sections at different depths, it was possible to analyze the effects of the freeze-thaw insult on the endothelial lining, and on the cellular and fibrillar components of valvular connective tissue. The first part of Table I summarizes the findings in sections from different frozen-thawed cultured valves, taken at arbitrary levels. The second section of Table I gives the results for the same valves when cut at different levels in transverse and flat planes. Both of the foregoing groups were then compared with control valves which were cultured in the same manner but not frozen. The histological findings are illustrated in Figures 2 to 6. Figure 7 is a section through a fresh valve (normal untreated control). Figure 8 shows a cultured dead valve which was destroyed by repeated freeze-thaw action in the absence of a protective agent.

TABLE 1: HISTOLOGICAL ANALYSIS OF CANINE VALVES

	Ref No: (a)	Multiple Section	Endothelial Lining		Collagen Bundles (e)	Nuclei of Collagen (f)	Connective Tissue (g)	Overall Interpretation (h)	
			Proliferation (c)	Loss (d)	Edema				
Frozen-Thawed (cultured for 9-12 days)			(b)						
Different valves*	1		1+	1+	2+	1+	0	V	Figure 1
	2		1	1	2+	2+	0	V	
	3		2+	0	0	0	0	V	Figure 2
	4		2+	1+	0	0	0	V	Figure 3(a,b,c)**
	5		3+	0	0	0	0	V	
	6		1+	1-2+	2+	1-2+	0	V	
Sections of one valve	7a	End on #1 slice	0	1	1	1	0	V	
	7b	End on #2 slice	1	3	1	2-3	0	V	
	7c	Flat section, #2 slice	1	0	1	2	1	V	
	7d	#5 slice	1	1	1	2+	1	V	
	7e	#7 slice	2	1	1	1	2-3	V	
Sections of a valve	8a	End on #1 slice	2	2	1	1-2	2	V	
	8b	#2 slice	1	1+	1	2	1	V	
	8c	Flat section #2 slice	2	1-2	1	2	2	V	
	8d	#4 slice	2	1	2	1-2	1-2	V	
	8e	#6 slice	1	2	1	2	1	V	
Control (cultured only for 9-12 days)									
Sections of one valve	9a	End on #2 slice	1+	1+	1+	1+	2+	V	
	9b	#3 slice	2+	0	0	0	2+	V	Figure 4
	9c	Flat section #2 slice	0	0	1	0	0	V	
	9d	#5 slice	1+	0	1	0	3+	V	

Table 1 - continued

Sections of a second valve	10a	End on #2 slice	1	1	1	2-3	1	V	
	10b	#3 slice	1-2	2	1	2	1	V	
	10c	Flat section #3 slice	1	1	0-1	0	0	V	
	10d	#6 slice	1+	0	0	0	0	V	
Sections of a third valve	11a	End on #1 slice	1	2	1	1	1	V	
	11b	#2 slice	1	1	1	1	1	V	
	11c	#3 slice	1	0	0	0	1+	V	
	11d	Flat section #3 slice	2+	0	1	1	2+	V	Figure 5
	11e	#4 slice	0	1	0	1+	1+	V	

(a) Histology slide library number
(b) Zero to four scale as defined for each column
(c) For proliferation, 0 implies normal, 4 implies increase in lining if cultured
(d) 0 = no loss; 4 = total loss
(e) 0 = normal; 4 = extreme edema
(f) 0 = normal number of nuclei; 4 = total loss
(g) 0 = normal; 4 = increase in number of cells in culture
(h) V = viable - an interpretative guess by an observer unaware of the type of valve being examined

Note: * Mitro, tricuspid and aortic are not distinguished. The table contains samples of all three valves which are considered to be architectually the same - see histology plates.

** Each section has a slice thickness of ≈6μ. The first slice is from the surface. Subsequent slices are spaced ≈240μ apart. Thus, the 3rd slice, for example, is taken at a depth of ≈500μ.

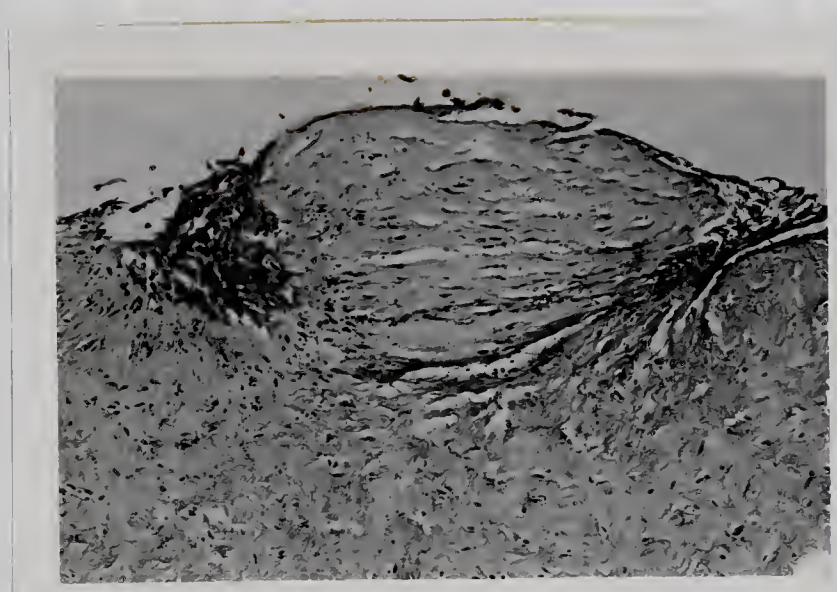

Figure 2. *Valve #1* (see Table 1). Section of preserved valve showing focal endothelial hyperplasia. X 65. Hematoxylin and eosin.

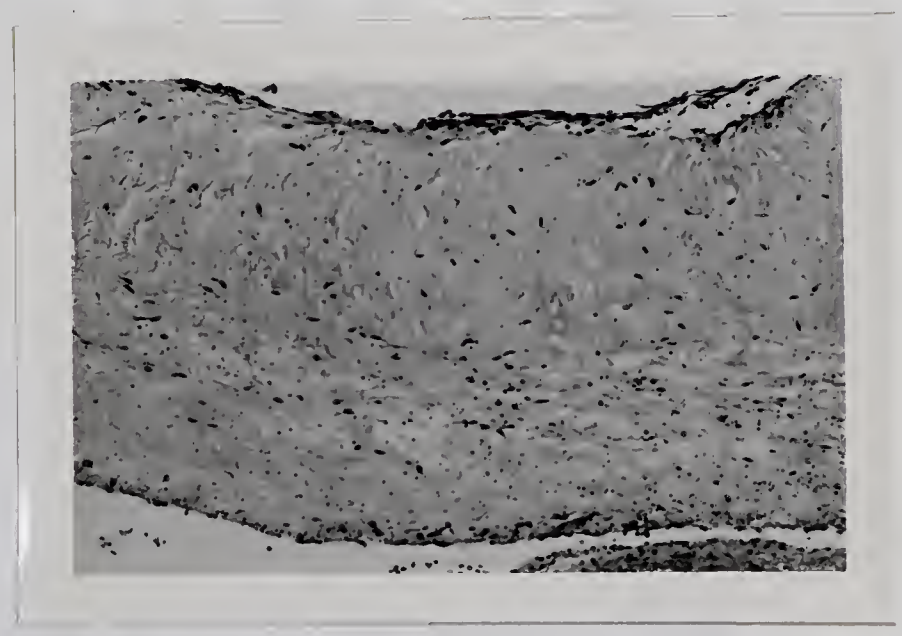

Figure 3. *Valve #3.* Preserved valve showing patchy endothelial cell loss, patchy endothelial cell hyperplasia and mild focal loss of fibrocytes in stroma. X 65.

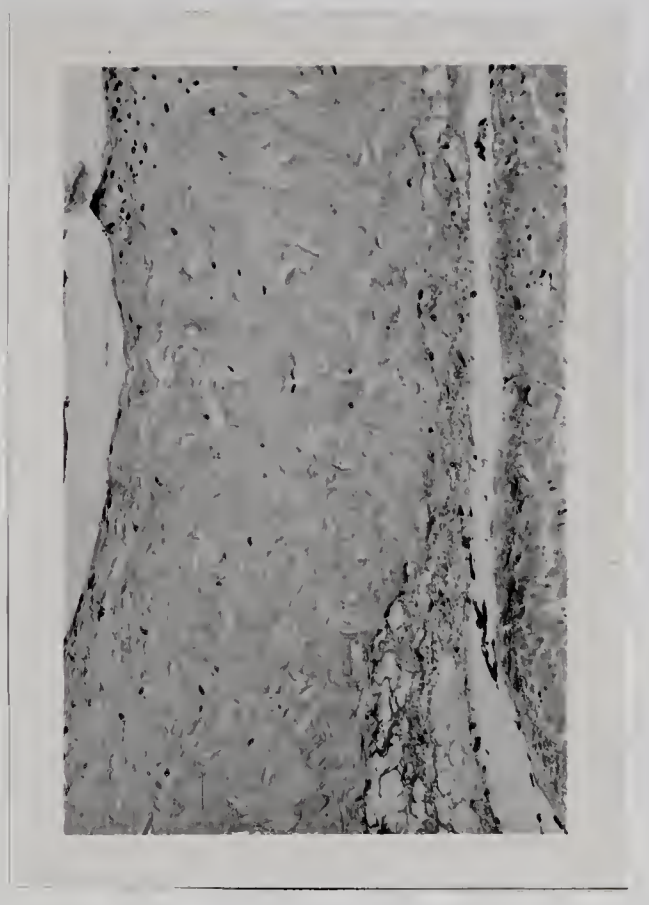

Figure 4a. *Valve #4.* Preserved valve. Note loss of stromal nuclei and patchy loss of endothelium. X 65.

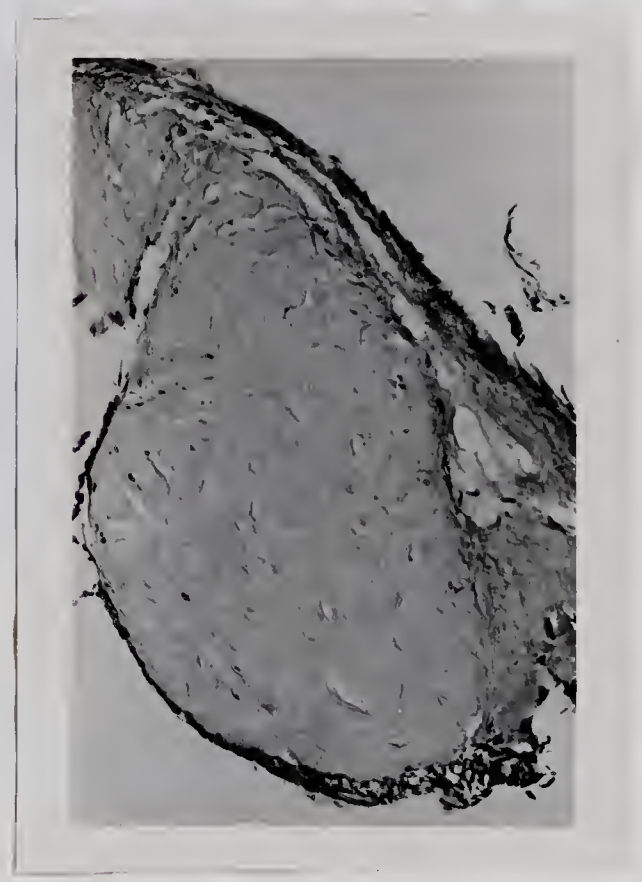

Figure 4b. *Valve #4.* Focal endothelial hyperplasia in combination with loss of stromal fibrocytes. X 65.

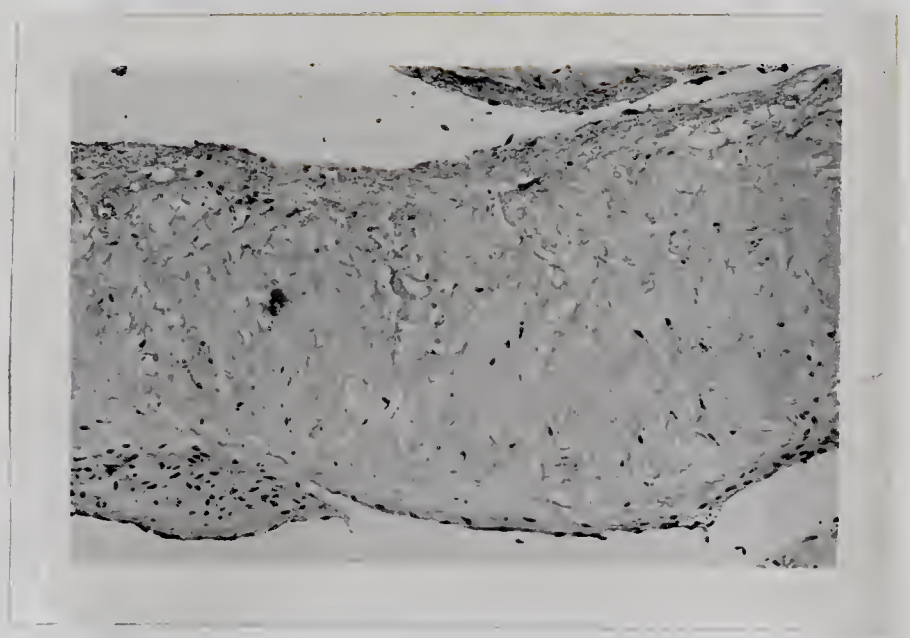

Figure 4c. *Valve #4.* A different area of the section of the valve. Endothelial cell loss on one side only. Mild focal loss of stromal nuclei also apparent. X 65.

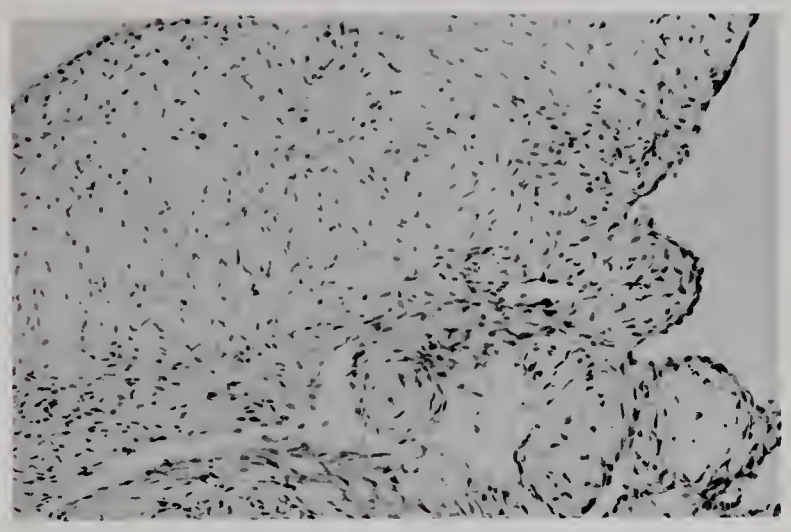

Figure 5. *Valve #9b.* Non-frozen cultured valve (control) showing hyperplasia of fibrocytes in stroma. X 65.

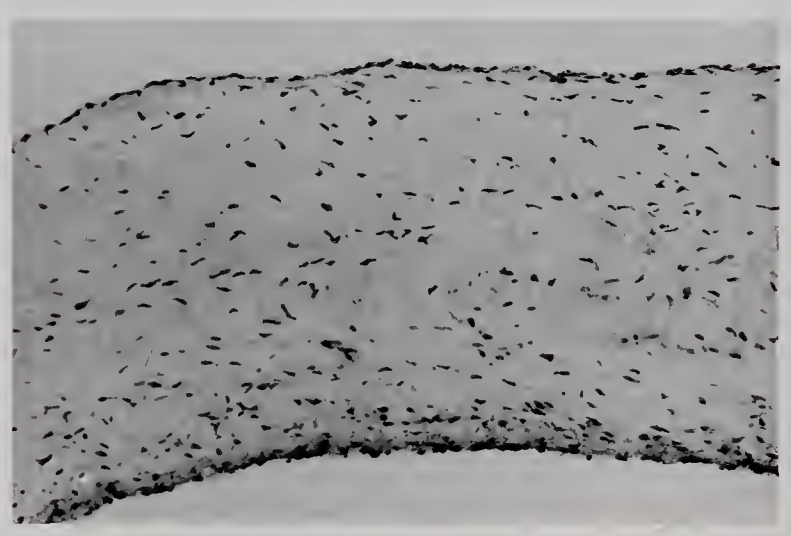

Figure 6. *Valve #11d.* Non-frozen cultured valve (control) with normal endothelium. X 65.

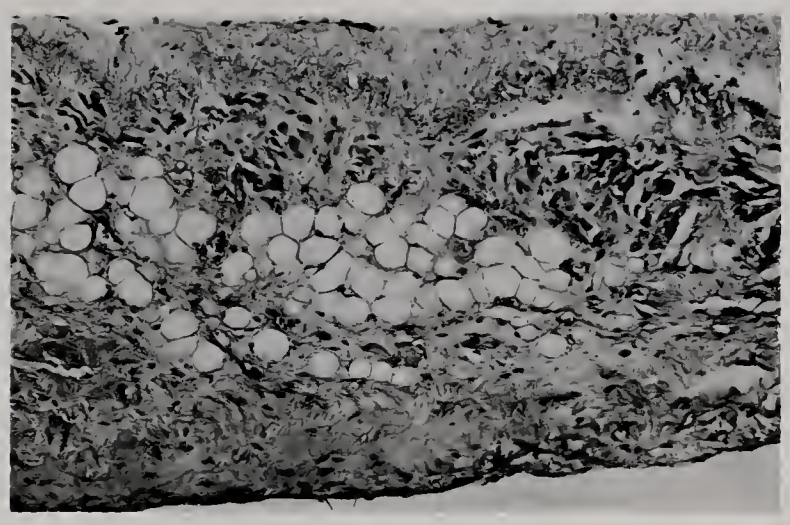

Figure 7. *Normal untreated control valve.* X 65.

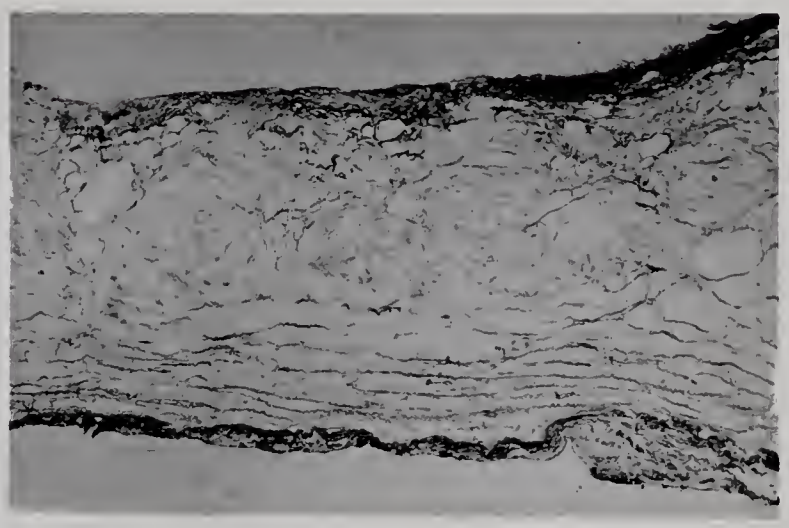

Figure 8. *Killed cultured valve.* X 65.

DISCUSSION

In this work, the objective has been to use the freeze-thaw procedure of previous writers (78)(79), who have reported successful *in vivo* grafts, in an effort to establish the extent of acceptable damage from cryopreservation. The results clearly show that the valves are viable, but that there is damage, in particular a loss of stromal fibrocytes, which may be in part due to the cryoprotective agent (Figure 4). The endothelial lining also suffers some damage (Figure 3, 4a, 4c). There was a persistance or multiplication of endothelial cells and stromal fibrocytes following the 8 days of culture (Figure 2, 5). Cell proliferation in culture was used as the absolute criterion of viability but it was not easy to distinguish endothelial cells from fibroblasts. Irreversible damage to both types of cells occurred in a patchy fashion. Overall quantitative losses of both cell types roughly correlate one with another, but when damage was minor, patchy endothelial loss tends to be more conspicuous. The local culture conditions, adhesion, medium and tissue growth stimulating factors, all have to be considered when looking at the patchy damage. Adhesion of the valve to the plate surface may not be uniform; therefore, proliferation may only occur where the valve is well stuck. Step sections appear to provide a better means of assessment of patchy damage in the cryopreserved valves than single random sections. The results presented are encouraging and indicate that an organized tissue ,even though simple ,can be cryopreserved. Further work is required on higher mammals, and human cadavers, to establish the

optimum cooling and thawing rates. Mazur (25) has shown that different types of cells have an optimum cooling and thawing rate for maximum recovery following thawing. The effect of different cryoprotective agents on heart valve tissue will also have to be looked at before optimal conditions are known for preserving valves for use in clinical grafting.

SUMMARY

Adult canine heart valves have been frozen to -196°C (0.5 to 0.7°C/min from 0 to -100°C), with 10% DMSO (v/v), stored, thawed at ≃ 150°C/min, and then cultured for 9 to 12 days. A histological analysis of sections through several valves indicates viability, but with a not inconsiderable loss of stromal fibrocytes and some damage to the endothelial lining. The practicality of freezing valve tissue for banking will have to be looked at very carefully because of the efficiency of mechanical prosthesis. However, demonstrating viability of heart valve tissue extends the range of tissues that are amenable to cryopreservation.

CHAPTER III

Using the mouse heart model to test microwave vs water bath warming and testing different cryoprotective agents on cardiac tissue.

INTRODUCTION

To date, no one has been able to obtain beating adult cardiac cells following the freeze-thaw insult (82) and ,for that matter, obtain beating adult cells in culture (83). If cardiac tissue is frozen to temperatures lower than -20°C the cells do not live when rewarmed (84)(85)(86)(87). Karow (9) has made the comment that due to Ramazzotto et al. (88) findings, if rat heart muscle was homogenized, frozen and rewarmed, then cytochrome oxidase activity decreased to 48.5% of the control, whereas in myocardium cubes, treated in the same way, the enzyme activity decreased to 79.4% of normal. It may be that adult mammalian hearts, by extrapolation from these results on small portions of adult myocardium, cannot be kept in the frozen state at temperatures lower than -20°C and at higher temperatures they can be kept for only 20 min (89)(90)(91)(92)(93).

The impossibility of preserving adult cardiac tissue may be due to the inability of adult tissue to adapt to stress, in contrast to fetal tissue. Adult tissue is also much more fully differentiated; possibly it can only function as a unit of many different cell types, each specifically designed to do one function within the complex syncytium of an adult beating heart. If one of the sub units (cell types) is not present, coordinated physiological function cannot be expected. Therefore, if an adult heart was homogenized or made into small cubes, normal function cannot

be expected because all of the sub units are not together to operate physiologically. Fetal tissue on the other hand, is tissue that is not fully differentiated and can adapt to stress more readily. Fetal cardiac cells will beat in culture (82), which may be due to the responsiveness of the cells to growth factors present in the culture media. With these possible explanations for failure (to date) there seemsto be no intrinsic reason why an adult heart, if preserved as a whole, could not function if proper freezing techniques were found.

In the hope of extending the range of cryopreserved tissue, the fetal and neonatal heart, both of which are an organized tissue of many cell types, were used. Once the system was established it could be subsequently used to test the effects of microwave thawing versus water bath, and also test different protective agents on cardiac cells. The means of assay was to measure the electrical activity of the transplanted heart. The electrical activity of the heart is due to the (changing potential) charge imbalance across the cell membrane of the myocardial cells. The fluids on the inside and outside of the cells are electrolyte solutions containing $\simeq$ 155 mEq/ℓ each of anions and cations. Intracellularly there is slight excess of negative ions (anions) and extracellularly there is an equal slight excess of positive ions (cations). These positive and negative charges give rise to the membrane potential. The sodium pump transports positively charged sodium ions to the exterior of the cell. Even though there is slow leakage of the sodium ions back to the interior, the build up of positive charges outside the

cell is enough to create a membrane potential of ≃ -85mv (resting membrane potential). If the membrane of the myocardial fiber remains undisturbed, the resting membrane potential remains at -85 mv, however, if there is a sudden increase in permeability to sodium, a sequence of rapid changes occur in a fraction of a minute (1 msec), before returning to the resting potential; this sequence is called the "action potential". It is initially accompanied by inrush of sodium ions into the cell and the inside of the cell may become positive, this is called "reversed potential" or "depolarization". Almost immediately after depolarization the pores of the membrane again become almost totally impermeable to sodium ions and the Na pump returns the inside of the cell negative (repolarization). When an action potential occurs there is a jump to as high as 105 mv which means that there is a reversal of potential or "overshoot" of 20 mv beyond the resting potential.

Each time an impulse is generated in any single fiber of the S-A node, it spreads immediately into the surrounding atrial muscle. The atrial muscle in turn conducts the signal in all directions at a velocity of ≃0.3m/sec. As the cardiac impulse travels through the atria, the atrial muscle contracts, forcing blood through the atrioventricular valves into the ventricules. The conductive system is organized so that the cardiac impulse will not travel from the atria into the ventricles too rapidly, this therefore allows time for the atria to empty the contents into the ventricles before ventricular contraction begins. It is the A-V node and its associated conductive fibers that delays this transmission of the cardiac impulse from

atria into ventricles. The Purkinge fibers that lead from the A-V node through the bundle of His (or Hiss) into the ventricles have functional characteristics that allow fast transmission of impulse through the entire ventricular system, which causes the ventricles to contract synchronously. A small proportion of the associated extracellular currents spread all the way to the surface of the body (Figure 9).

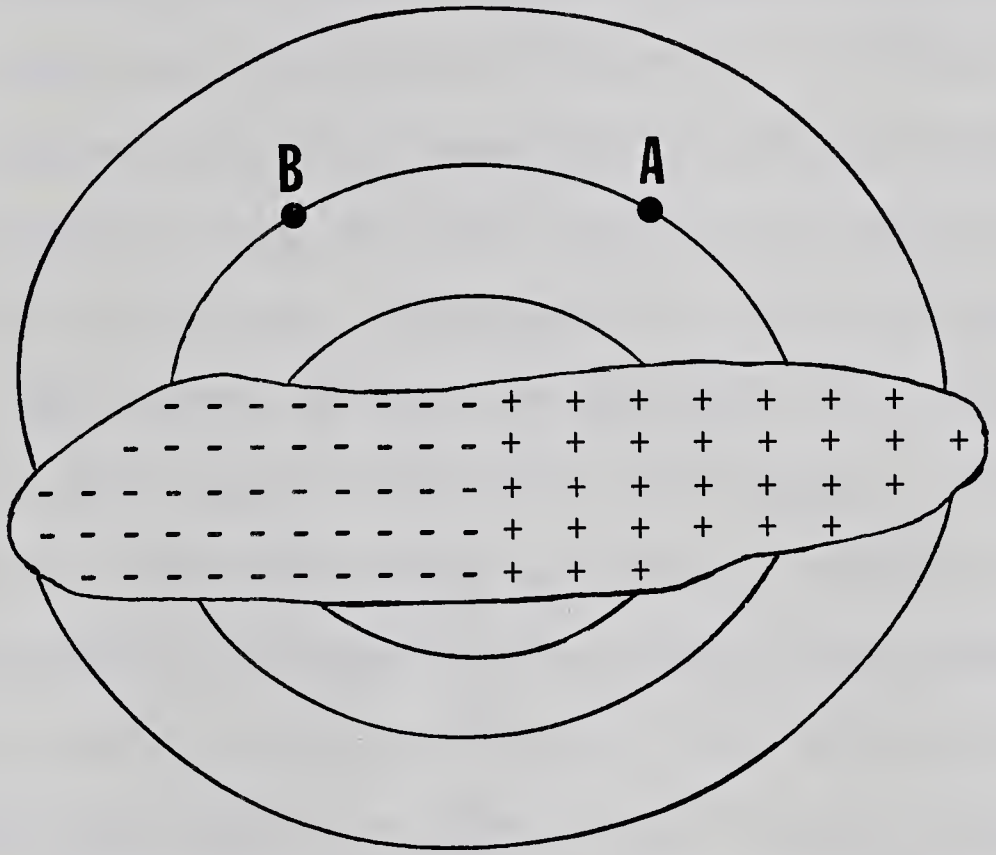

Figure 9. *Cardiac muscle.* Current flow through the fluids surrounding a strip of cardiac muscle that has been depolarized at one end. The depolarized end has negative extracellular potential relative to the polarized end.

There are large eliptical pathways of current that spread into the surrounding extracellular fluid due to the two areas of opposite potential. If an electrode is placed on the negative side of the cardiac muscle and one on the positive side, there will be a potential difference between the two areas. If the electrodes are placed some distance away from the hearts or cardiac muscle (A & B),

but within the field of the flowing electrical current, a potential difference will still be recorded. If the line through points A and B is considered as a resistor, the electrolyte solution being conductive but still having a reasonable amount of resistance to the flow of current, there will be a potential between A and B. As point A and B move closer together, less and less potential difference occurs. Another factor that affects the potential differences is the areas that are depolarized and polarized. The larger the areas, the more current that flows through the resistance; therefore, maximum potential occurs when 1/2 of the cardiac muscle is depolarized and the other 1/2 of the cardiac muscle is still polarized.

Now consider the heart in the chest cavity surrounded by body fluids which conduct electricity very readily. The heart is suspended in a conductive medium, and when one portion of the ventricle becomes electronegative in respect to the remainder of the heart, electrical current flows from the depolarized area (-) to the polarized area (+) in large circular routes, through the whole body. In the normal heart, the current flows primarily from the base towards the apex, during almost the entire cycle of depolarization. Therefore if a meter is connected with its positive electrode towards the apex and its negative electrode towards the base, the meter will record positively during the entire cycle. If two electrodes are placed on the surface of the body (points A and B) the flow of current around the heart will cause a potential difference between the two electrodes, because A is nearer the negative part of the ventricle than is electrode B. In doing ECGs on patients

the electrodes are placed on the Rt and Lt arm and left leg, thereby measuring the potential difference between these points. The ECG tracing that results is called the P, QRS and T waves. The P wave is caused by the electrical current generated as the atria depolarize prior to contraction. QRS is caused by currents generated when the ventricles depolarize prior to contraction, and T waves are caused by current generated as the ventricles recover from the state of depolarization.

The electrical current generated by the cardiac muscle during each beat of the heart changes potentials and polarity in less that 10 m sec, therefore equipment is needed that will respond to these changes. The potential difference to be measured is of the order of a millivolt; therefore, a sensitivity of a fraction of a millivolt is needed. Amplifiers with high gain and with low internal noise should be used. Interference arising from power line is frequently a source of difficulty.

It was necessary to put the mouse in a shielded cage in order to obtain a coherent noise free signal. Short leads from the cage to the input of the high gain amplifier were also used. An alternate method would be to used a difference amplifier (94). Power line noise is due to currents that exist in the ground connection associated with all amplifiers, receiving energy from the power line. In a difference amplifier the ground terminal is not used as one of the pair of input terminals connected to the points where the potential difference is sought, but is connected to an indifferent point such

as the right leg. The input terminals are connected to an opposite pair of electrodes on the Rt and Lt arm or in our case on each side of the grafted heart. Therefore, power line interference appears as a so called "common mode signal" between these terminals and ground. The difference amplifier is designed to attenuate the common mode signal while amplifying the input (electrocardiogram). The ratio of amplifier gain for the desired signal to gain for common mode signal is called common mode rejection. Since common mode signals may be of an order of magnitude larger than the electrocardiogram, a common mode rejection of 10,000 is desirable. Some ECG machines eliminate the ground connection by using transistorized equipment that operates on batteries.

A problem that is present at the clinical level and was present on the mouse model was muscle tremor or ear twitch. Muscle tremor artifacts contain frequency components which extend far beyond those of the ECG; therefore, the signal to noise ratio can be improved by a choice of filters which eliminate as much of the high frequencies as possible without sacrificing the ECG signal. ECG signals contain frequency components exceeding 2000 Hz but most conventional ECGs do not pass components much above 60 to 100 Hz, therefore a restricted bandwidth can be used to record the ECG. One thing that has to be watched at the clinical level is not to miss some small notches which can be clinically significant.

The S-T segment and T wave are low frequency phenomena, therefore the amplifier must pass frequencies as low as 0.1 Hz to obtain these portions of the ECG.

The electrodes used to record from the surface of the skin are an important part of the measurement of any biopotential measurement such as the ECG (Figure 10).

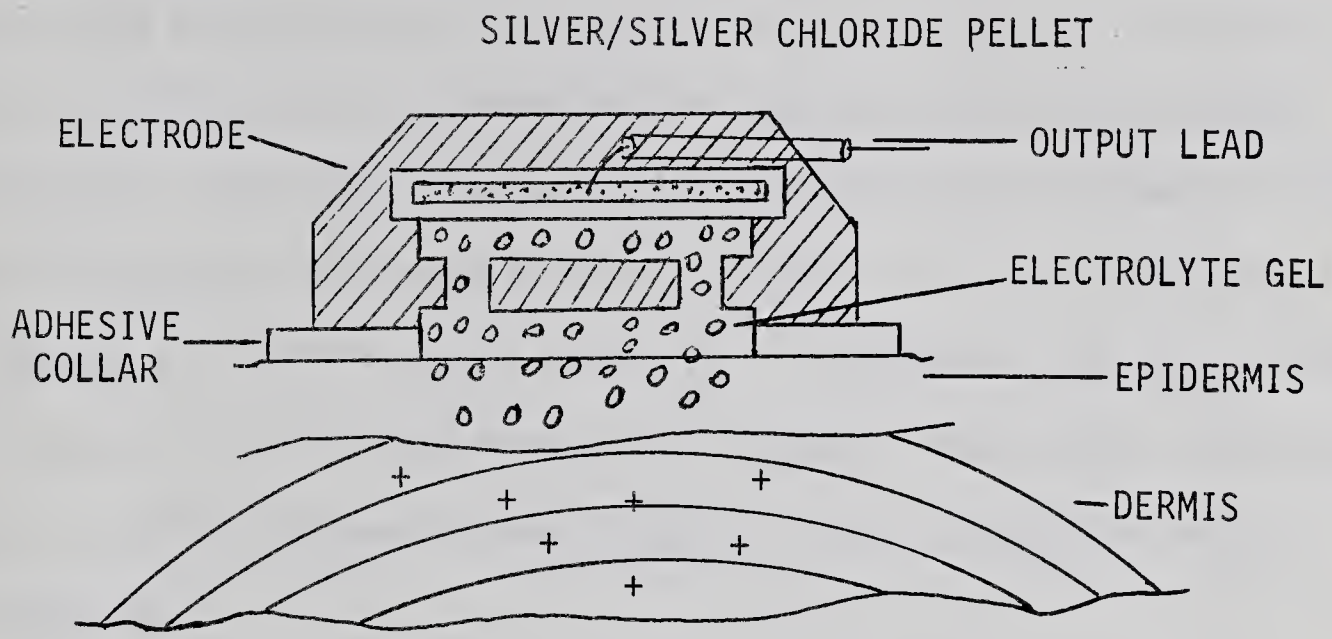

Figure 10. *ECG electrode.* Silver/silver chloride electrode with disposable adhesive collar, showing action of electrolyte gel on skin.

Skin is a multilayered structure exhibiting a trans-cutaneous impedance of the order of magnitude of 1 to 100 KΩ. This impedance is highly capacitive and resistive, with almost no inductive components. The epidermis (flattened dead cells) has a very high electrical resistance while the dermis (cuboidal viable cells) may be considered a volume conductor. The best measurement of voltage at the surface of the skin requires that the biopotential be transferred to the amplifier without attenuation, distortion or drift. This is best accomplished when interface (source impedance) is as low as possible. The input impedance of the amplifier should be high, at least 100 to 1000 X the source impedance, which in this case is the impedance between the dermis

and the amplifier input. When the ratio of amplifier input impedance to source impedance is high, very little power is transferred from the skin to the amplifier. This implies that a minimum of current per unit area (current density) flows through the skin electrode and then to the amplifier input. The wire or cable between the electrode and the amplifier must be shielded because a large amount of noise will be generated if even a minimum of electrons are induced to flow through an extremely high input impedance. This is obvious when it is remembered that the voltage is equal to the current times the impedance (Ohm's Law), therefore put the high impedance input stage as close as possible to the electrode.

There are many types of electrodes, stainless steel plates, etc, which are strapped to the limbs. The problem with metallic electrodes,when used on dry skin, is that they have an impedance of ≃ 100,000 Ω in the frequency band of the ECG which is .1 Hz to 100 Hz. The electrical skin impedance can be lowered by using a highly concentrated salt (3M) paste between the electrode and the skin. The paste can lower the electrode-skin impedance to ≃ 1000 Ω. Another method used is to rub the skin until red (remove some epidermis) or puncture the skin with a pin before applying the paste thereby making a better contact with the dermis. The system that was used in the mouse model was to use pin electrodes and penetrate right through the ear thereby making a very good contact with the dermis. The solder joints were placed inside the wire cage to further eliminate outside noise. With the above setup

it was possible to record the electrical activity of the transplanted hearts with little noise interference.

Using the above model it was possible to show that organized tissue, more complex than heart valve tissue,could be cryopreserved. The model also showed that myocardiac cells are better preserved with certain cryoprotective agents than others. Microwave was also shown to be as effective for thawing the samples as water bath agitation.

FREEZING AND THAWING OF FETAL AND NEONATAL MOUSE HEARTS

The following sections describe the method used in freezing and thawing fetal and neonatal mouse hearts, with different protective agents and freezing rates. The hearts were thawed in either a microwave (MW) or a conventional water bath (WB) from -196°C to either 4°C or room temperature. Following the freeze-thaw insult, the hearts were implanted into syngeneic recipient adult (6 weeks or older)mice. Electrical activity of the grafted heart was studied up to 120 days, the actual period depending upon the objective of the experiment. The hearts were examined histologically after removal. The implant survives by diffusion of nutrients but does not work as a pump. The formation of the diffusion paths was also studied as the implanted hearts do not grow under the "no load" situation. All readings of this histology were done by JD*, who was unaware of what was done to the hearts being examined.

Freezing. Pregnant Balb-C mice were used throughout these experiments. When using a fetus of seventeen to nineteen days gestation,

* Dr. J.B. Dossetor, Chairman of Transplant Immunology, U/A.

a midline incision was made on the mother and fetuses and placenta removed by hysterotomy (Figure 11).

Figure 11. *Pregnant mouse.* A Balb-C mouse at nineteen days gestation showing the fetus inside the uterus.

Taking the placenta insured the fetus staying alive for a longer period of time following hysterotomy (Figure 12). Each fetus was placed in a saline solution to prevent it from drying during removal of the other fetuses. Each mother had seven to nine fetuses. The heart was removed from the fetus using two pairs of forceps; one under the sternum and the other to open the rib cage and expose the chest cavity. The lungs remain collapsed, therefore, if care is taken to insure minimum bleeding, the hearts continue to beat, making them easy to identify. Once identified, the tips of the forceps were placed under the atria at the base (Figure 13). The heart was

placed in chilled culture solutions which limited the warm ischemic time.

Figure 12. *Eighteen day fetus.* The eighteen day fetus is seen with the attached placenta.

When neonatal hearts were used, pregnant mothers were allowed to go to term and hearts were removed from neonatal mice aged 1 to 9 days. The mouse was anaesthetized and the rib cage opened on the left side of the sternum. Neonatal mice have inflated lungs, making it more difficult to remove the heart. Once again, after identifying the heart, the tip of a small forcep was placed on each side of the atria at the base, and the heart plucked out.

Figure 13. *Fetal heart.* The fetus with chest cavity opened and the forceps on each side of the atria at the base of the heart.

There were varying amounts of atrial tissue but in all cases the ventricles were intact. The excised heart was placed in a chilled culture solution.

The chilled culture solution not only limited the duration of warm ischemia but washed blood out of the chambers as the heart continued to beat for a short period of time after being placed in this media.

The hearts were then transferred from this initially chilled solution to one of three solutions: A) Eagle's Minimum Essential Medium (MEM) containing Hepes buffer, 10% (v/v) fetal

calf serum, 1% (v/v) glutamic acid, penicillin (100 units/cc) and streptomycin (100 µg/cc); B) McCoy's 5a containing Hepes buffer, 10% (v/v) fetal calf serum, 1% (v/v) glutamic acid, penicillin (100 units/cc) and streptomycin (100 µg/cc) and C) Cross solution (95)(no fetal calf serum or other proteins were present).

Different protective agents were added to the above solutions at different concentrations and at different rates.

*Dimethylsulfoxide (DMSO)** was used with solution A in 10, 5 and 2.5% (v/v) concentrations and Solutions B and C in 10% (v/v) concentrations. This protective agent was always added in a density gradient, that is, the DMSO was added over twenty minutes at 4°C to the final concentration indicated. An additional ten minutes were given at this final concentration to insure complete equilibration of the solution with the heart tissue. Continuous agitation of the solution was used to insure good mixing.

*Glycerol*** was used with solution A at a 10% (v/v) concentration. This protective agent was also always added in a density gradient but the length of time varied from 45 to 90 minutes. If glycerol was added over 20 minutes to the final concentration of 10%, an additional 25 minutes was given for equilibration giving a total of 45 minutes. Glycerol, added over 30 minutes to a final concentration of 10% was then followed by an additional 30 minutes in one set of hearts (total 60 minutes) and 60 minutes in another

* [$(CH_3)_2$ SO MW = 78.13 Fisher Scientific]

** [($CH_2OHCHOHCH_2OH$) MW = 92.1 Fisher Scientific]

set of hearts (total 90 minutes) because it was felt that it may take longer for the glycerol to permeate myocardial cells.

*Methanol (methyl alcohol)** was added to solution A in 10 and 5% (v/v) concentration. Methanol was always added over 20 minutes at 4°C or 37°C to the above concentration with 10 additional minutes allowed for equilibration. Again there was continuous agitation of the solution while adding the protective agent.

*Ethylene glycol (EG)*** was used with solution A in 15, 10 and 5% concentration. As above, EG was added over 20 minutes at 4°C and an additional 10 minutes given for equilibration.

*Dextran**** was dissolved in solution A for 24 hr to a final concentration of 15 and 10% (v/v). Hearts were added directly into the solution at 4°C and left there for 30 min before freezing.

*Pluronic***** was used in solution A in 10% concentration. Hearts were placed into the 37°C solution, and 30 minutes allowed for equilibration.

* [(CH_3OH) MW = 32.04 Fisher Scientific]

** [($HOCH_2OH$) MW = 62.07 Fisher Scientific]

*** [General formula $(C_6H_{10}O_5)_n$ Average MW = 17,700D Sigma Chemical Co.]

**** [General formula $HO - (CH_2CH_2O)_a \left| \begin{matrix} CHCH_2O \\ | \\ CH_3 \end{matrix} \right|_b (CH_2CH_2O)_c - H$

average MW 8350D BASF Wyandotte Corporation]

Control groups of mouse hearts were used. In each particular case the protective agent was added to the hearts and these hearts were not frozen but implanted into syngeneic mice and followed for electrical activity. The experimental hearts that had the protective agent were placed in a 15 ml glass bottle along with 5 ml of the above mentioned solutions (Figure 14). The small bottles were capped and placed in a prechilled freezing unit (Linde BF-4-1)(Figure 15). The Linde unit was calibrated for each freezing run. Once all the hearts were in the cooling chamber with one sample used as a reference for the controller,they were frozen at different freezing rates as described below. Hearts frozen in DMSO, methanol, ethylene, pluronic and dextran were frozen at mean rates between 0.5 to 0.7°C/min. When the latent heat of fusion was given off, the machine was put on override to limit the time the sample was exposed to the elevated temperature. This compensation took ≃ 5 min. The machine was then put on automatic and a freezing rate of 0.5 to 0.7°C/min down to -100°C. The temperature between the freezing point and -100°C was monitored every 5 min on a temperature potentiometer and a record kept (Figure 16).

The hearts frozen in glycerol were frozen at 0.5 to 0.7, 20 and 200°C/min. The reason for this was to try to maximize survival. Once hearts (regardless of protective agent) had reached -100°C, they were transferred to liquid nitrogen vapor and cooled at 10 to 20°C/min down to -196°C where they were stored for 1 to 10 days.

In one group of mouse hearts, the above procedure was followed except that hearts were placed in solution A which had no protective agent. This group was used as a control to determine

what percentage,if any, would survive without a protective agent.

Figure 14. *Heart inside a 15 ml vial.* The heart is shown in a 15 ml glass vial, with 5 ml of protective agent.

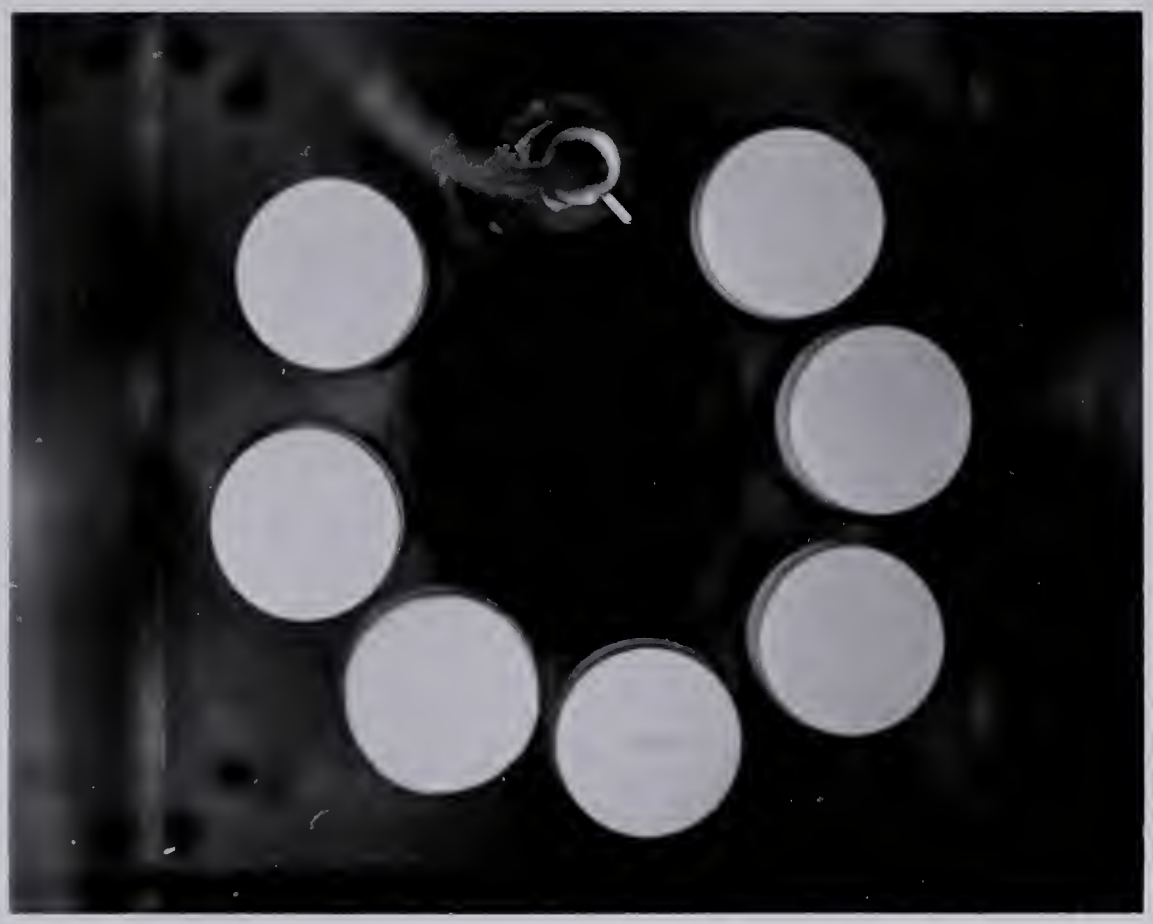

Figure 15. *Freezing chamber.* Inside of the freezing chamber is shown with the thermocouple which runs the controller. The capped samples can also be seen.

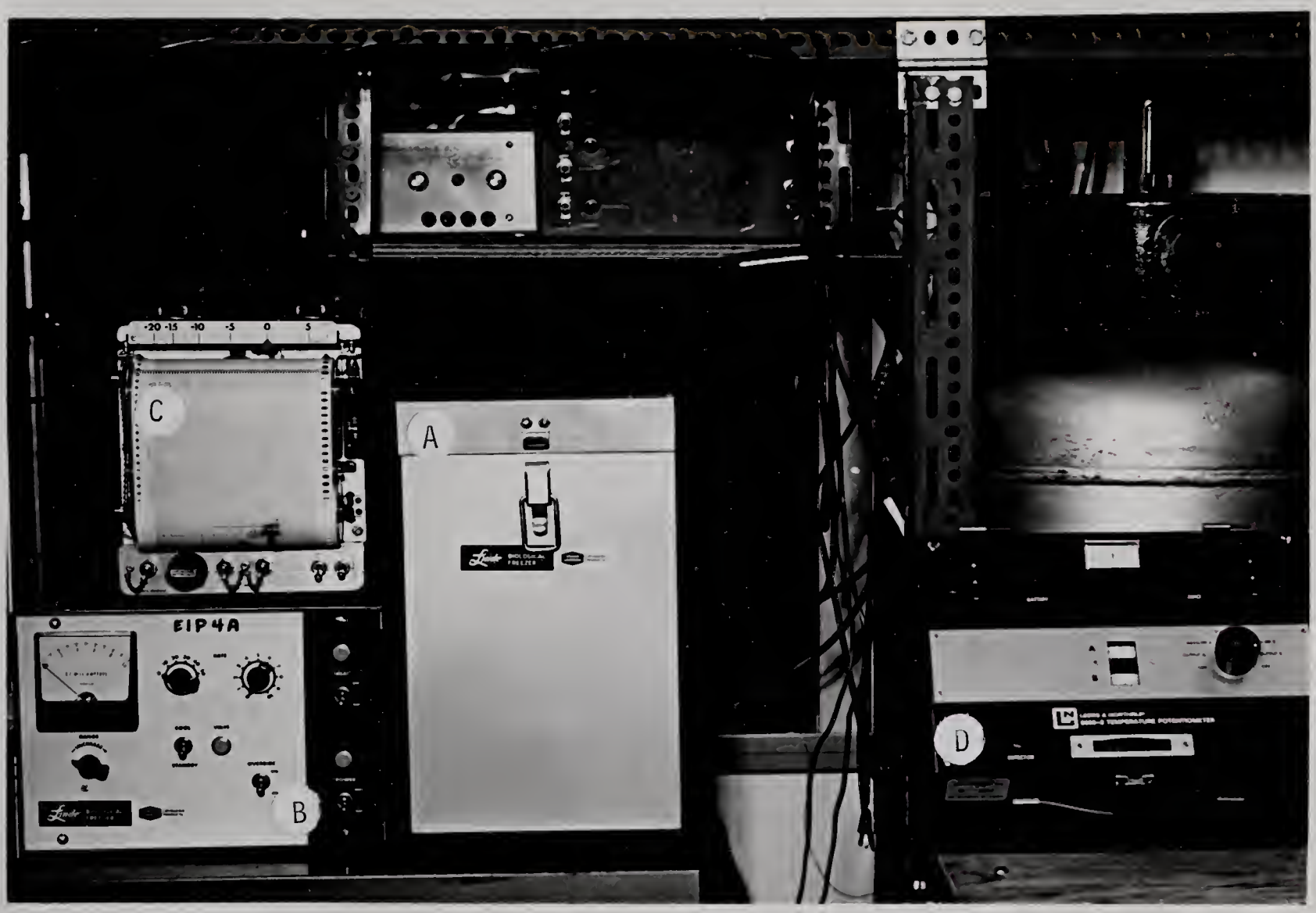

Figure 16. *Freezing system.* The hearts are placed inside the cooling chamber (A). The freezing rate is controlled by the controller (B) and the temperatures of the samples are recorded on the paper recorder (C) or by the potentiometer (D).

Thawing. 5 ml samples, each containing a frozen heart, were thawed either by placing them in a 25°C water bath or by using a 2450 MHz, 0 to 2 kw microwave system (Figure 17).

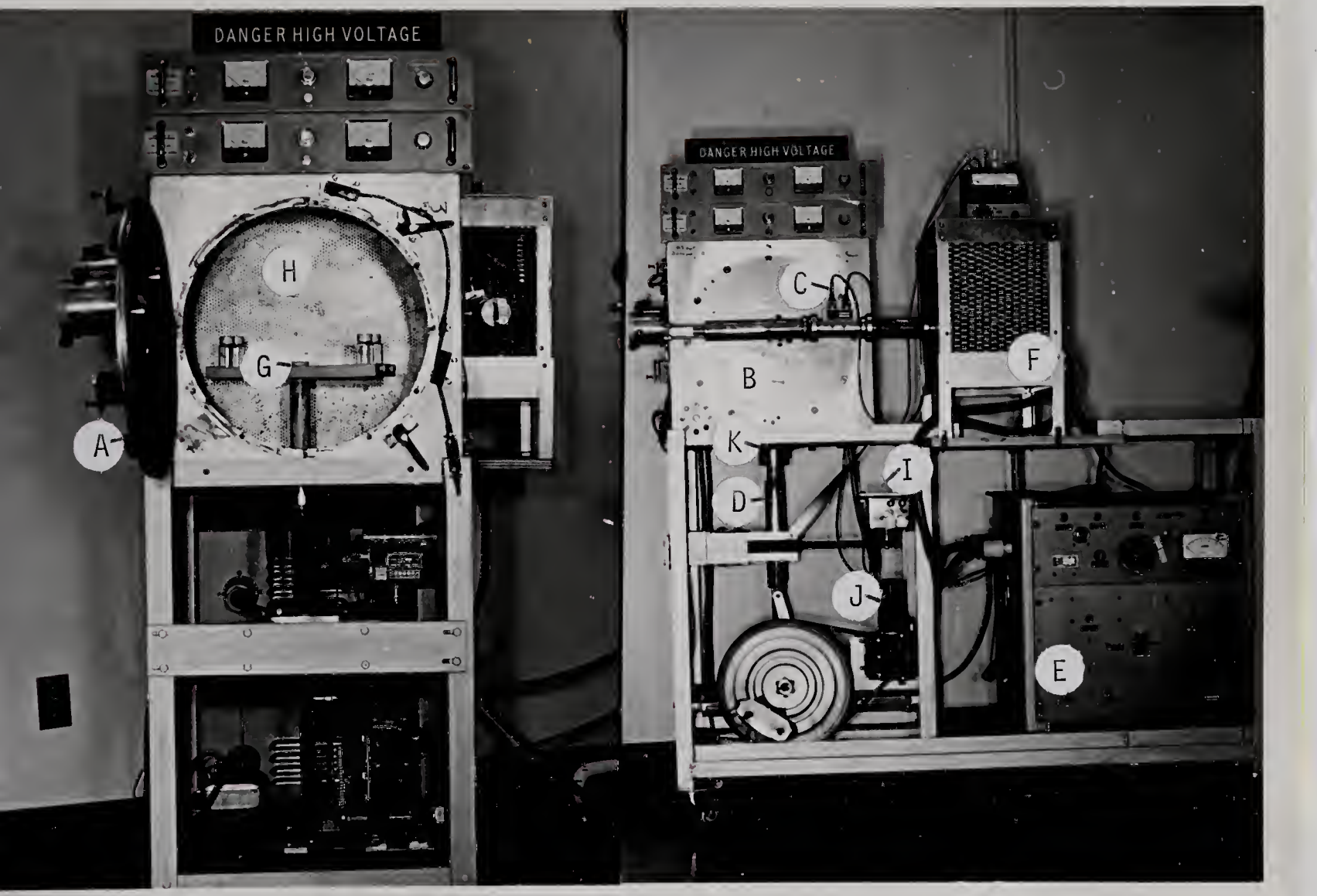

Figure 17. *Microwave thawing system.* A - cavity door, with interlocks and tuning posts. B - side wall with antenna and power coupling unit. C - power monitor on transmission line. D - mechanical for oscillating turntable. E - magnetron power supply and control systems. F - 2 kw (Philips) 2450 MHz magnetron encased with protection systems. G - turntable rack for sample holders. H - mesh facing on inside cavity wall to increase wall loss and enhance coupling. I,J - variable speed dc driving shaft through wave trap K independently adjustable in speed.

The microwave heating system consisted of a cavity having dimensions 48.5 x 38.5 cm, which gave rise to a large and fairly evenly distributed number of modes of resonance over the bandwidth of the magnetron, when the cavity is very lightly loaded (97). The stripline coupling the transmission line B to the cavity in Figure 17 is not shown in the Figure, but is one of several antennae tested. The original design for this family of multimode coupling arrays used is due to Johnston (98). One in particular gave satisfactory results for a wide range of small frozen loads in the cavity, provided these loads were moved through the electromagnetic fields in the cavity in a rapid fashion, the sample following a spinning set of elliptical orbits. Figure 17 shows the method of rotation used: the rotating turntable driven by motor I, is oscillated by motor J through a flywheel and lever. Although the method is rather crude and an arbitrary predetermined set of path lengths and velocities was used, the method has proven to be surprisingly effective even for large organs (99). Frozen samples were placed on the teflon rotating arm and oscillated up and down. Power was fed to the cavity by the radiator from a 2450 MHz magnetron. The power output could be varied from 0.14 to 2.0 kw. Stationary water samples were included in the cavity to increase the load, thereby protecting the magnetron. Although a high percentage of the energy was still reflected back to the magnetron, the technique provided a useful method for uniform heating of samples of different shapes and sizes although the phenomenon remains unexplained, the small sample, when rotated in the fashion described, absorbs the energy stored in the resonant

cavity. In these experiments, mean heating rates of 200°C/min were used to thaw the hearts from -196°C to 10° ± 10°C. The electric field strength experienced by the hearts is assumed to vary from zero to values near breakdown (30,000 v/cm) during thawing.

Tissue samples were agitated while being thawed in the water bath at thawing rates around 150°C/min. In most cases the samples were thawed to just 4°C to limit the subsequent warm ischemia.

Reimplantation. Hearts were placed on ice (4°C) following thawing before reimplantation into syngeneic mice, six to sixteen weeks of age. The recipient mice were anaesthetized with 0.08 - 0.15 ml of one in four dilution of dibutol (60 mg/ml), intraperitoneally, the dose being judged by the weight of the mouse. About five minutes later, the right ear was cleaned with alcohol and a small pocket formed by blunt dissection (Figure 18). One frozen-thawed heart was placed in the pocket (Figure 19). The air from the pocket was expressed and the incision wiped with alcohol. The left ear was marked, using holes ranging from one to five, to identify each mouse in a particular cage.

Assay of graft function. The graft could not be assessed as a pump; however, electrical activity was evaluated by electrocardiography and contractibility could be seen directly through the skin of the external ear with a dissecting microscope (Figure 20).

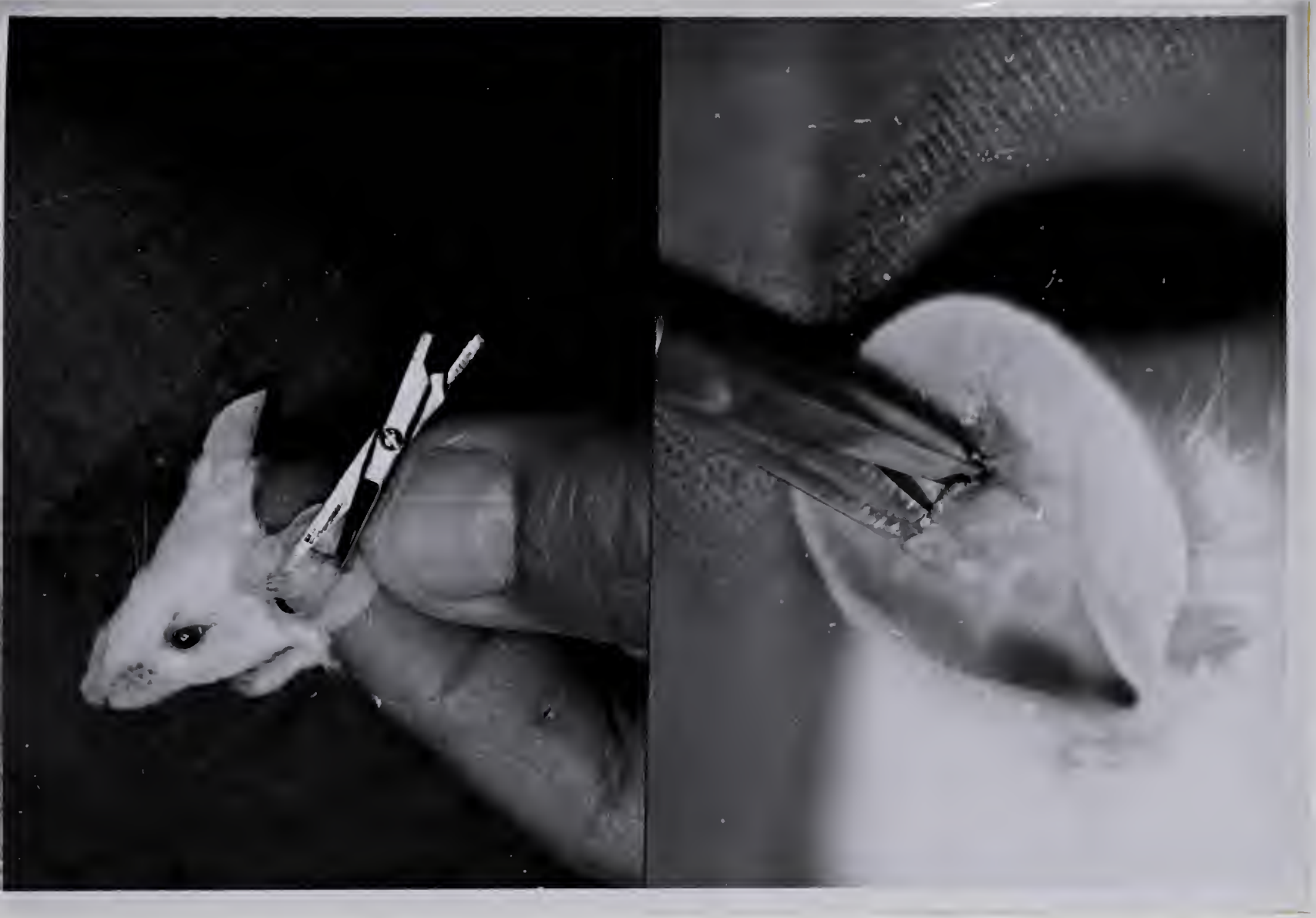

Figure 18. *Transplant pocket.* The ear of the mouse is seen with small pocket formed by blunt dissection.

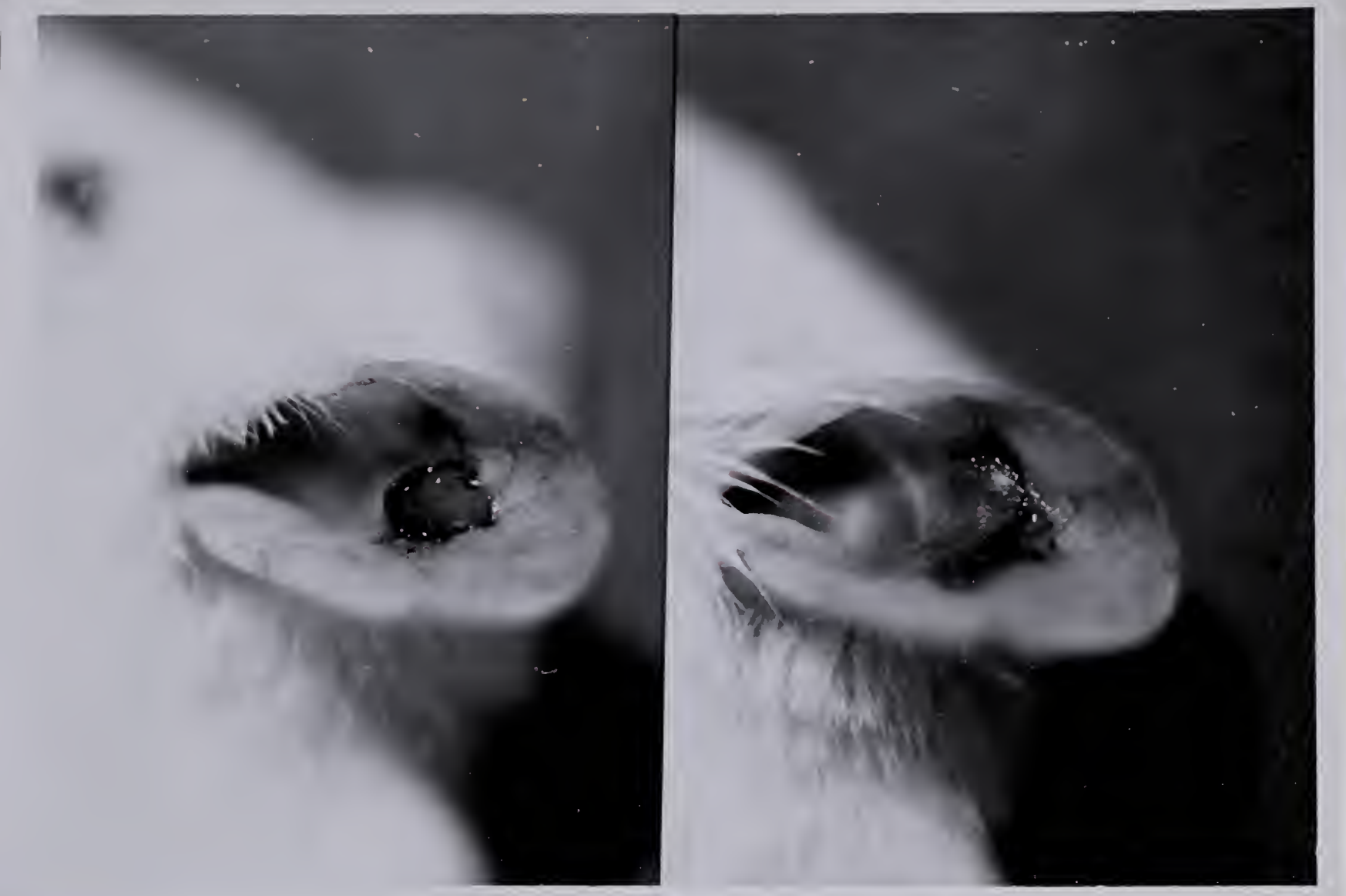

Figure 19. *Transplanted heart.* The frozen-thawed heart seen in the opening of the small pocket (left). Pocket closed and wiped with alcohol (right).

Figure 20. *Grafted heart.* The beating heart as seen under the dissecting microscope. X 4.

The test animal was put to sleep with dibutal. The mouse was placed in a fine mesh copper cage which was grounded to the base of the recorder, making sure no gound loop occurred. The leads from the cage to the input of the amplifier were short and also shielded. The short leads and isolation cage were used to minimize any external noise from the ECG tracing. Pin electrodes were used to record the electrical activity by placing them on each side of the graft (Figure 21). To ensure good electrical contact the needle was pushed through the ear, thereby making contact with the dermis and lowering the ear to electrode resistance to ≃ 1000 Ω(100). The electrodes were soldered to wires which went to terminals on the

inside wall of the isolation cage. The output of these terminals were fed to the input of the high gain amplifier having an input impedance of 5 meg ohms. The output of the amplifier was fed to a strip chart recorder and oscilloscope (Figure 22).

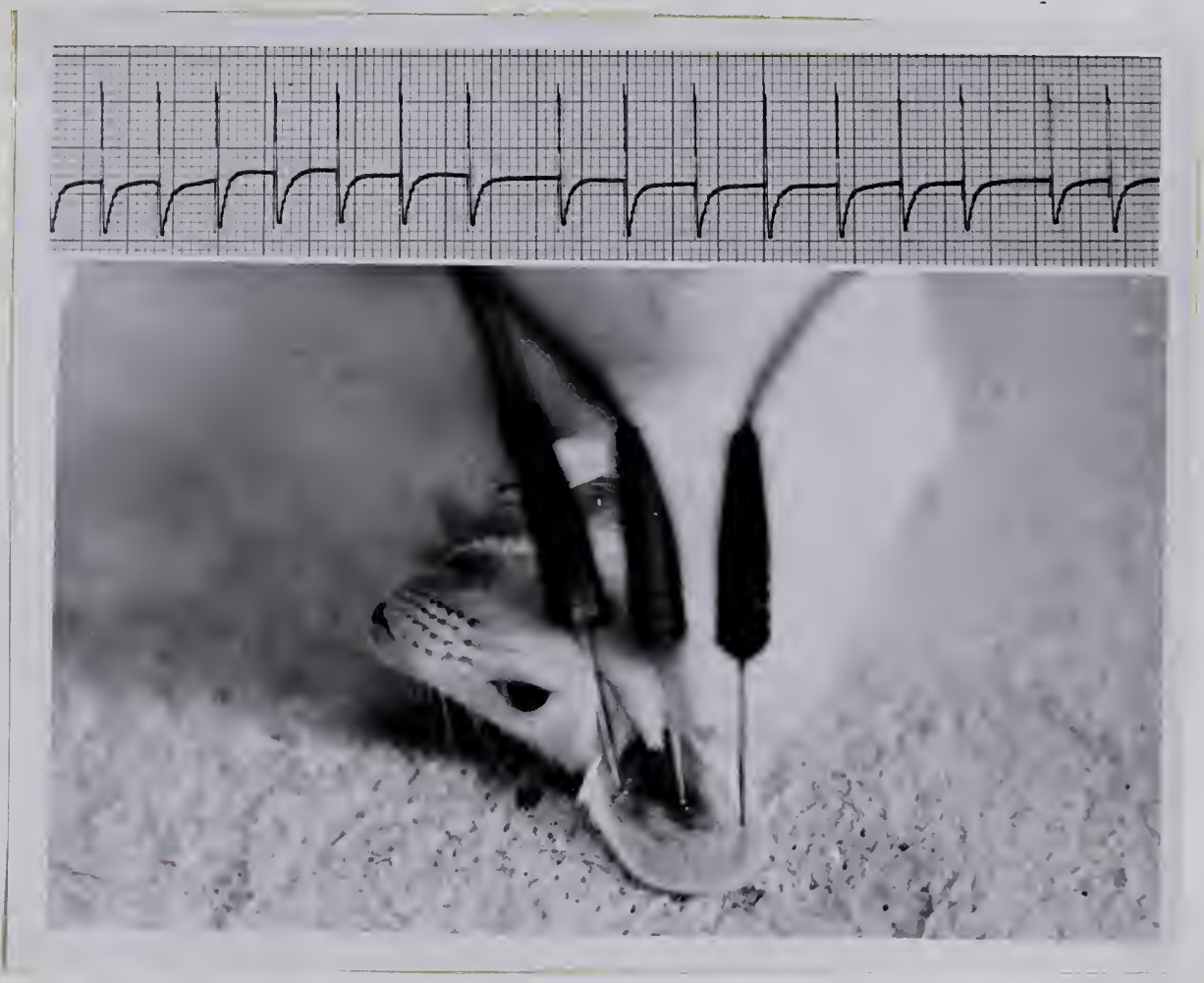

Figure 21. *Pin electrodes.* The pin electrodes can be seen on either side of the heart, with the 3rd used as a reference electrode.

The beats of the hearts were first followed on the scope and when a good tracing seen, a sample was taken on the paper recorder (Figure 23). To prove that the recording was just the grafted heart and not the adult recipient's heart beats, the ear could be cut off from the animal, while the pin electrodes were still in place and a recording made (Figure 24). The grafted hearts were tested every seven days for periods of 30 to 90 days. Following this test period, the ear

was cut off and the graft removed for histology.

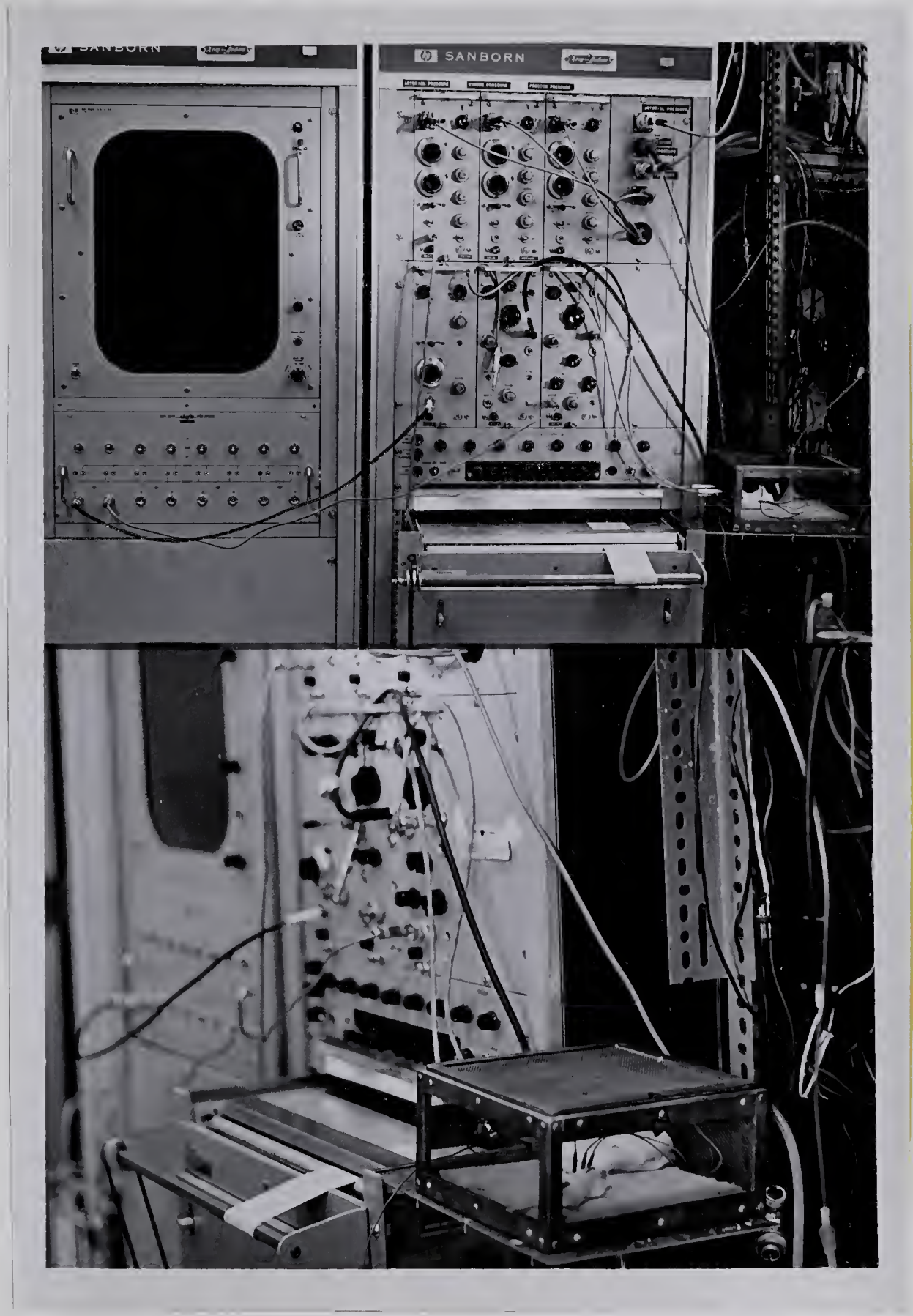

Figure 22. *ECG monitoring system.* The mouse in the copper cage with the output of the pin electrodes fed into a high gain amplifier, its output is fed into a scope or paper recorder.

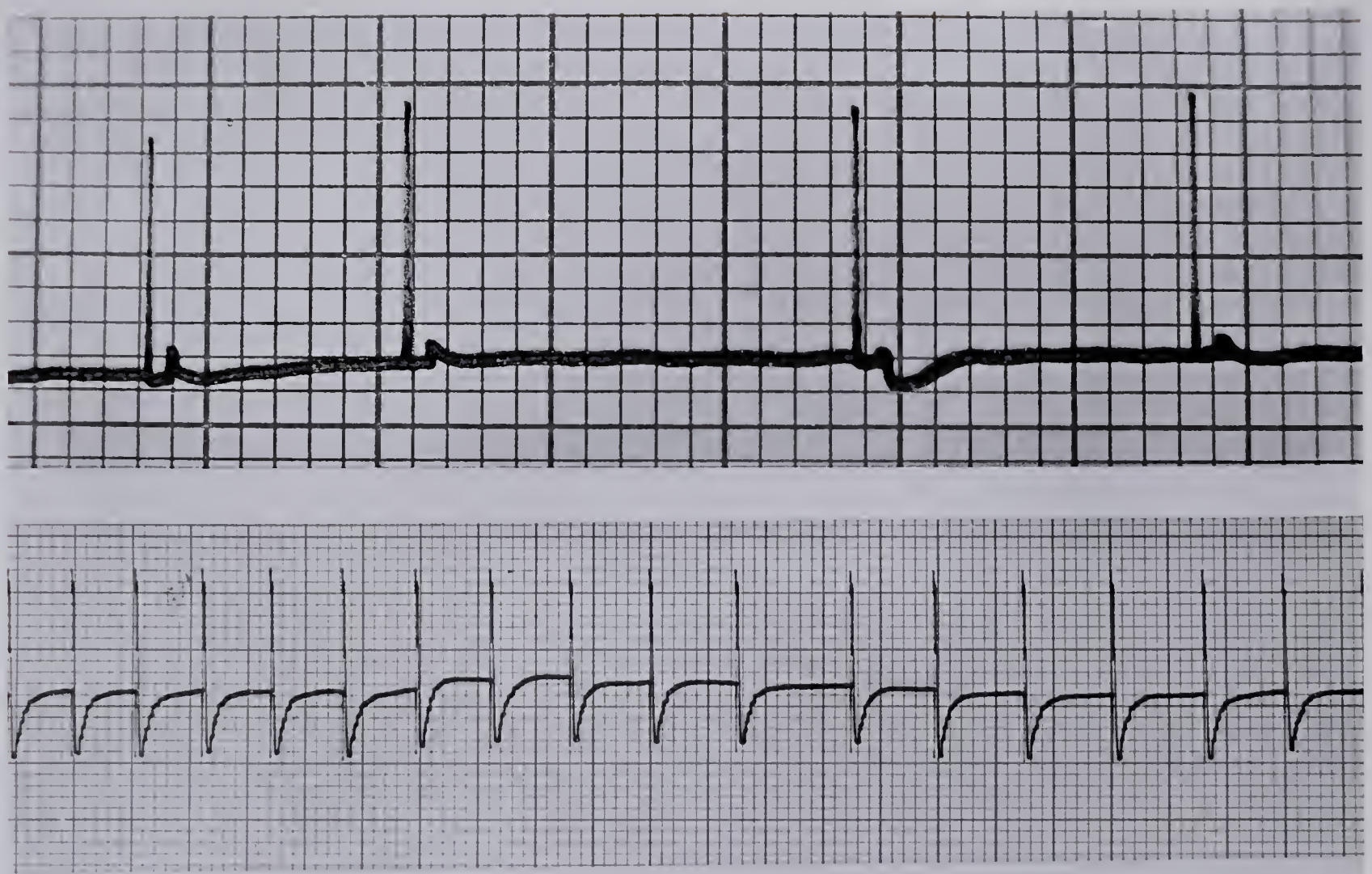

Figure 23. *ECG tracing.* A positive ECG tracing of a frozen-thawed heart.

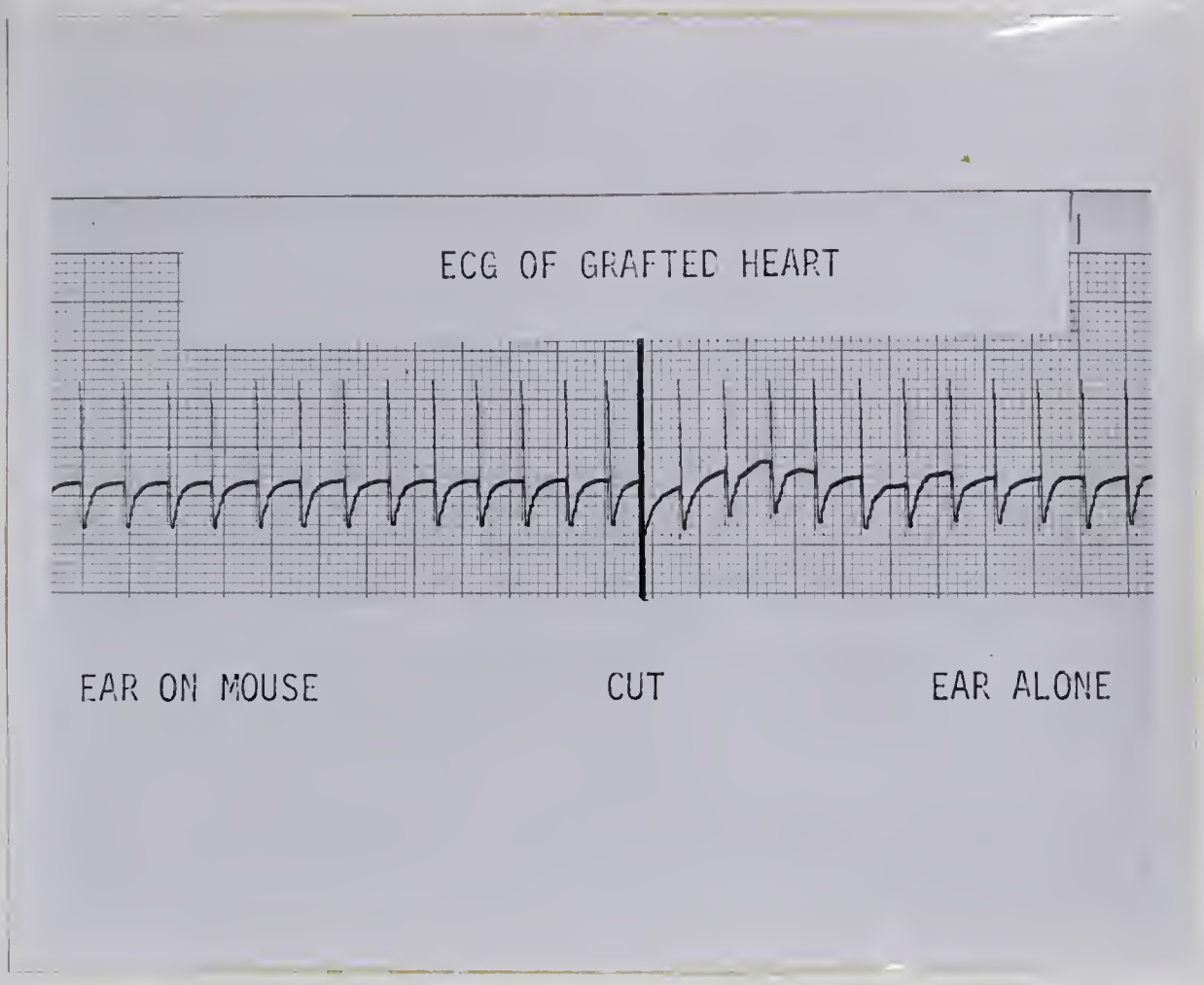

Figure 24. *ECG tracing of cut off ear.* To ensure that the recording was that of the grafted heart,the mouse was connected to the ECG machine and a tracing made; the ear was cut off and the tracing continued. After 30 min the beat strength decreased to zero.

HISTOLOGY

Routine hematoxyline and eosin stained preparations were prepared for assessment of tissue damage, in comparison with controls, using a + to ++++ grading system: + was normal, and ++++ was complete loss of structure. Syncytium, nuclei and fibre cross striation were graded separately. Arbitrary criteria of damage had to be revised as 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 bio-chemical substances (which are released into the blood which attracts cells and causes inflammation).

Each slide was assigned a general overall grading: v - "viable," nv - "not viable". Viable is used here to indicate the cardiac cells looked as if they could live if reimplanted; another very arbitrary assessment but carried out by individuals who had no knowledge of what had been previously done to each heart.

EXPERIMENTAL RESULTS ON FETAL AND NEONATAL MICE

Microwave vs water bath warming. The results are given in Table II.

Immersion Fluid	Time (hours) stored at -196°C	Thawing Method	Electrical Activity 6-15 days		16-25 days		>30 days	
A	72	MW	16/27	59%	16/27	59%	12/27	44%
A	72	WB	8/10	80%	7/10	70%	8/9*	89%
B	72	MW	7/7	100%			6/7	86%
B	216	WB	6/11	55%			6/11	55%
C	72	MW	0/5		0/5		0/5	
C	72	WB	0/5		0/5		0/5	
D	216	WB	0/6		0/6		0/4*	

MW: microwave thawing
WB: water bath thawing
* : change in denominator - mice died

Table II. *Microwave vs water bath warming.* The number, and percentage, of syngeneic ear-implanted fetal hearts, surviving 6th and 55th days after implantation. These initial experiments show comparison between four solutions used for freeze preservation, and two methods of thawing.

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. In previous experiments (101) electrical activity of similar transplants had existed for periods in excess of one year.

The recovery of electrical activity for the frozen-thawed hearts is shown in Table II. Such activity was monitored at intervals

over the period of 6 to 30 days after implantation. As in the control groups those hearts which had survived the initial period after grafting continued to beat in excess of 90 days. There were no differences in electrical activity between control implants and those which had survived freeze-thawing, when analysed for the nature of the electrical complexes or their rate.

The results show that solutions A and B, Eagle's MEM or McCoy's 5a, both also containing 10% fetal calf serum, 10% dimethly-sulfoxide, and Hepes buffer, permit significant survival, in contrast to solution C (Cross solution with 10% DMSO but not containing Hepes buffer or fetal calf serum) and solution D (Eagle's MEM, with fetal calf serum and Hepes buffer but not containing DMSO). Thus, if the results for solution A and B are pooled, the overall survival between 6 - 15 days is 37 of 55, or 67%, and at more than 30 days is 32 of 54, or 59%. No survival was obtained with solution C or D.

From these results, it is concluded: a) that both DMSO and fetal calf serum may be important components for survival of fetal mouse hearts exposed to freeze-thaw injury; b) that microwave thawing is as effective as thawing in a water bath, and 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 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 time of graft implantation.

Fetal and neonatal hearts frozen in different protective agents. As control, Balb-C mice received direct syngeneic ear transplants of unfrozen, neonatal hearts. These control hearts were implanted after the addition of the various protective agents over a period of 30 min at 4°C. These hearts usually showed normal electrical activity by the 5th day of the transplant and continued for periods in excess of 30 days. The graft was then removed for histological studies.

The recovery of the electrical activity of the frozen-thawed hearts after treatment with different concentrations of various protective agents is shown in Table III. 15% and 10% concentration of EG (v/v) are protective, but the time for resumption of electrical activity following transplantation is longer than for the controls, and the amplitude of the beat is approximately 50% of the control. 5% EG only gave 36% recovery when followed for more than two weeks after implantation with the amplitude of the beat being 1/4 of the control.

10% and 5% (v/v) methanol added at 4°C and 37°C over 30 min was not toxic to the controls, but these concentrations offered no cryoprotection. DMSO was most protective at 10% concentration with the amplitude of the beat being the same as the controls. 5% DMSO gave electrical recovery of 87% and 85% in the first and second week with a fall to 50% of the control in the third week. The contraction strength of these hearts was less than the controls. DMSO at a concentration of 2.5% only gave 1/3 the protection of 10% DMSO. Dextran (mean MW = 17,700D) gave 89% recovery in the controls

TABLE III. FETAL HEARTS FROZEN IN DIFFERENT PROTECTIVE AGENTS

Protective Agent	Temperature Protective Agent Added	Electrical Activity 5-7 days		Beat Strength** Mean	10-15 days		Beat Strength Mean	20-30 days		Beat Strength Mean
			%			%			%	
Control 15 % EG	Added at 4°C	20/20	100	3.89	15/16*	94	3.6	13/13	100	3.53
Control 5% EG	"	10/10	100	3.2	4/9	90	3.3	6/9	66	2.5
Frozen 15% EG	"	16/27	59	1.6	19/20	95	1.8	16/17	94	2.1
Frozen 10% EG	"	15/17	88	2.4	14/14	100	2.1	11/12	91	1.88
Frozen 5% EG	"	3/21	16	1.0	8/21	36	1.0	8/18	36	1.16
Control 10% Met.	Added at 4°C	8/8	100	3.63	8/8	100	3.29	8/8	100	3.75
Control 5% Met.	"	10/10	100	3.6	10/10	100	4.0	10/10	100	4.0
Frozen 10% Met.	"	0/22	0		2/18w	3.7	1.0	0/18	0	
Frozen 5% Met.	"	0/14	0		0/11	0		0/5	0	
Control 10% Met.	Added at 37°C	18/19	84	2.69	15/18	83	2.53	13/17	76.5	2.54
Frozen 10% Met.	"	2/24w	8	1.0	0/23	0		0/23	0	
Control 10% DMSO	Added at 4°C	11/11	100	3.73	7/7	100	4.0	4/4	100	4.0
Control 5% DMSO	"	7/8	87.5	3.71	8/8	100	3.5	7/7	100	3.33
Control 2.5% DMSO	"	8/8	100	3.0	4/4	100	3.2	3/3	100	4.0
Frozen 10% DMSO	"	12/12	100	3.56	12/12	100	3.13	12/12	100	3.9
Frozen 5% DMSO	"	12/13	87.5	1.5	11/12	85	2.6	5/8	50	3.0
Frozen 2.5% DMSO	"	6/25	24	1.3	9/24	37.5	1.0	6/20	30	1.2
Control 15% Dex.	Added at 4°C	8/9	89.1	3.9	7/8	85.5	3.3	5/6		3.5
Frozen 15% Dex.	"	0/14	0		0/14	0		0/10	0	
Frozen 10% Dex.	"	0/14	0		0/14	0				
Control 10% Plur.	Added at 37°C	8/8	100	3.1	6/8	75	3.2	7/8	87	3.7
Frozen 10% Plur.	"	0/15	0		0/12	0		0/12	0	

* Change in denominator (mice died)

** 5 - 10 mm = 1^+
11 - 20 mm = 2^+
21 - 30 mm = 3^+
31 or over = 4^+

tested in the first week, but neither 15% or 10% gave cryoprotection to frozen-thawed samples. Pluronic F68 (mean MW = 8,350D)(96) added at 37°C was not toxic to the controls but again 10% gave no cryoprotection to fetal hearts. Glycerol at 10% concentration was not toxic when added over 90 and 60 min at 4°C, and 30 min at 37°C (Table IV). The amplitude of the beat was comparable to that of the other controls. The 10% glycerol added over 45, 60 and 90 min at 4°C gave poor survival of the frozen-thawed hearts with the amplitude of the beat in those that had electrical activity being 1/3 of the controls. The glycerol added over 90 min at 4°C gave a little stronger beat. Variation of freezing rates of 20°C/min and 200°C/min with glycerol (added over 60 min at 4°C) also gave poor recovery. It was only when 10% glycerol was added at 37°C (over 30 min) that good recovery comparable to DMSO and EG occurred with the amplitude of the beat being a little stronger than EG but not as strong as the DMSO preserved hearts (Table IV)(Figure 25).

Neonatal mice hearts from day 1 to 9 were frozen in 10% DMSO (Table V). After implantation of neonatal hearts day 1 to day 5, good electrical activity was seen at week 1 but this then declined in the second and third week. The strength of the beat was less than the fetal controls, except the day 1 neonatal with 5% DMSO giving the same beat strength. Implantation of heart tissue obtained 6, 7 and 9 days after birth was associated with good recovery of electrical activity only if the hearts were sectioned in half before being transplanted. This probably indicates that poor recovery was due to inadequate diffusion of metabolic nutriments into those larger hearts

TABLE IV. FETAL HEARTS FROZEN IN GLYCEROL

Protective Agent	Temperature and Time Protective Agent Added	Electrical Activity 5-7 days	%	Beat Strength** Mean	10-15 days	%	Beat Strength Mean	20-30 days	%	Beat Strength Mean
Control 10% Gly	Added over 90 min at 4°C	6/6	100	2.4	5/5	100	3.8	5/5	100	3.8
Control 10% Gly	Added over 60 min at 4°C	24/24	100	3.1	14/14	100	3.6	13/14	93	3.7
Control 10% Gly	Added over 30 min at 37°C	23/24	96	3.5	20/23	87	3.5	17/20	85	3.5
Frozen 10% Gly	Added over 45 min at 4°C	4/10	40	1.0	5/10	50	1.3	3/10	30	1.0
Frozen 10% Gly	Added over 60 min at 4°C	1/8	11	1.0				4/9	44	1.0
Frozen 10% Gly	Added over 90 min at 4°C	2/13	15	1.0	6/13	46	1.6	4/11	36	1.8
Frozen 10% Gly at 20°C/min	Added over 60 min at 4°C	7/21	33	1.0	5/19	33	1.0	6/13	46	1.0
Frozen 10% Gly at 200°C/min	Added over 60 min at 4°C	6/28	21	1.0	4/22	18	1.0	2/16	13	1.0
Frozen 10% Gly	Added over 30 min at 37°C	17/31	55	1.6	28/29	97	2.7	25/27	93	2.5

** 5 - 10mm = 1^+
11 - 20mm = 2^+
21 - 30mm = 3^+
31 or over = 4^+

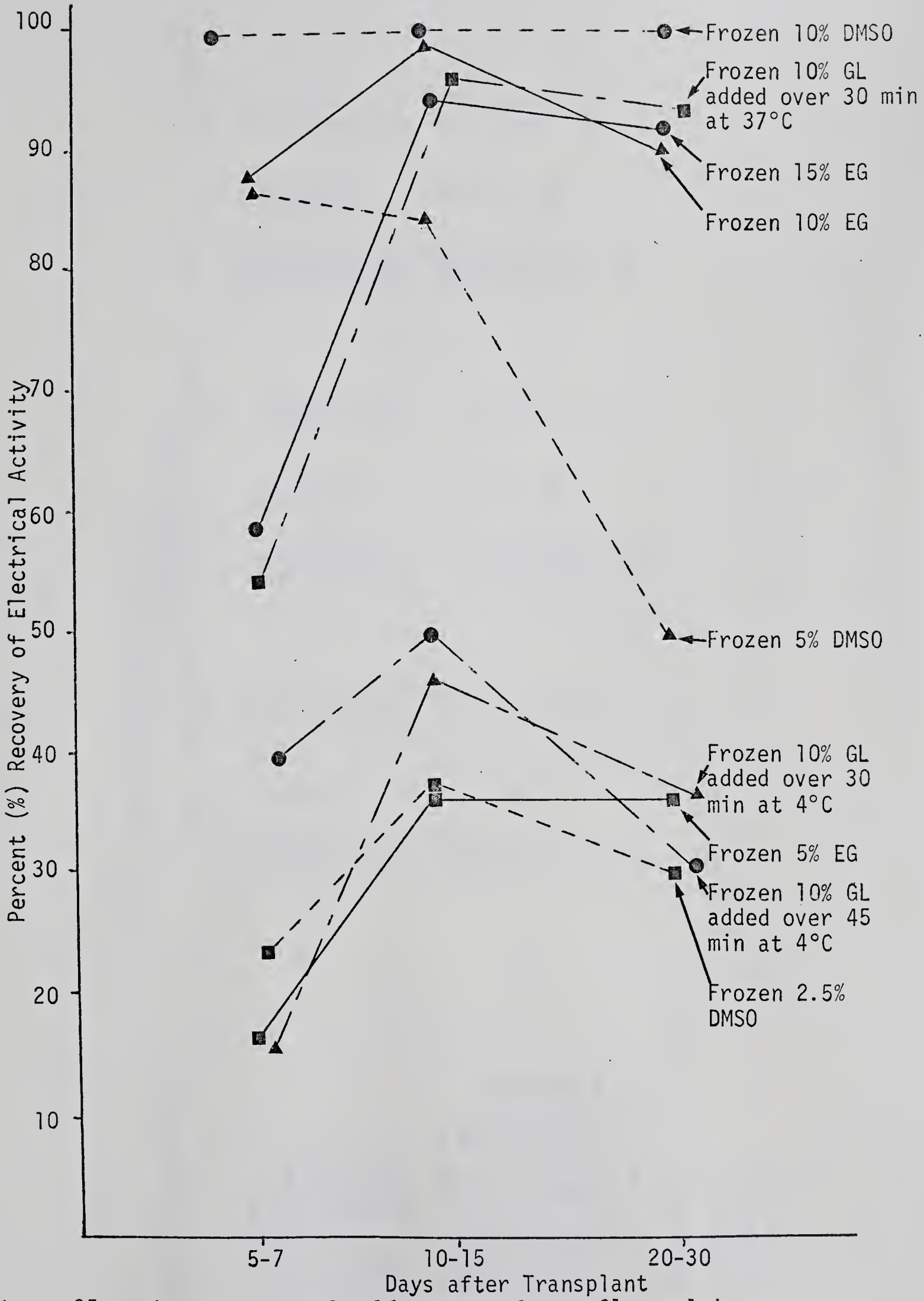

Figure 25. *Plot of parts of Tables III and IV.* Glycerol is as protective as DMSO and EG when added at 37°C.

TABLE V. NEONATAL HEARTS FROZEN IN 10% DMSO

Protective Agent Added at 4°C	Electrical Activity 5-7 days		Beat Strength** Mean	10-15 days		Beat Strength Mean	20-30 days		Beat Strength Mean
		%			%			%	
Control 1 day neo.	21/23	91	2.8				10/19	52	2.8
Control 1 day neo.*	8/8	100	3.7	7/8	87	3.5	6/8	75	3.3
Control (WH) 2 day neo.	9/11	82	2.1	6/11	54	2.5	4/11	32	2.5
Control (WH) 5 day neo.	9/10	90	1.8	5/10	50	1.0	7/10	70	2.0
Control (WH) 6 day neo.	9/12	62	2.1	9/10	87	2.5	4/9	42	2.3
Control (SH) 6 day neo.	8/8	100	2.5	8/8	100	2.4	7/7	100	2.1
Control (WH) 7 day neo.	1/3		2.0	0/3			0/3		
Control (SH) 7 day neo.	3/4		3.0	3/4		3.0	3/4		3.0
Control (SH) 9 day neo.	6/6		2.33	6/6		2.5	6/6		4.0
Frozen 10% DMSO 1 day neo.	22/25	88	2.35				16/24	66	2.6
Frozen 10% DMSO 2 day neo.	11/16	69	1.6				8/16	50	2.5
Frozen 10% DMSO (WH) 5 day neo.	6/9	67	1.2	3/7	43	1.0	0/6	0	
Frozen 10% DMSO (WH) 6 day neo.	1/6		1.0	2/5		1.0	1/4		1.0
Frozen 10% DMSO (SH) 6 day neo.	5/8	62	1.2	4/6	66	1.0	2/5	40	1.5
Frozen 10% DMSO (WH) 7 day neo.	1/4		1.0	2/4		1.0	1/3		1.0
Frozen 10% DMSO (SH) 7 day neo.				8/8	100	1.4	4/8	50	1.2
Frozen 10% DMSO (WH) 9 day neo.	1.4		1.0	0/3			0/3		
Frozen 10% DMSO (SH) 9 day neo.	3/5		1.0	2/5		1.0	2/5		1.0
Frozen 10% DMSO - Adult septum	0/11			0/9			0/9		

* 5% DMSO added before implantation.

** 5 - 10mm = 1^+
11 - 20mm = 2^+
21 - 30mm = 3^+
31 or over = 4^+

and removal of metabolic end products. In the frozen-thawed group the same trend is seen. Good recovery occurred with 1 to 5 day old neonatal hearts, with a decrease in the second and third week following transplantation. The 6, 7 and 9 day neonatal hearts gave good recovery only if they were cut in half before transplantation, and even then the beat strength was decreased as compared to the controls. Thus, even post-natal larger hearts survive the freeze-thaw insult when supplied with sufficient nourishment to sustain tissue vitality.

Examples of histology slides are shown in Figure 26, 27, 28 and 29. Frozen-thawed hearts that had good electrical activity had good histological integrity when compared to control histology for each group. Large hearts showed an outer rim of viable cell with an inner necrotic centre. Non-viable hearts showed overall necrosis of the graft with oscification in some areas.

The internal carotid of some of the mice were cannulated and the vessels injected with microfil. Figure 30 shows the circulation of the ear with the small capillary supplying nutrients to the transplanted heart.

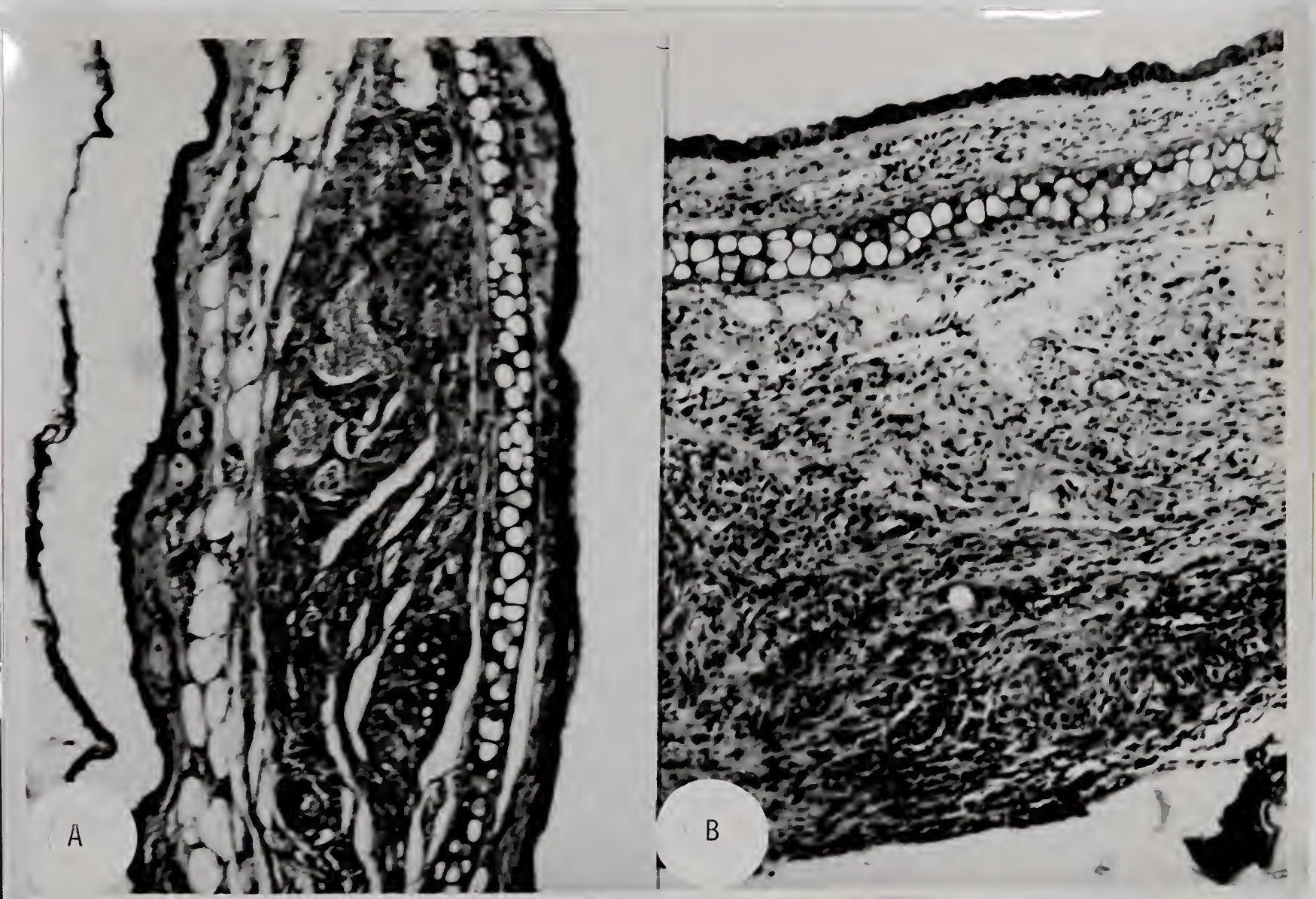

Figure 26. *Glycerol (10%) added to control and frozen-thawed fetal hearts.* A) Frozen in 10% glycerol added at 37°C. *Interpretation* - syncytium - 3, nuclei - 2. General comment - "Probably viable". B) Control with 10% glycerol added at 37°C. *Interpretation* - syncytium - 3, nuclei - 1. General comment - "Viable". X 100 . Hematoxyline and eosin.

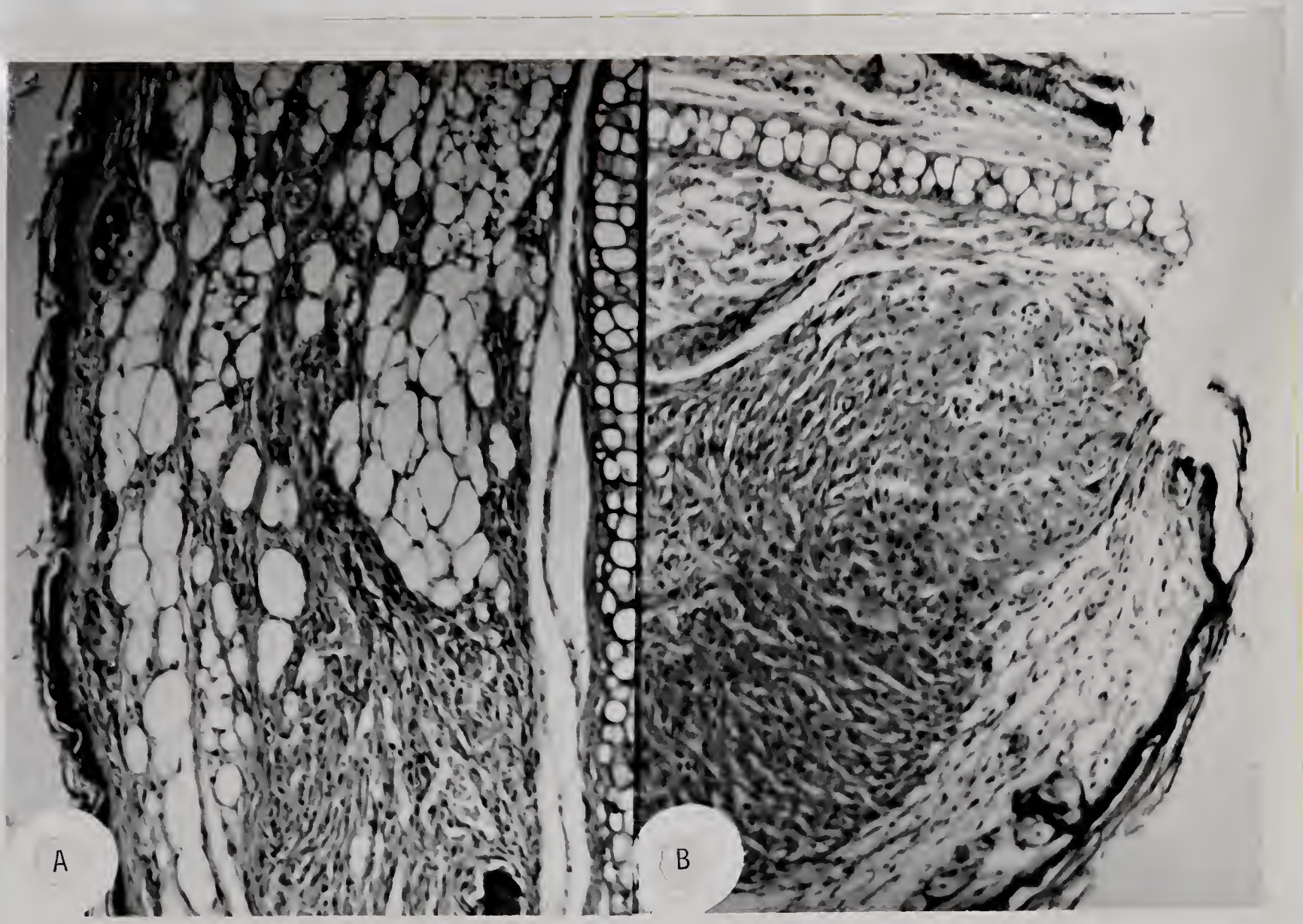

Figure 27. *Control and frozen-thawed fetal hearts with 15% EG added.* A) Fetal heart frozen in 15% EG. *Interpretation* - syncytium - 1, nuclei - 2. General comment - "Viable". B) Controls with 15% EG added. *Interpretation* - syncytium - 2, nuclei - 2. General comment - "Probably viable". X 100. Hematoxyline and eosin.

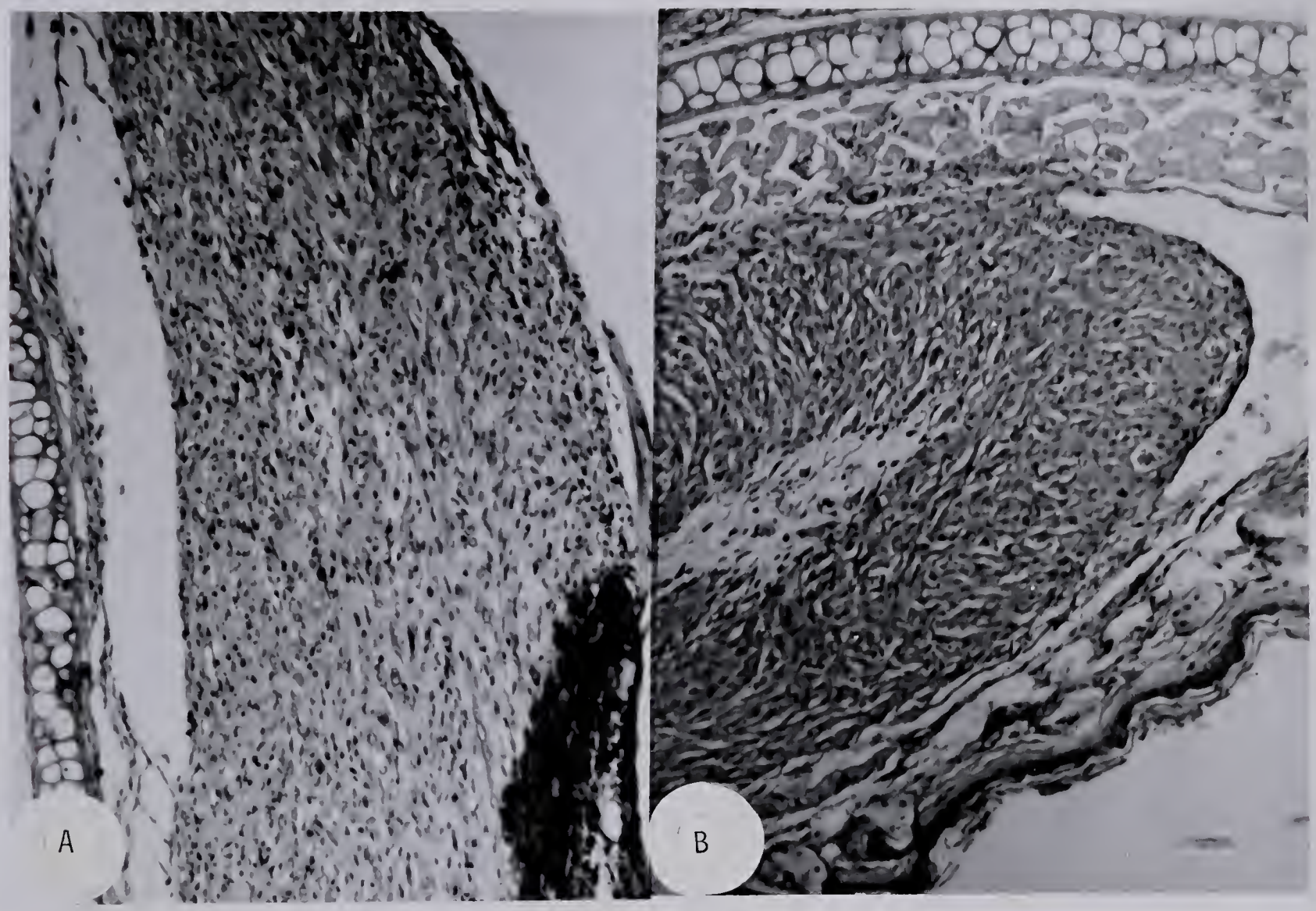

Figure 28. *DMSO (5%) added to controls and frozen-thawed fetal hearts.* A) Frozen in 5% DMSO. *Interpretation* - syncytium - 2, nuclei - 2. General comment - "Viable". B) Control with 5% DMSO added. *Interpretation* - syncytium - 2, nuclei - 2. General comment - "Viable". X 100. Hematoxyline and eosin.

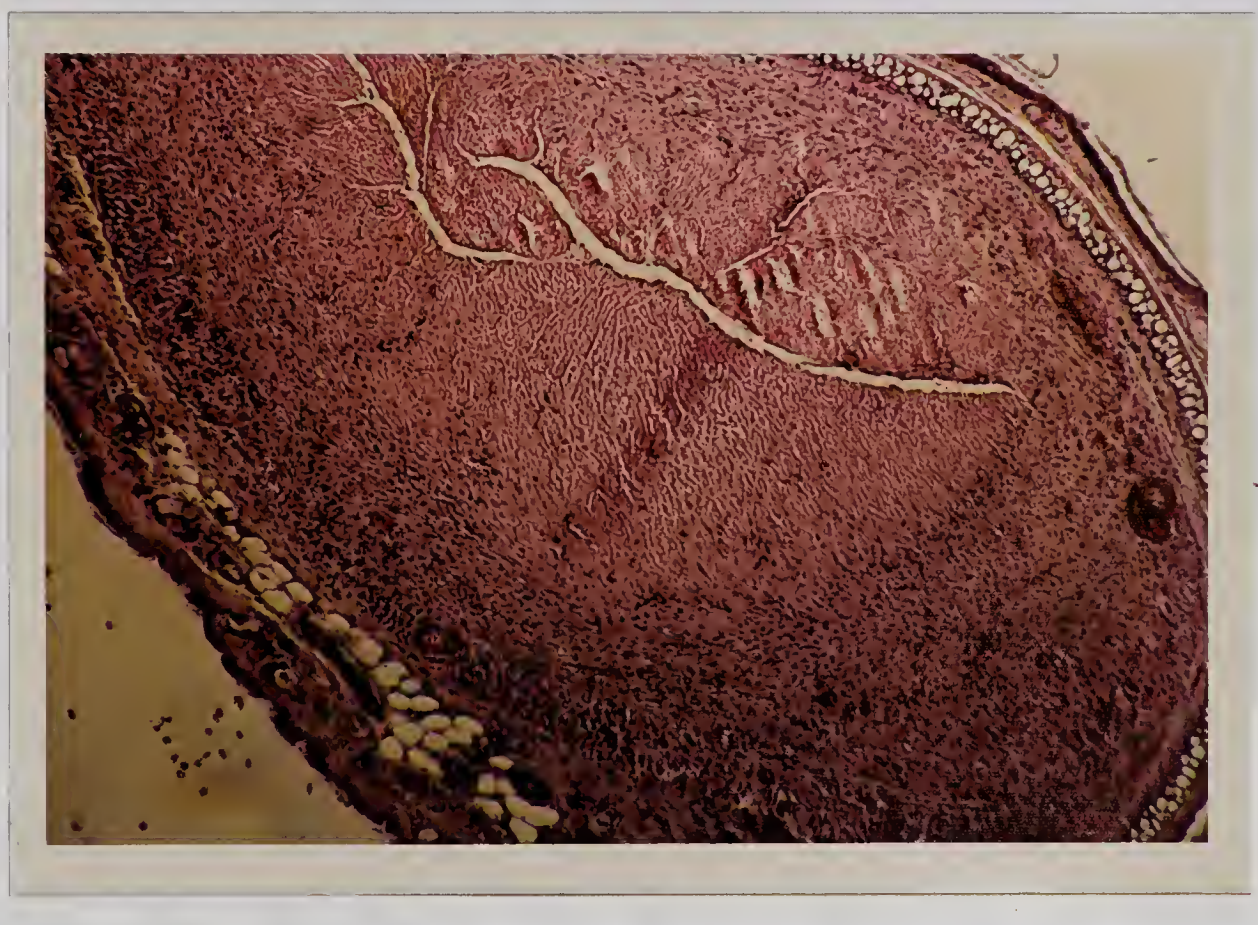

Figure 29. *Nine day neonatal control whole heart.* A large whole heart with a necrotic centre and viable outer layer of cells. *Interpretation of outer layer* - syncytium - 3, nuclei - 2. General comment - "Viable", "Inner is Necrotic". X 100. Hematoxyline and eosin.

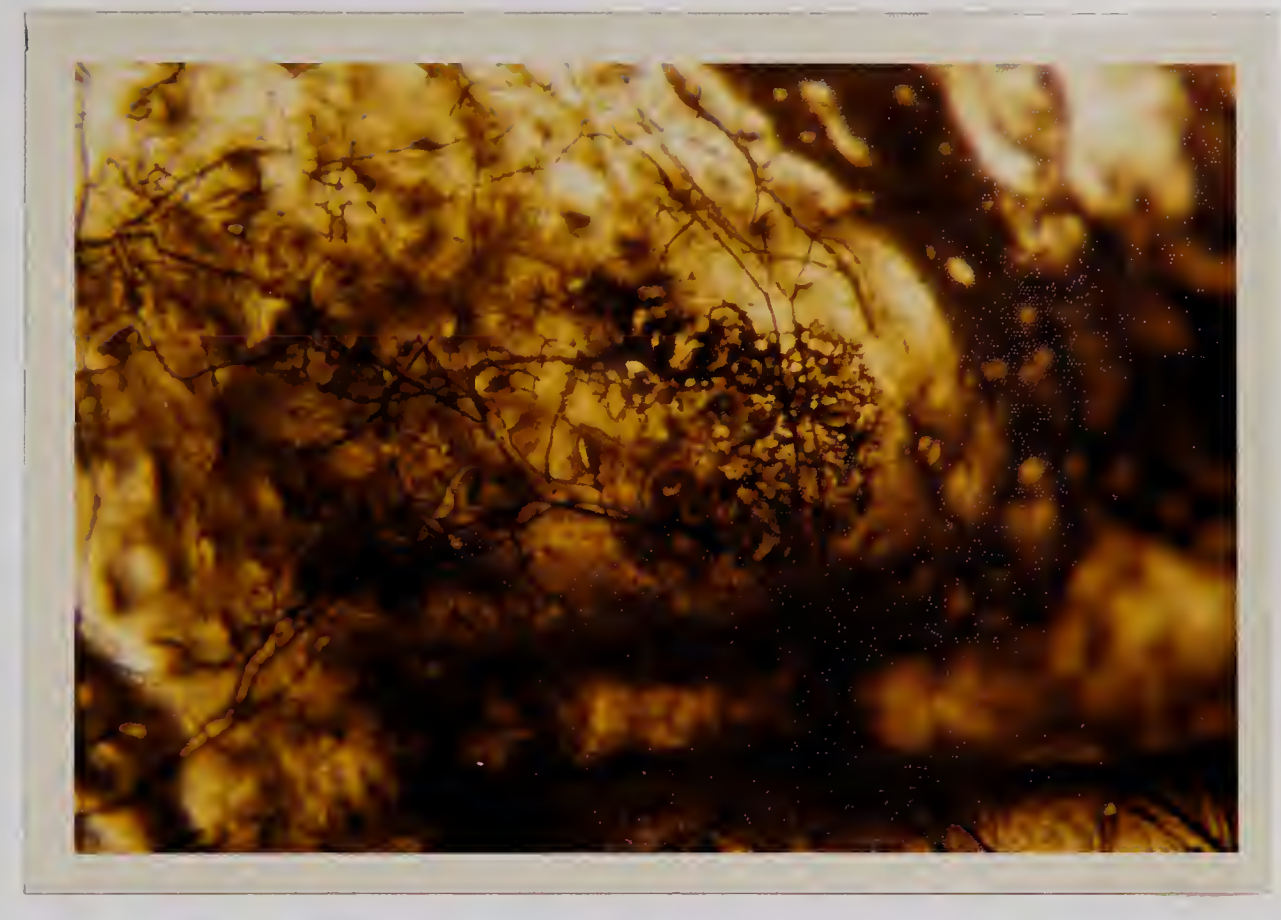

Figure 30. *Microfil injected heart.* The circulation of the ear is seen with the fine network going into the tranpslanted heart. X 5.

DISCUSSION

In our search to establish possible methods for the preservation of mammalian organs in a deep frozen state, and their subsequent recovery, we have previously concluded that electromagnetic energy in a limited frequency range is essential for both the uniformity of thawing and the control of the thawing rate (99). From a consideration of the penetration depth of the wave, frequencies in the range from 500 to 5000 MHz (5 to 50 X 10^8 cycles per sec) are of possible interest; it was arbitrarily decided to chose a convenient frequency in the middle of this range, 2450 MHz. It has been shown that microwave thawing at this frequency is an acceptable method for recovering tissue culture cells from liquid nitrogen storage (102) and that, in the case of canine kidneys, perfused with, for example, 10% (v/v) DMSO and fluorocarbon, uniformity of heating from -79°C to 20 ± 10°C is possible (99). The microwave insult is not inconsiderable in these cases: to achieve thawing rates above 100°C/min in an adult organ, the absorbed power density is of the order of 10 watts/gm. The electric field strength associated with the wave corresponds to an average value just less than 100 v/cm; its actual value for any thawing rate depending upon as yet unknown dielectric quantities, which others are also attempting to measure.

The electrical field strength variation experienced by a thawing heart will vary widely as the 1 mm organ in the 5 ml sample is rotated randomly through the resonant electromagnetic fields of the cavity, which is resonant in many (>20) modes. Only a very

small fraction of this stored energy is absorbed in the heart and the solution in which it is frozen; corona discharge was observed in parts of the cavity, indicating the presence of high local electric field strengths. The absorption of energy in the sample increases very rapidly above -10°C; in a microwave cavity it was not possible with the equipment available to monitor the rate between, for example, -60 and -15°C (possibly the most critical range in freezing and thawing). Consequently a mean rate (from -196°C to +10°C) slightly above that possible in a water bath was established in order to make a comparison: the rates for the water bath and the microwave thawed hearts sould be about the same in the -60 to -15°C range.

The results reported in this thesis show that the survival of electrical activity of frozen fetal mouse hearts occurs with microwave thawing in a multicellular system. This is apparent from studying a limited number of hearts. There is no significant difference between the two methods of thawing. However, solution A and B are clearly significantly different from C and D and this was the basis for using the model to test different cryoprotective agents.

The model has proven to be very useful for the testing of different protective agents on myocardial syncytium. As shown in the literature (9), EG and DMSO are protective even at low concentrations. Glycerol is as protective as DMSO and EG but only if it is added at 37°C, however, 5 and 10% DMSO gives a stronger beat following implantation. This would seem to indicate that, at the lower temperature, glycerol does not permeate cells even if 90 min

is allowed for equilibration. The other agents were not cryoprotective; this could be due to a lack of diffusion of Dextran and F68 into the tissue. None of the protective agents were toxic to control transplants. In neonatal hearts, size became a critical factor, good survival being obtained only if larger hearts (day 6 - 9) were cut in half. Histological examination showed that the larger 6, 7 and 9 day old whole heart implants had central necrosis but apparently viable outer zones in those hearts with measureable electrical activity (Figure 29). The DMSO, EG and glycerol protected hearts had histological integrity that was the same as the controls.

SUMMARY

Hearts removed from 17 - 19 day fetal and 1 - 9 day neonatal mice were frozen in liquid nitrogen and tested for electrical activity after rewarming. After exposure to various cryoprotective agents, hearts were cooled at 0.5 - 0.7°C/min and at 20 and 200°C/min to -100°C and then stored in liquid nitrogen for periods between 72 and 216 hours. Exposure to controlled microwave at 2450 MHz or immersion in a water bath at 25°C was used in thawing. Histological examinations were performed after electrical activity (QRS) had been studied for periods in excess of 30 days after subcutaneous implantation into the ear of syngeneic adult mice. EG, DMSO and glycerol appear equally protective provided that glycerol is added at 37°C. Methanol, although non-toxic at those concentrations

which will protect tissue culture cells, does not offer protection to this type of organized tissue. None of the high molecular weight compounds tested offered cryoprotection in this system. Diffusion of both protective agents and nutrients have been shown to be limiting factors in the survival of frozen-thawed neonatal hearts between 6 - 9 days of age.

CHAPTER IV

ISLETS OF LANGERHANS FROZEN TO -196°C

INTRODUCTION

The last and most complex tissue looked at was the islet of Langerhans from adult animals. This organ has the complex function of regulating the blood glucose. Of particular interest and importance would be technique to preserve function, after freeze-thawing, the cells in the islets that are responsible for insulin secretion, the beta cells. Long term preservation of adequate numbers of histo-compatible islets is a prerequisite to any attempt to manage diabetes mellitus in man by islet transplantation. If organ size (or tissue thickness) represents the diffusion limit either for cryoprotective agents or nutrients, other organs in this size range give hope for banking. In the progression of organ size and complexity (fetal heart $\simeq 1X10^6$ cells, islet $\simeq$10,000 cells), the islets are within these boundaries, and are of extensive clincial significance. This Chapter describes the freezing and thawing of islets, using perifusion as the *in vitro* test and implantation as the *in vivo* test.

METHOD AND MATERIALS

In all experiments, non-fasted Wistar male rats ranging in weight from 200 to 260 gm were used. In all experiments, control rats, anaesthetized with dibutal (0.04 mg/gm weight), had the islets harvested as in the experimental group. The islets from these animals should have optimum insulin secreting potential to which the experimental groups can be compared.

Isolation of Islets of Langerhans. The islets were harvested from the pancreas using a modification of the method of Lacy and Kostianovsky (72). The rat was anaesthetized with dibutal, the abdomen was shaved and painted with iodine. At laparotomy the duodenum was first immobilized to facilitate cannulation of the common bile duct. A ligature of 4-0 silk was tied where the common bile duct enters the duodenum and a snap attached at the end of the tie for subsequent traction. The duct was exposed just below the junction of the hepatic duct and a 4-0 silk placed around the duct, for subsequent use in holding the cannula in place(Figure 31). With fine iris scissors, the exposed area, of common bile duct was incised, after applying tension by the distal duct suture. A PE10 polyethylene cannula was introduced and tied into the duct with 4-0 silk. 15 ml of Hank's balanced salt solution (HBSS) at 4°C was injected into the pancreas via the cannula. This distends the pancreas and disrupts the acinar tissue (Figure 32). The distended pancreas was dissected out of the animal and placed in chilled HBSS. Using small scissors, the pancreas was minced up into small 2 - 4 mm fragments. The minced tissue and solution were then transferred to a 50 ml conical centrifuge tube, placed on ice, and allowed to settle for two minutes. Supernatant was removed and the tissue washed twice with 30 ml of chilled HBSS, allowing 2 min for sedimentation of the fragments between washes. Following the final wash, there would always be 2.5 to 4.0 ml of minced tissue. If the sample was non-sterile, 16 mg of collagenase (Type I Sigma) was added. If the sample was sterile, collagenase(12 mg per cc in HBSS)was passed through a 0.22 μ

millipore filter and then 2 cc added to the minced tissue. The conical tube containing the minced pancreas and collagenase was transferred to a 37°C water bath and agitated vigorously by hand until the acinar tissue was digested from the islets. This disruption took approximately 15 to 18 min. The digestion process was stopped by adding 30 ml of chilled (4°C) HBSS to the tube and then placed on ice to allow sedimentation. Following this 4 - 5 min sedimentation period, supernatant was removed and the tissue resuspended in 10 ml of chilled HBSS and decanted into a 15 ml conical centrifuge tube and 5 min allowed for sedimentation. The supernatant was removed leaving ≈ 1 ml of digested acinar tissue and islets.

A discontinuous density gradient centrifugation was used to separate acinar tissue from islets. Clinical grade dextran of average molecular weight (170,000 D) was dissolved in HBSS in concentrations of 32, 24, 18 and 10%(w/v). In sterile experiments, dextran was autoclaved and then placed in the refrigerator for 24 hr. Digested tissue was resuspended in 32% dextran solution to a volume of 7 ml. Two ml of 24% dextran was carefully layered on top to give a total of 9 ml, followed by 2 ml of 18% (to 11 ml volume) and then to 2 ml of 10%, giving a total of 13 ml. The tube was centrifuged for 15 min at 800 g. During this the islets usually moved up to the 10 and 18% dextran interface, with a large number also at the 18 and 24% interface. Islets at these interfaces were usually very clean, though some nodes and ductil elements were found at the 18 - 24% interface. The isolated islets were then transferred to the 50 ml

conical tubes and washed with Minimum Essential Medium (MEM) containing 10% fetal calf serum (FCS) (v/v) and pencillin (100 units/cc), steptomycin (100 µgm/cc) and glutamine (1%) (v/v). This harvest procedure took approximately 90 min.

Islets used for culture were harvested under sterile conditions. Pancreatic excision and acinar digestion was done under sterile conditions, using sterile glassware and all solutions passed through a 0.22 µ millipore filter.

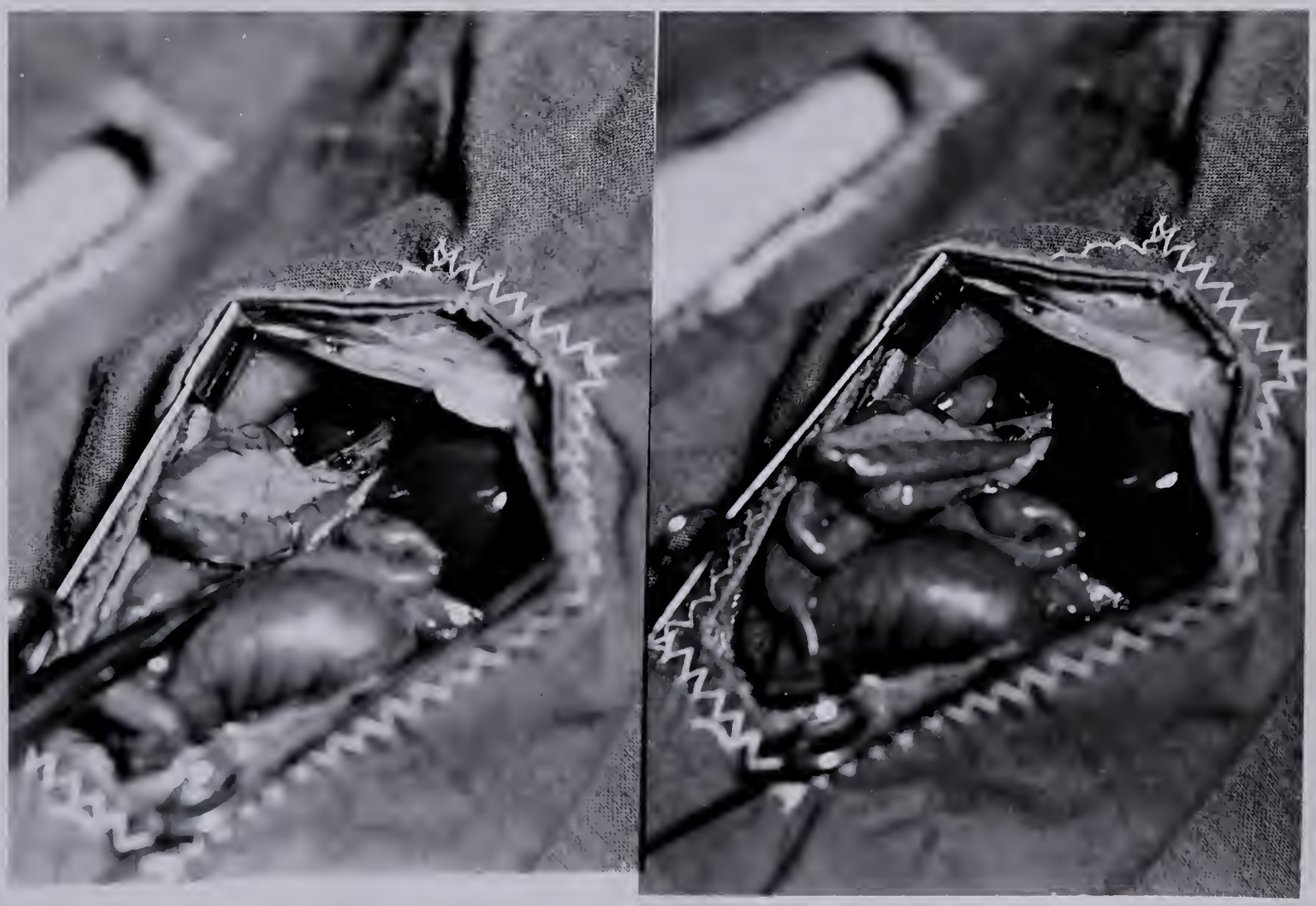

Figure 31. *Common bile duct isolated and cannulated.* The Rt picture shows the exposed common bile duct. The left picture, the PE10 polyethylene tubing tied in place in the duct.

Figure 32. *Distended pancreas.* The two pictures show the pancreas distended with 15 ml of HBSS.

Following the final wash of relatively pure islets, the 50cc tube was centrifuged at 1000rpm for 10 min to form a pellet of islets on the bottom. Supernatant was removed to 9.25 ml or 9 ml, depending on the concentration of DMSO to be used. Islets were resuspended in the supernatant, DMSO was then added at 4°C until reaching required concentration of 7.5% or 10% (v/v). The islets in 10 ml of solution were placed in small freezing vials and

slowly frozen (0.5 - 1°C/min) down to -100°C and then at 20°C/min to -196°C (This system is the one described in Chapter II). Samples were stored at -196°C for periods of 1 - 7 days, followed by thawing in a 37°C water bath. The DMSO was removed from the islets by a stepwise dilution over a period of 30 min at 4°C to a final concentration of .75% DMSO. The diluted islets in some cases were incubated for 48 hr for supposedly optimum metabolic recovery.

Perifusion of isolated islets of Langerhans. The technique of Lacy et al. (103) was used for perifusion of isolated islets of Langerhans. The term perifusion denotes supplying nutrients to an organ not via the vascular system, but surrounding the organ with the perfusate and allowing the nutrients to diffuse into the cells . The major component of the apparatus (Figure 33) is a stainless steel 25 mm reusable millipore filter chamber into which the experimental isolated islets are placed, using 24 mm, 5 micron pore size filter paper. To the outlet of the chamber is attached a piece of silastic tube (2mm ID) which passes through a peristaltic pump calibrated to give a flow rate of 1 ml/min; to the inlet is attached a similar piece of tubing which asperates the perifusion solution from a beaker. The chamber, tubing and beaker are all in a 37°C water bath. The priming volume of the system is ≈ 3 ml and therefore 3 min intervals are required for a change in the perifusion solution to reach the end of the outlet tube which was used as the site of sampling.

Two perifusion solutions were employed; both consisting of HBSS with albumin (0.5%) (v/v) but where one had 30 mg % glucose, the

other had 300 mg %. 30 mg % glucose solution was used for the first 60 min of perifusion, during which time the islet beta cells are believed to become "stabilized" with no stimulus for insulin release. At 60 min the perifusion solution was changed to the 2nd solution which contained 300 mg % glucose in HBSS and albumin. This concentration of glucose is a strong stimulus for secretion of insulin. The whole perifusion sequence was carried out at 37°C and the pH of the solution maintained at 7.3 to 7.4 with bicarbonate. If the solution became too alkaline, a mixture of 95% air and 5% CO_2 was bubbled through it.

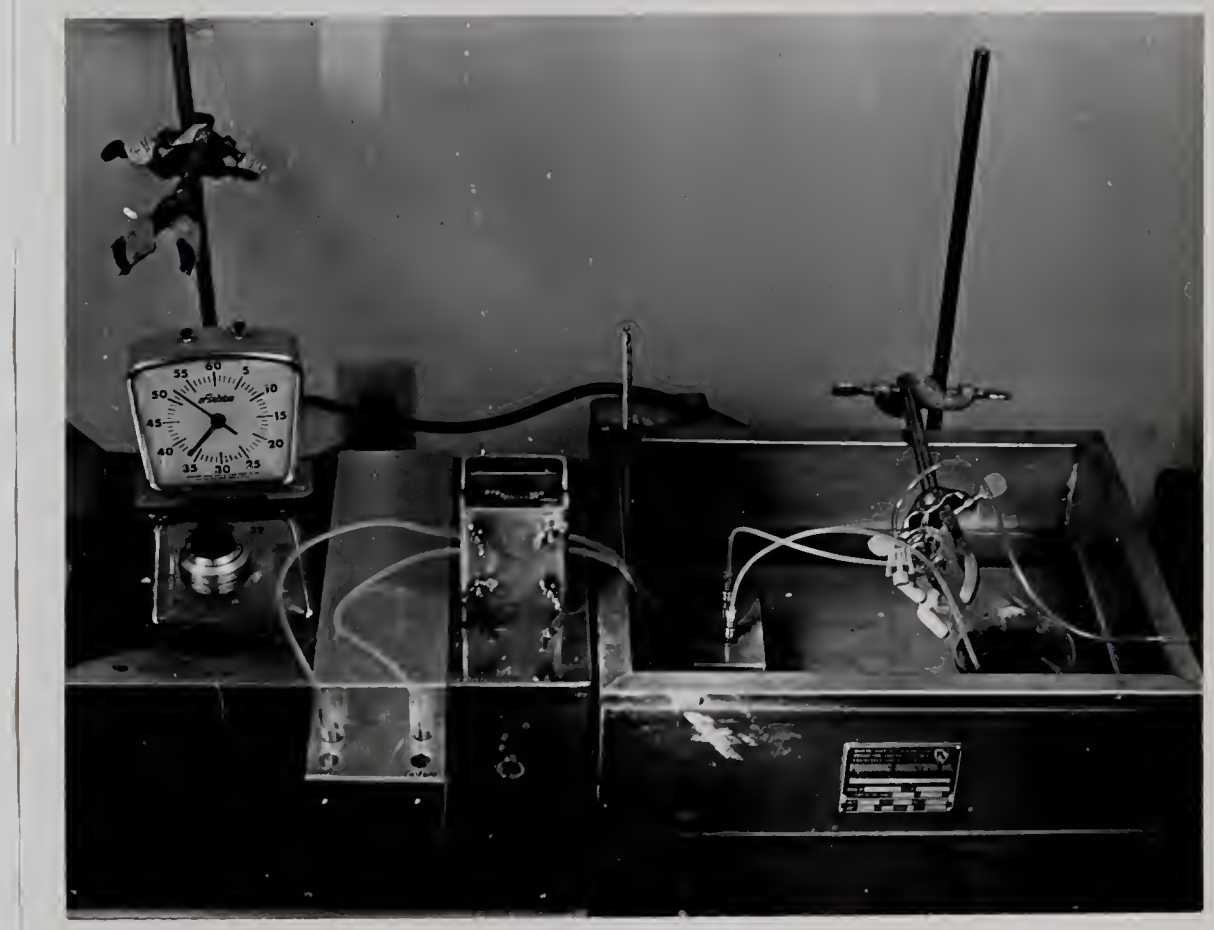

Figure 33. *Perifusion system.* The inlet of the filter holder (with islets) is connected with a silastic tube that goes to the flask holding the perfusate. The outlet goes through the pump and into the collection tubes. The filter holder and perfusates are in a 37°C water bath.

For each study, isolated islets that had been frozen-thawed and cultured for two days, or islets that had just been frozen and thawed, were perifused in parallel with fresh islets. Islets were placed on the millipore filter paper via the inlet of the chamber. Care was taken to insure that all the air was removed from the system.

Samples during perifusion were collected from the outlet tubing as shown in Figure 33. Two perifusion sequences were employed. The one in Figure 36 shows a double stimulation, in that islets were perifused with 30 mg % glucose for 60 min, then challenged with 300 mg % glucose for 80 min, followed by a change to a non-stimulating level of glucose for 45 min and then rechallenged for 100 min. The 2nd perifusion sequence consisted of 60 min non-stimulating perifusion followed by 300 mg % glucose insulin challenge for 130 min, giving a total of 190 min perifusion. Background insulin levels were determined on the solutions before the onset of perifusions. All samples were immediately frozen following collection for subsequent insulin assay.

Following termination of perifusion, the filter was removed from each chamber and covered with 1% Neutral Red Stain(in HBSS solution) for 10 min after washing with HBSS, stained islets were counted under a dissecting microscope at 15 X magnification. In controls, individual islets were stained an intense red, and appeared globular (Figure 34). Frozen-thawed islets, in some cases, did not take up stain as readily as control. Only darkly stained globular islets were counted in these experiments. Counting was performed independently by two individuals

and then averaged.

Figure 34. *Stained islets.* Islets that have been stained with neutral red.

Insulin determination. The content of insulin in each sample taken during perifusion was determined by a modification of the radioimmunoassay technique of Hales and Rundle (104), using the reagents and protocol of the Schwartz/Mann Insulin Radioimmunoassay Kit, with some modification.

The principle of this radioimmunoassay is that the unlabelled insulin in, say, a perifusion sample reacts with specific insulin antibody. This complex is then made into a larger complex by the second antibody, made against the insulin antibody when the

latter is used as an antigen. All this occurs in the first period of 6 hr at 4°C. This larger complex remains in suspension and its insulin molecule (unlabelled) can exchange with I^{125} insulin molecules, competitively in proportion to concentration. This competitive interchange occurs during the 18 hr period at 4°C. After this second refrigeration period the larger complexes are precipitated by centrifuging. The amount of insulin in the unknown is found by comparing its specific radioactivity to a standard curve, which is prepared by adding varying amounts of unlabelled insulin to the appropriate quantities of labelled insulin and antibody binding site. The reagents used in the Schwartz/Mann Radioimmunoassay Kit:

1) 0.04M phosphate buffer

2) 0.04M phosphate buffer with 500 mg bovine albumin powder, Fraction V, in 100 ml.

3) Insulin (Human) standard solution 200 μU/ml

4) Insulin binding reagent which is porcine insulin antiserum raised in guinea pigs, and precipitated with anti-serum to guinea pig immunoglobulin (IgG) raised in rabbits.

5) Insulin(^{125}I) is porcine insulin 1.25 mg/ml and labelled with 0.125 μCiI^{125}

6) Perifusion sample

PROCEDURE

The assay was carried out in 12 X 75 mm polystyrene test tubes. Polystyrene tubes have no affinity for the antibody under the conditions of the assay and also can be safely

taken through the centrifugation step. All aliquoting of reagents were performed with Oxford Sample Micropipets.

The initial stage in the assay was to combine unlabelled human insulin standards or unknown samples with the binding agents and an appropriate amount of phosphate buffer to make the volume of the reaction mixture ≃ 200 µl. The volumes added are shown in Table VI. Tube 1 determined the background radioactivity of this system. Tube 2-9 were used to construct the standard curve, which was performed in triplicate. Tube 10 represented the unknown perifusion samples, measured in duplicate. Each full assay involved 200 tubes consisting of 4 background, 24 standards and 172 unknowns. These reaction mixtures were incubated for 6 hr at 4°C before adding insulin (^{125}I) to allow for large complexes to form in solution, thus 100 µl of insulin (^{125}I) was added to each tube which was then placed in the refrigerated 4°C Gamma counter (Packard Gamma Scintillation Spectrometer,model 578) for a further 18 hr incubation period to allow I^{125} insulin to interchange competitively with insulin in the large complex and duplicate counts made on each tube to obtain total counts per minute (TCPM). Following the 18 hr, 1 ml of phosphate buffer was added to each tube and vortex mixed. The tubes were then centrifuged at 4500 g for 20 min at 4°C. This was used to precipitate the antigen-antibody complexes. The supernatant was gently decanted off and the tubes recounted 3 times in the gamma counter to give residual counts per minute (RCPM).

Tube #	Insulin Content (μU)	Insulin Standard (μl)	Binding Reagent (μl)	Phosphate Buffer (μl)	TCPM corrected	RCPM corrected	% Bound
1	0			200	1040 = background		
2	trace		100	100	4572	11877	38.5
					4257	11777	36.1
3	0.4	2	100	100	3840	12183	31.5
					3697	12017	30.8
4	1.0	5	100	100	3507	12144	
					3376	12402	27.2
5	2.0	10	100	100	2417	12103	20.0
					2559	12300	20.8
6	3.0	15	100	100	1922	12457	15.4
					1857	12316	15.1
7	5.0	25	100	100	1286	12325	10.4
					1375	12360	11.1
8	10.0	50	100	100	674	12030	5.6
					757	12119	6.2
9	20.0	100	100	50	429	12147	3.5
					324	11629	2.8
10	unknown	?	100	100			

Table VI. *Experimental protocol.*

Calculations for amounts of insulin in experimental samples.

The total counts and the residual counts per min are now known for each tube. The RCPM in tube 1 is the background radioactivity of the assay. The average was taken and subtracted from the total CPM and the RCPM to give corrected total CPM and corrected residual CPM. To find the percentage of labelled insulin bound to the binding reagent divide corrected residual CPM by corrected total CPM and multiply by 100.

$$\text{Percent Bound} = \frac{\text{Corrected Residual CPM X 100}}{\text{Corrected Total CPM}}$$

The standard curve is constructed by plotting on semilogarithmic graph

paper the percent bound vs the known standard insulin (μU/tube) added in tubes 2 to 9 (Figure 35).

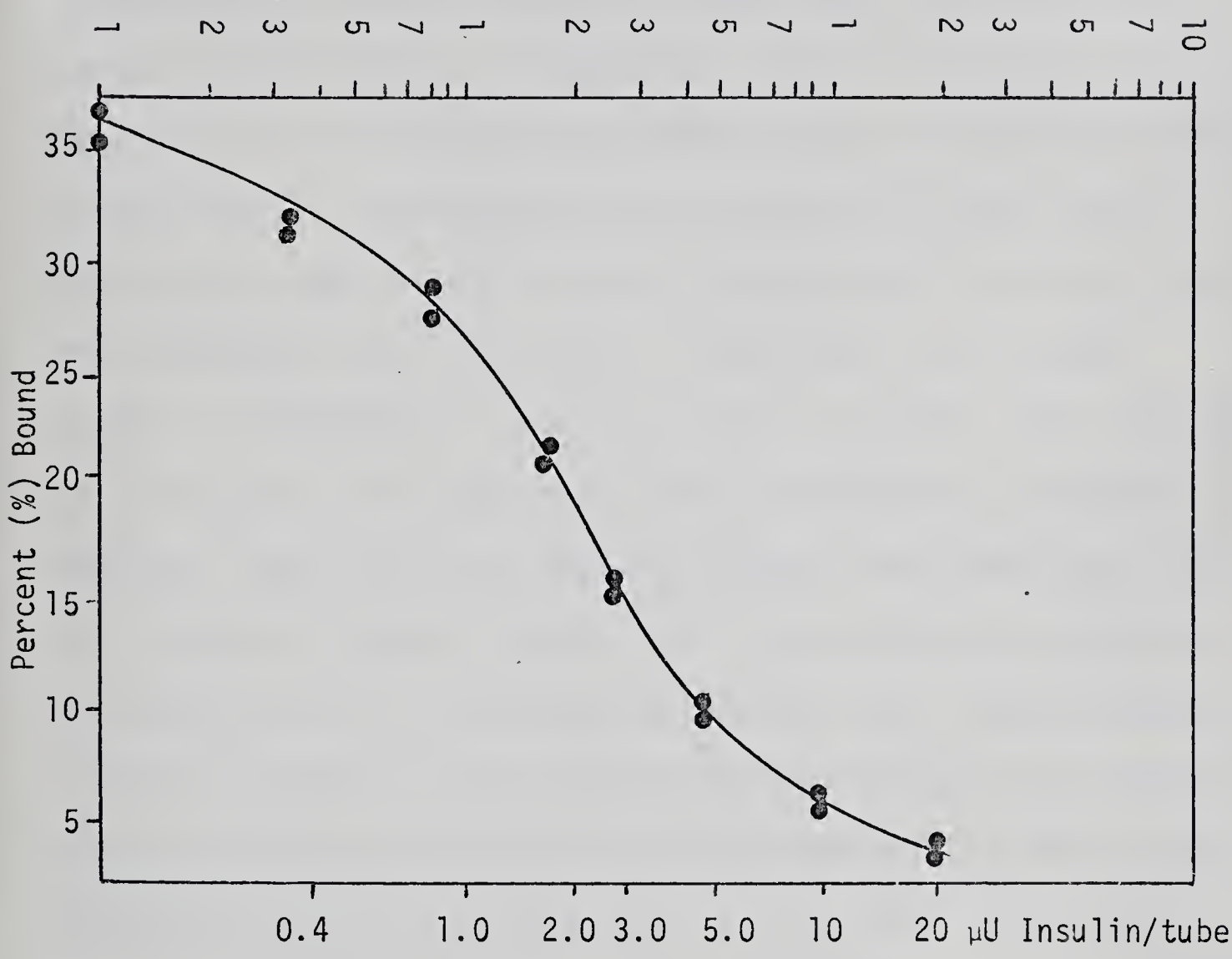

Figure 35. *Standard Curve*. Values taken from Table VI.

The percent bound for the perifusion samples was compared with the standard curve and the amount of insulin (μU/tube) was found. A dilution was used which was 1/40 of the unknown, therefore each unknown was muliplied by 40 to get the insulin concentration (μU/ml). The final concentration value for each perifusion samples

was taken as the average of the duplicate samples which were used to get insulin secretion (μU/islet/min).

Transplantation. One Lewis rat made diabetic by injecting streptozotocin (65mg/kg) into the femoral vein, was examined for several weeks following the injection. The rat was placed in a metabolic cage and urine volume, urine glucose and weight loss were measured daily. Blood glucose was also done on a weekly basis. This rat was then transplanted with frozen-thawed islets that had been harvested from 4 Lewis rats. These islets were frozen slowly in 7.5% DMSO and stored at -196°C for several days, followed by thawing in a 37°C water bath. DMSO was removed by a stepwise dilution. The islets were grouped together, spun down slowly and the supernatant removed leaving 3 ml. The pellet was resuspended and drawn up in a 3 cc syringe and placed on ice. The peritoneal cavity of a diabetic rat, anaesthetized with dibutal, was opened by midline incision and the portal vein identified. The islets were injected through a 22 gauge needle into the liver via the portal vein. Once the needle was removed, gel foam and pressure was applied for 1/2 hr to stop bleeding. The abdomen was closed in two layers and the post-operative clinical course closely observed using a metabolic cage to permit urine collection. Urine volume, urine glucose and weight were measured daily and blood glucose was done weekly.

Electron microscopy. An electron microscopic study was done on a group of islets harvested in the usual manner. The control group had DMSO added slowly over 30 min to a final concentration of 7.5% at 4°C. The protective agent was slowly removed by a stepwise

dilution to a final concentration of 0.75%. These samples were then fixed in gluteraldehyde and prepared for electron microscopic examination. The frozen group also had DMSO added to 7.5% and then slowly frozen to 0.5 to 0.7°C/min to -100°C and then at 20°C/min down to -196°C where they were stored for 24 hr. The samples were then thawed in a 37°C water bath and DMSO slowly removed to 0.75%. These islets were processed for electron microscopy in an identical manner by Dr. T.K. Shnitka.

RESULTS

Perifusion. From the experimental procedure outlined, two measurements were made: 1) The number of islets of Langerhans after staining by neutral red. 2) The insulin concentration in each tube obtained from the standard curve, subsequently corrected for both perifusion flow rate (1 ml/min)and background insulin content, to enable insulin secretion rate to be calculated, using the formula, below.

Insulin secretion (μU/islet/min) = Total insulin concentration - background insulin concentration (μU/ml) X flow rate (ml/min) ÷ number of islets.

If plots are made of insulin secretion against time, comparison can be made of the dynamics of insulin secretion of each experimental groups of frozen-thawed, frozen-thawed-cultured, killed, and control. The mean secretion value of several experiments of identical protocol were grouped together. Figure36 shows the responses obtained when double stimulation with glucose was used to compare the function of

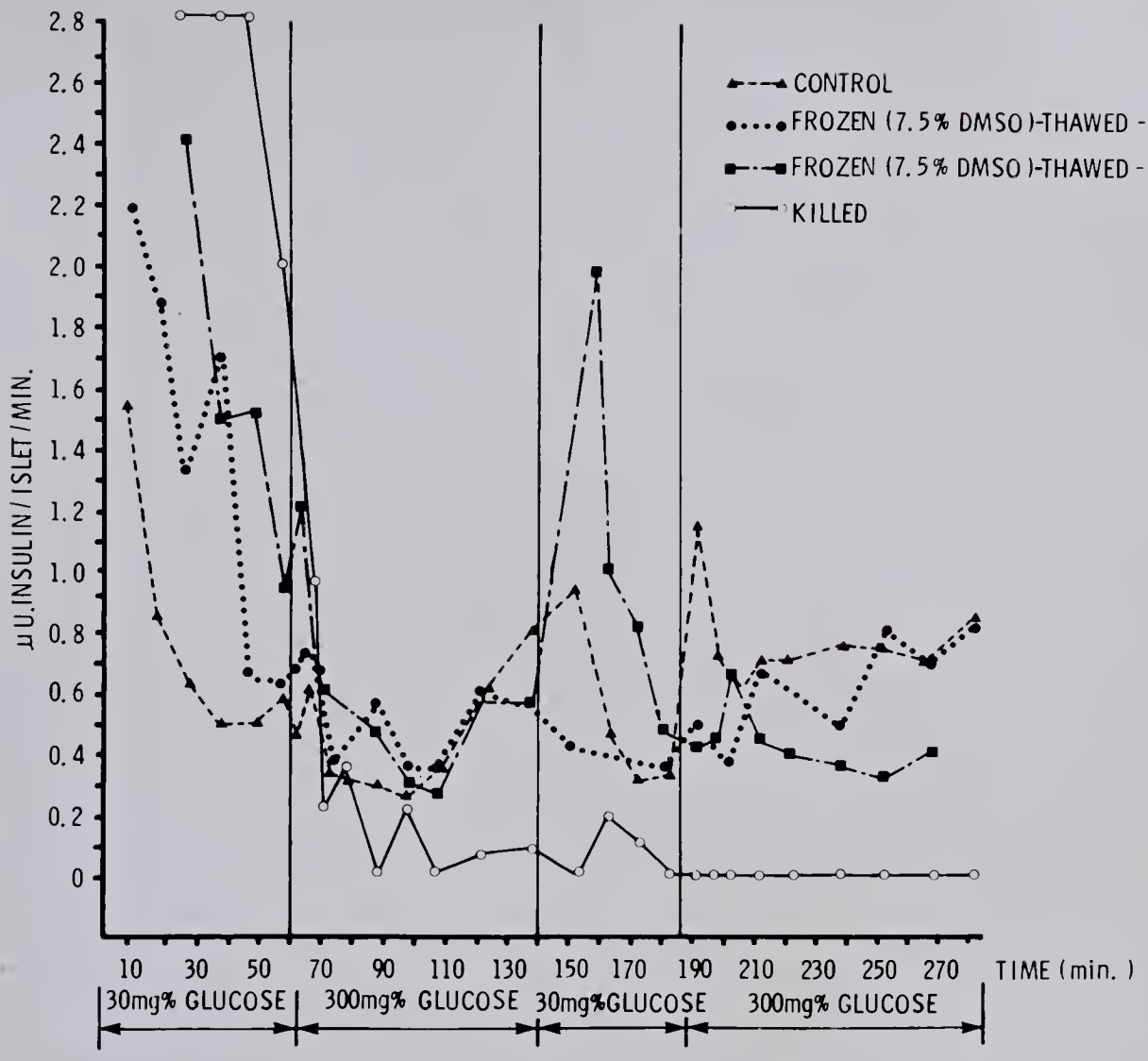

Figure 36. *Double stimulation (30 - 300 mg%) of islets.* Normal fresh islets (▲- - -▲) ± 0.03 < SE < ± 0.34: Frozen (7.5% DMSO) - thawed-cultured (●···●) ± 0.05 < SE < ± 0.34: Frozen (7.5% DMSO) - thawed-non-cultured (■-·-■) ± 0.25 < SE < ± 1.6: Killed islets (○——○). SE = Standard error.

two cryoprotected groups of islets. Each group was cryoprotected by 7.5% DMSO but in one group, after thawing, there was a period of 48 hr of tissue culture prior to perifusion and glucose challenge. Each curve was obtained from several experiments. Islets which had been cultured for 48 hr after thawing were secreting as well as the controls, even after 270 min of perifusion and better than those which had not been cultured.

Insulin secretion rates from unfrozen fresh islets, killed islets, and islets which had been cryoprotected by either 7.5% or 10% DMSO (before freeze-thawing and subsequent culture) are compared in Figure 37. The curves in every case are the mean of several experiments. A single high glucose challenge was used. The frozen (7.5% and 10% DMSO)-thawed and cultured group secreated insulin at the same rate as the controls. The frozen (7.5% DMSO)-thawed-non-cultured group secreted 50% less insulin than the control. Islets which had been killed, had an initial washout and a low no insulin response. It is therefore apparent that frozen-thawed-cultured islets retain insulin secretion that is comparable to the normal control response, while the non-cultured group has shown less response to the glucose challenge.

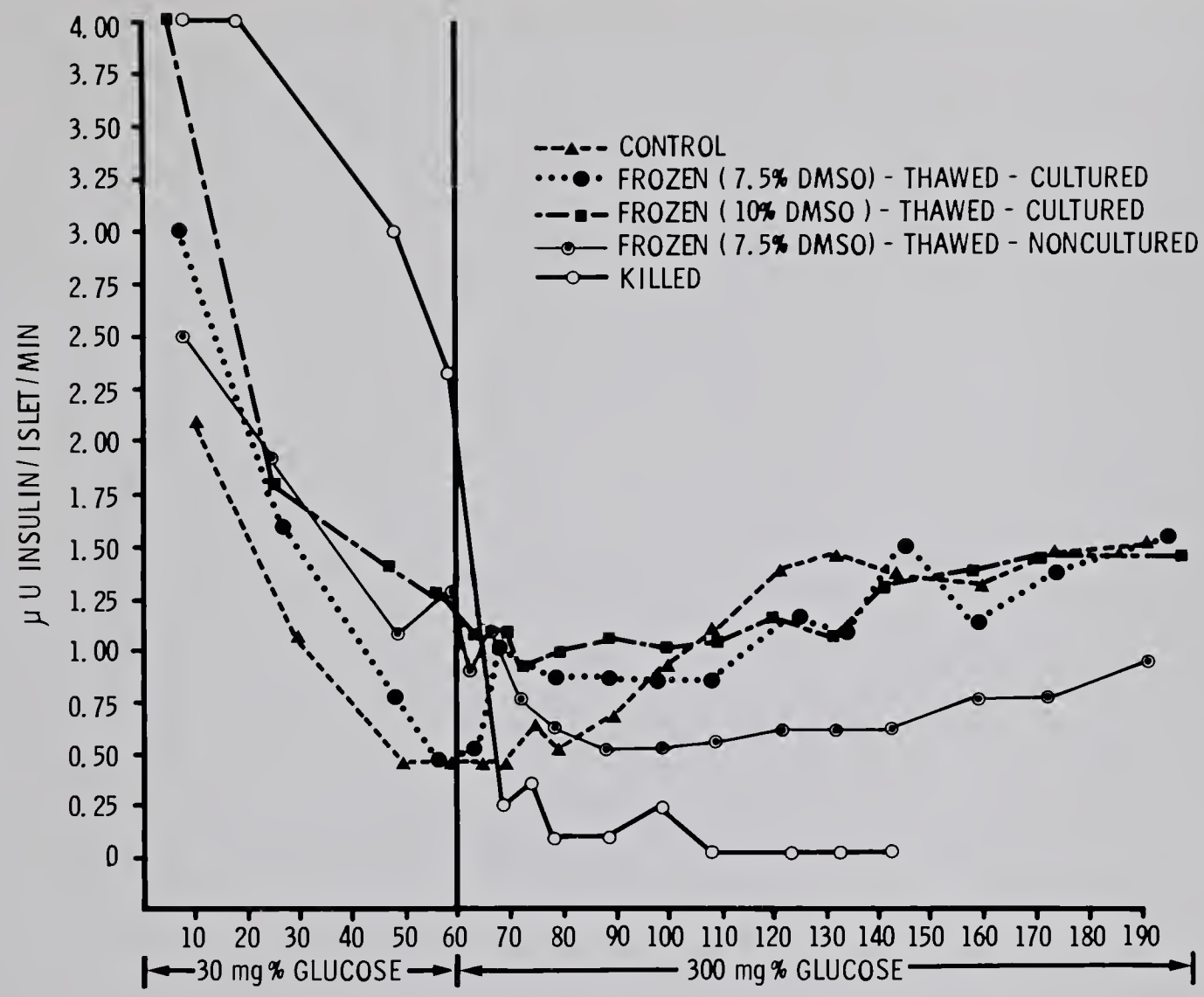

Figure 37. *Single stimulation (30 - 300 mg% glucose) of islets.* Normal fresh islets (▲---▲) ± 0.12 < SE < ± 0.2: Frozen (7.5% DMSO)-thawed-cultured (●···●) ± 0.03 < SE < ± 0.36: Frozen(10% DMSO)-thawed-cultured (■-·-■) ±0.04 < SE < ± 0.22: Frozen(7.5% DMSO)-thawed-non-cultured (⊙——⊙) ± 0.08 < SE < ± 0.25: Killed (○——○). SE = standard error.

Electron microscopy. The electron microscopic findings are given in Figures38 to 44. The beta cells are more numerous than alpha cells and tend to be concentrated in the centre of the islet. The beta cell secretory granules are contained in dilated membranous sacs. In some species, the granules are round, but in other, including man, they appear to be composed of one or more rectangular to polygonal crystals. The granules are extremely dense and at high magnification they have a periodic internal structure. The region between the dense crystal and the limiting membrane is of low density. The beta cells have a round central nucleus that is relatively smooth in contour. The Golgi apparatus is in a juxtanuclear position, and dense amorphous material was seen in some of the Golgi vesicles. Mitochondria are larger and more numerous than those of the alpha cells but the endoplasmic reticulum and ribosomes are not as prominent. The endoplasmic reticulum is composed of short cisternae or vesicular profiles except in cells with few granules, where the cisternae are elongated and occur in stacks. Lysosomes and lipofascin pigment granules are relatively abundant in beta cells. The beta cells are the source of the hormone insulin, a small protein molecule that lowers blood glucose.

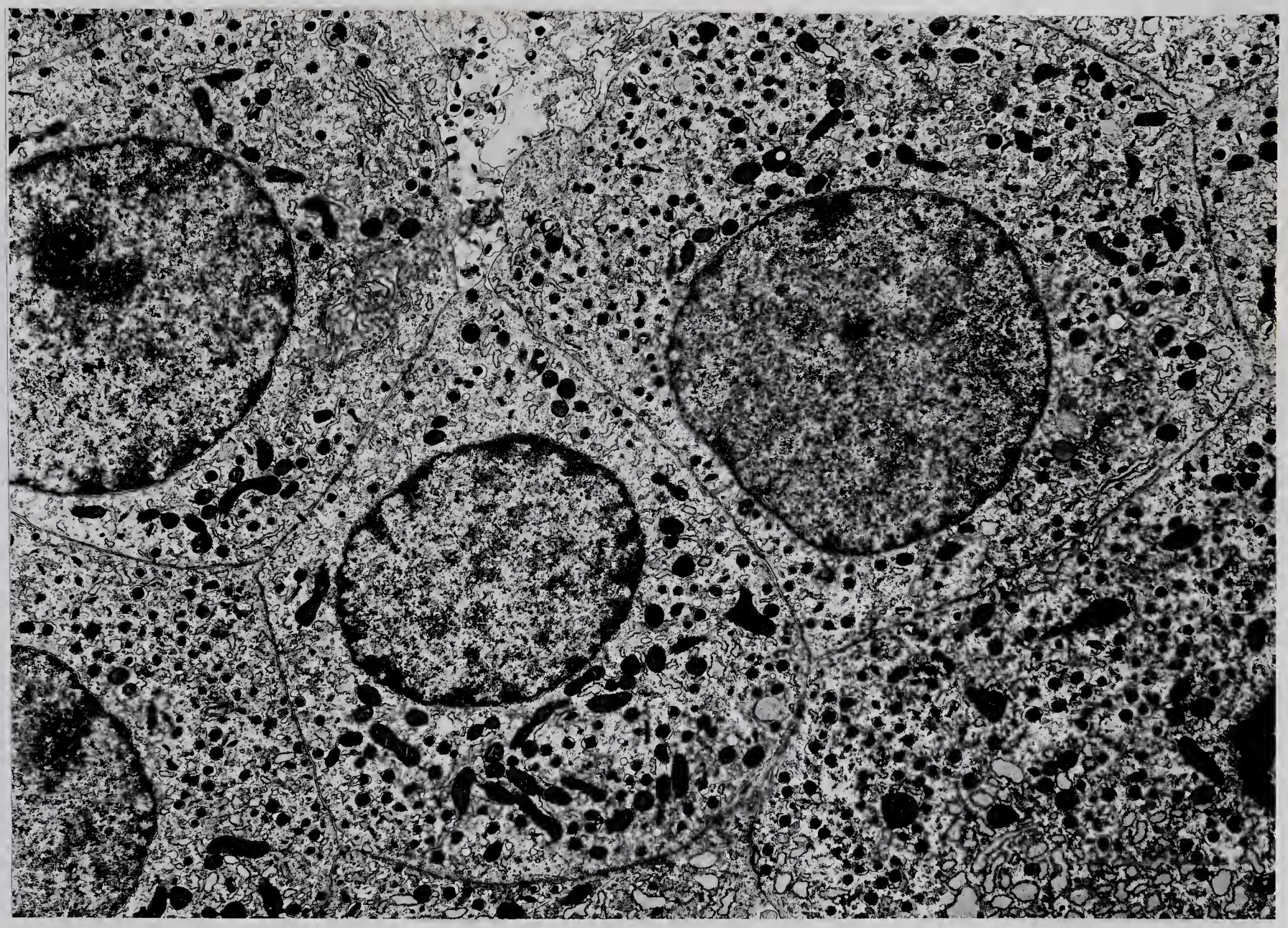

Figure 38. *Normal harvested islets.* DMSO was added slowly over 30 min to a final concentration of 7.5% (v/v) at 4°C. Following this addition of DMSO it was slowly removed and then prepared for electron microscopic examination. Plate 1.

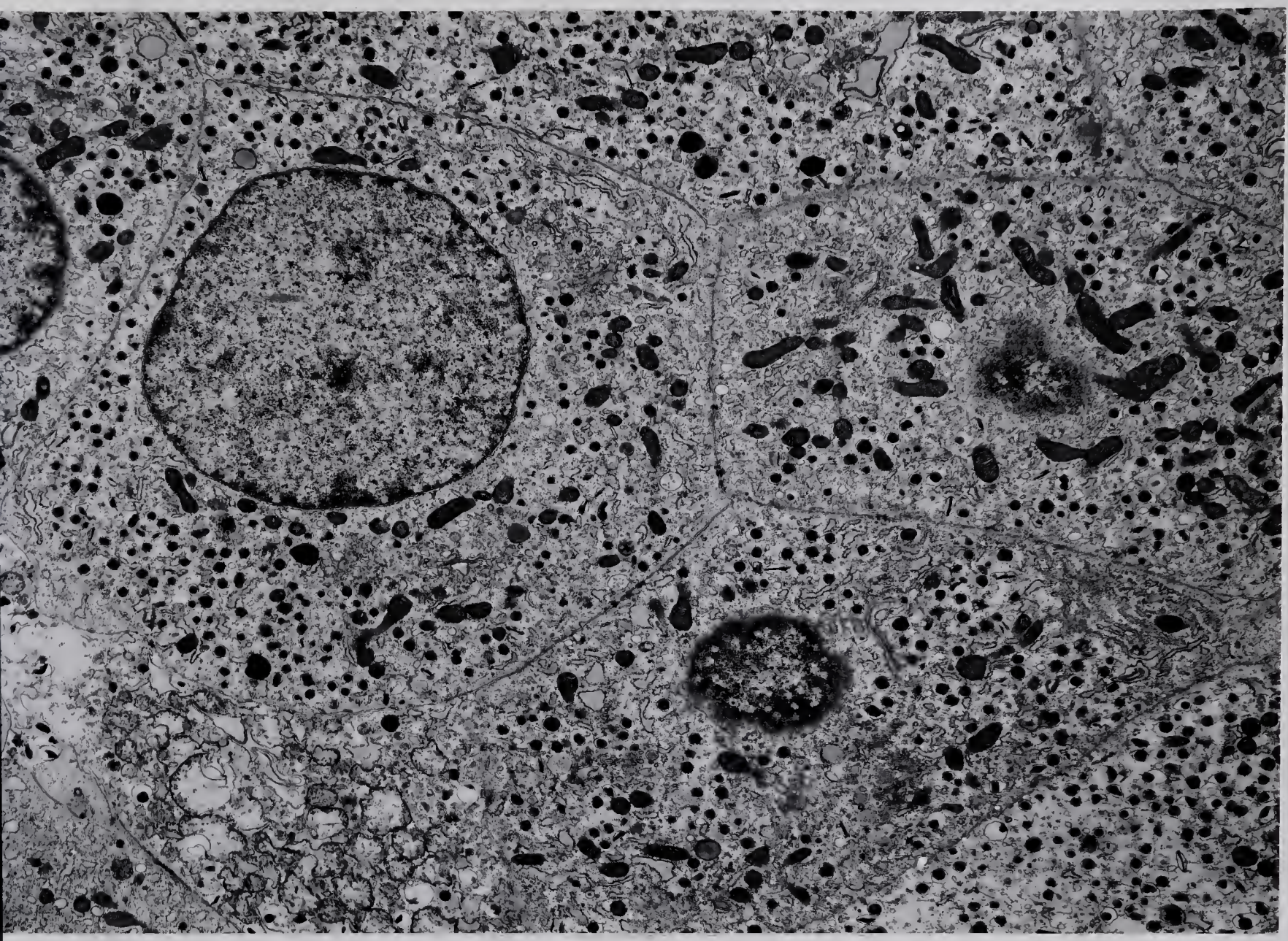

Figure 39. *Normal harvested islets.*
As described in Figure 38. Plate 2.

Interpretation of Figures 38 and 39. The beta cells show excellent preservation of the fine structure, and they cannot be distinguished from similar cells that have been fixed *in situ* without prior treatment. The plasma membranes are intact and there is no evidence of either swelling or disruption of mitochondria, cisternae of endoplasmic reticulum, components of the Golgi apparatus, or

ground substance of the cytoplasm. The secretory granules are not altered in size or number. There is also no evidence of lysis of the electron-dense core of these granules, or enlargment of the surrounding "clean zone". The inner and outer nuclear membranes are intact with no clumping of the chromatin material.

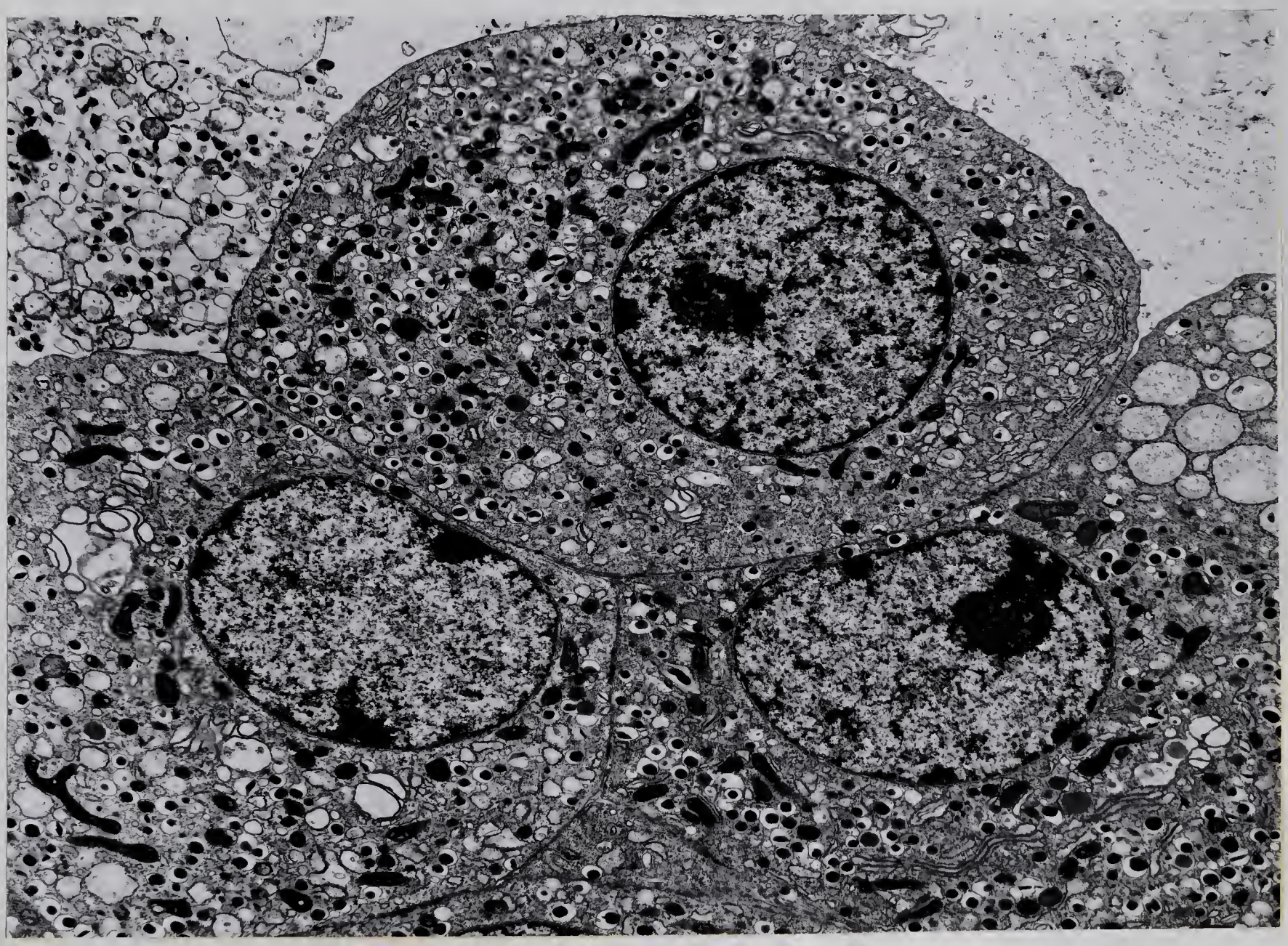

Figure 40. *Normal harvested islets.*
As described in Figure 38. Plate 3.

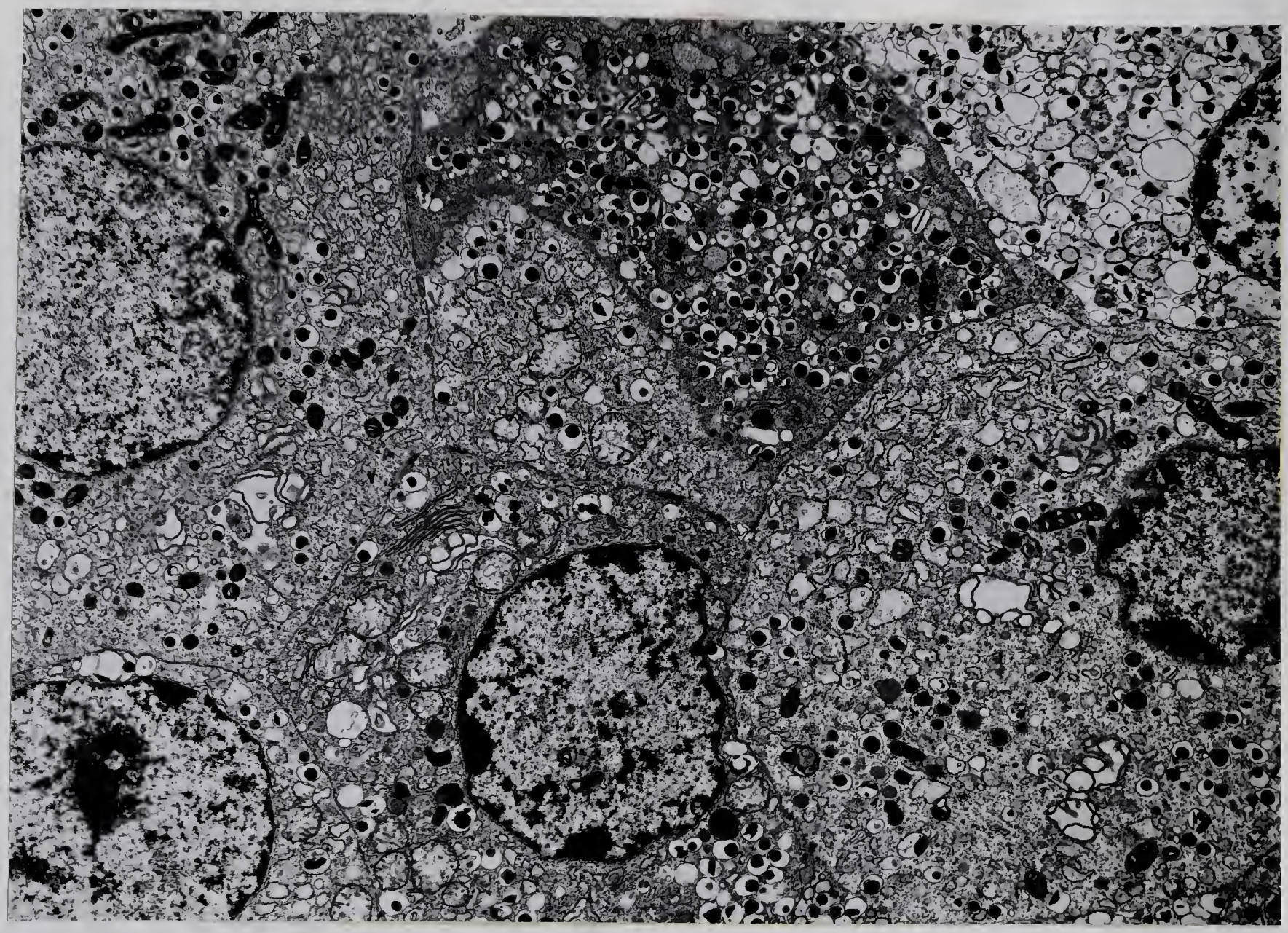

Figure 41. *Normal harvested islet.*
As described in Figure 38. Plate 4.

Interpretation of Figure 40 and 41. There are mild patchy ultrastructural changes in some beta cells in this group. There was swelling of the mitochodria and cisternae of the rough endoplasmic reticulum, some lysis of the central cores of some of the beta cell granules, therefore increasing the number of central cores with an angular rod like or rhomboidal outline. There was mild peripheral margination of the nuclear chromatin. In Figure 40 a dark beta cell can be seen which is due to dehydration.

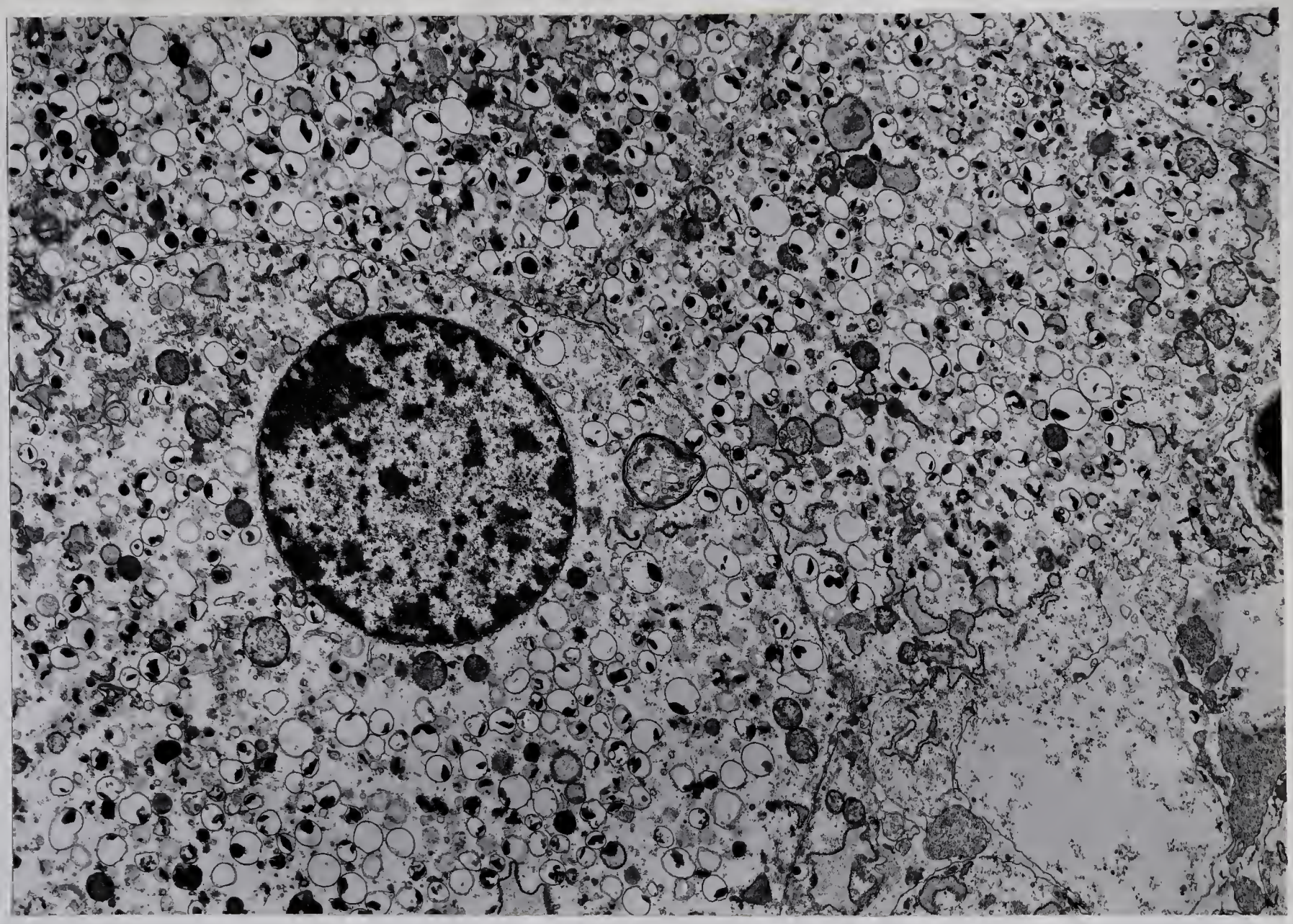

Figure 42. *Frozen-thawed islets.* These islets have been harvested as described with DMSO added slowly over 30 min to a final concentration of 7.5% (v/v), frozen slowly (0.5 - 0.7°C/min) to -100°C and then at 20°C/min to -196°C and stored. The frozen samples were then processed for electron microscopic examination. Plate 1.

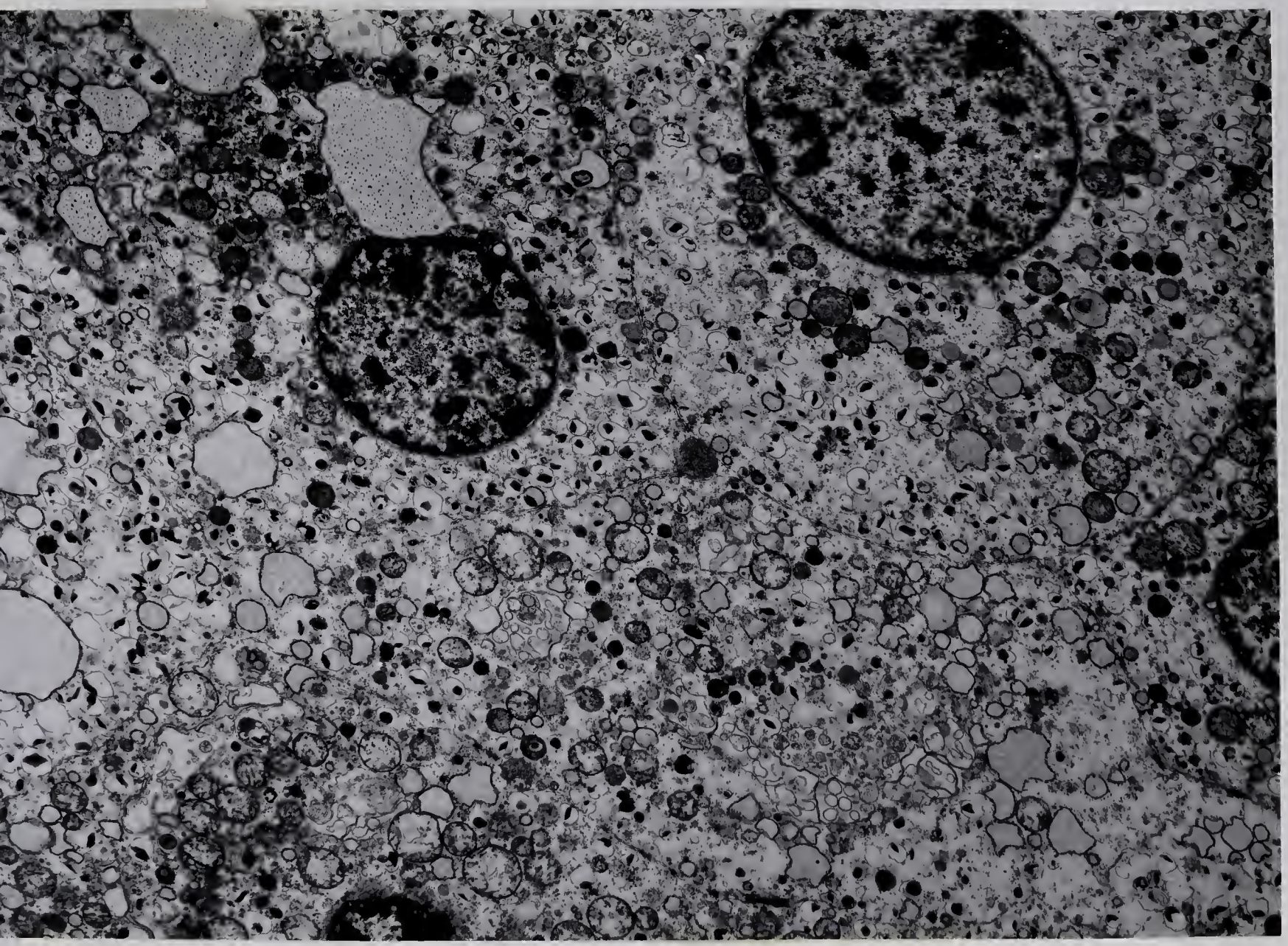

Figure 43. *Frozen-thawed islets.* As described in Figure 42. Plate 2.

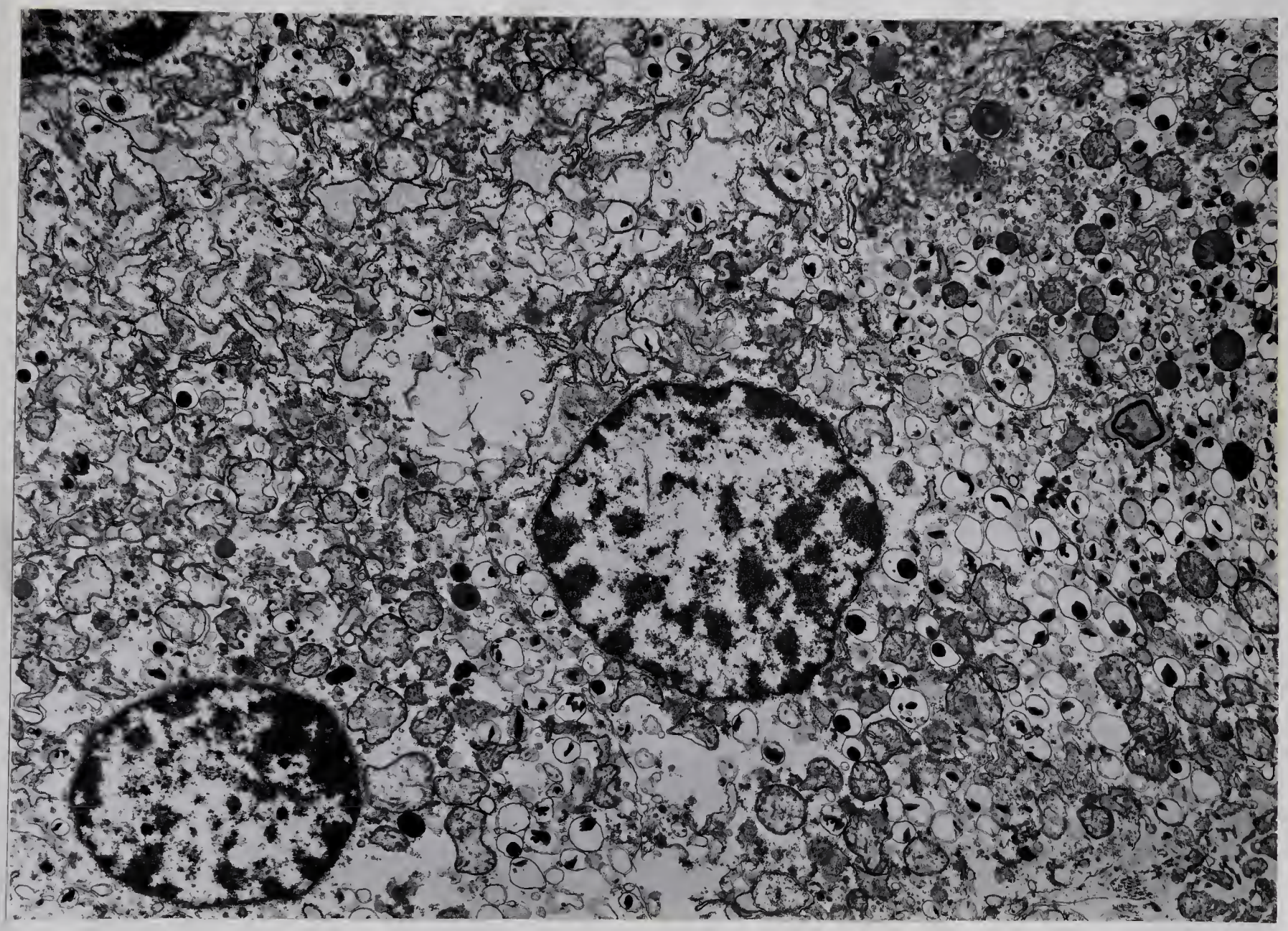

Figure 44. *Frozen-thawed islets.* As described in Figure 42. Plate 3.

Interpretation of Figures 42, 43, and 44. There was patchy ultrastructural changes due to the freeze-thaw insult, with patchy 1 to 2^+ swelling of the mitochondria and 2 to 3^+ swelling of cisternae of the rough endoplasmic reticulum and ground substance of the cytoplasm. The nuclear chromatin showed moderate clumping and peripheral margination, which may have been due to the fixative

of electron microscopic technique. About 50% of the beta cell granules have 2 to 3^{+} lysis of the central cores and moderate enlargement of the clear spaces surrounding these central cores. The plasma membranes and membranes of the rough endoplasmic reticulum show occasional small defects at sites of rupture.

Islet transplant in rat. The results of daily urine volume, urine glucose and weight gain were recorded for 10 days before transplantation of the islets and 16 weeks following the transplant (Figure 45).

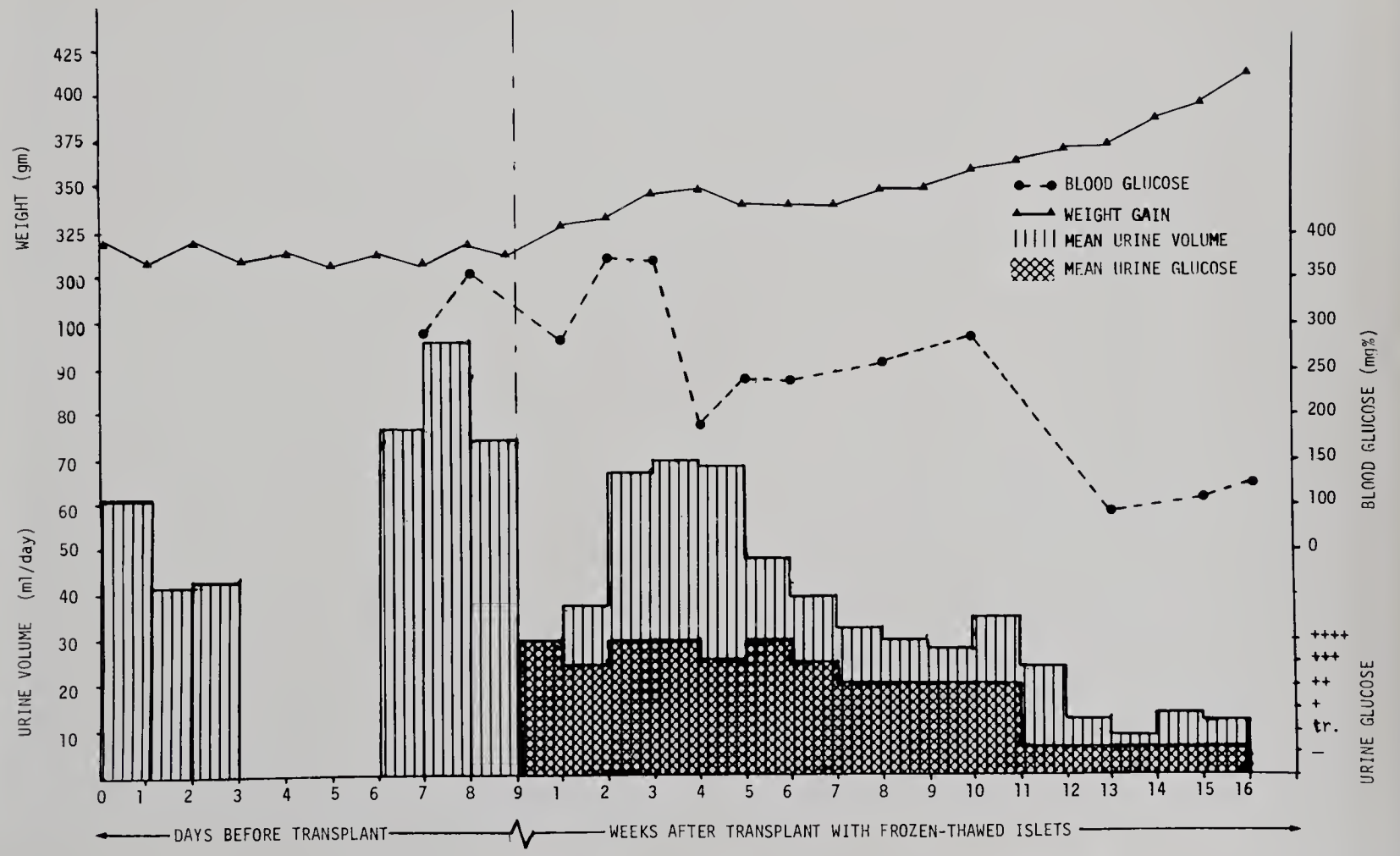

Figure 45. *Condition of diabetic rat.* The one transplanted rat was followed for 16 weeks after transplantation. The urine glucose,urine volume, blood glucose and weight gain are shown.

The dip stick method was used to measure urine glucose (Diastix). This gave a crude semi-quantitative indication of the amount of glucose in the urine (negative implying no glucose in the urine, trace = 1/10%, + = 1/4%, ++ = 1/2%, +++ = 1%. ++++ = 2%). In the weeks following the transplant, a mean was taken of the urine volume, weight gain and urine glucose (2^+, 3^+, 4^+ would give a mean of 3^+). The blood glucose was taken before transplantation and periodically after as shown in the graft. The general condition and body weight of the rat improved (Figure 46).

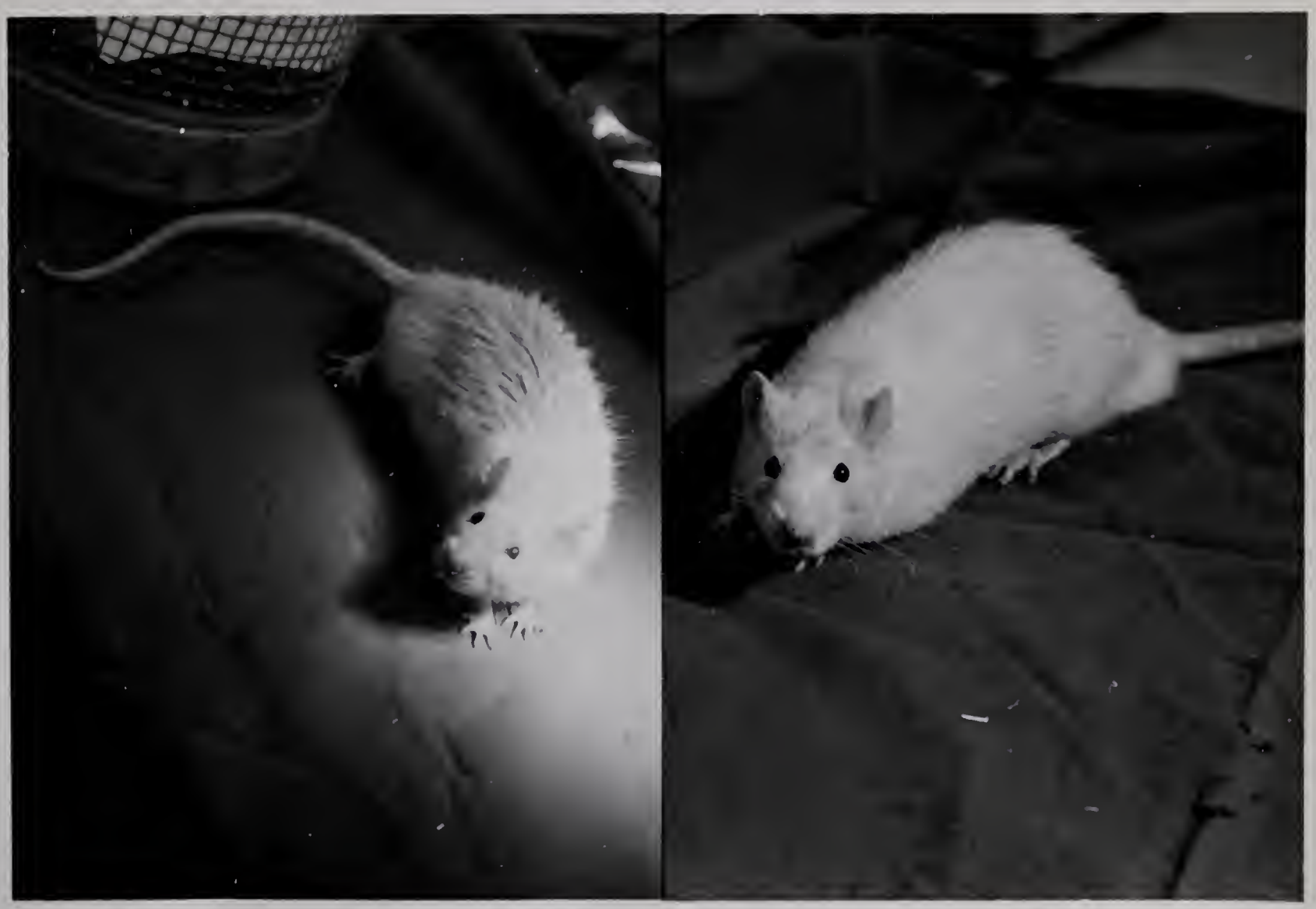

Figure 46. *Transplanted rat.* Lt picture shows the transplanted rat (Weight - 340 gm) at the 5th week. Rt picture taken at the 16th week after transplantation (Weight 415 gm).

At the end of the 16th week the cured rat was sacrificed and the liver and pancreas removed for routine histological examination. Serial sections were made on the liver to try to locate the transplanted islets (Figure 47 & 48).

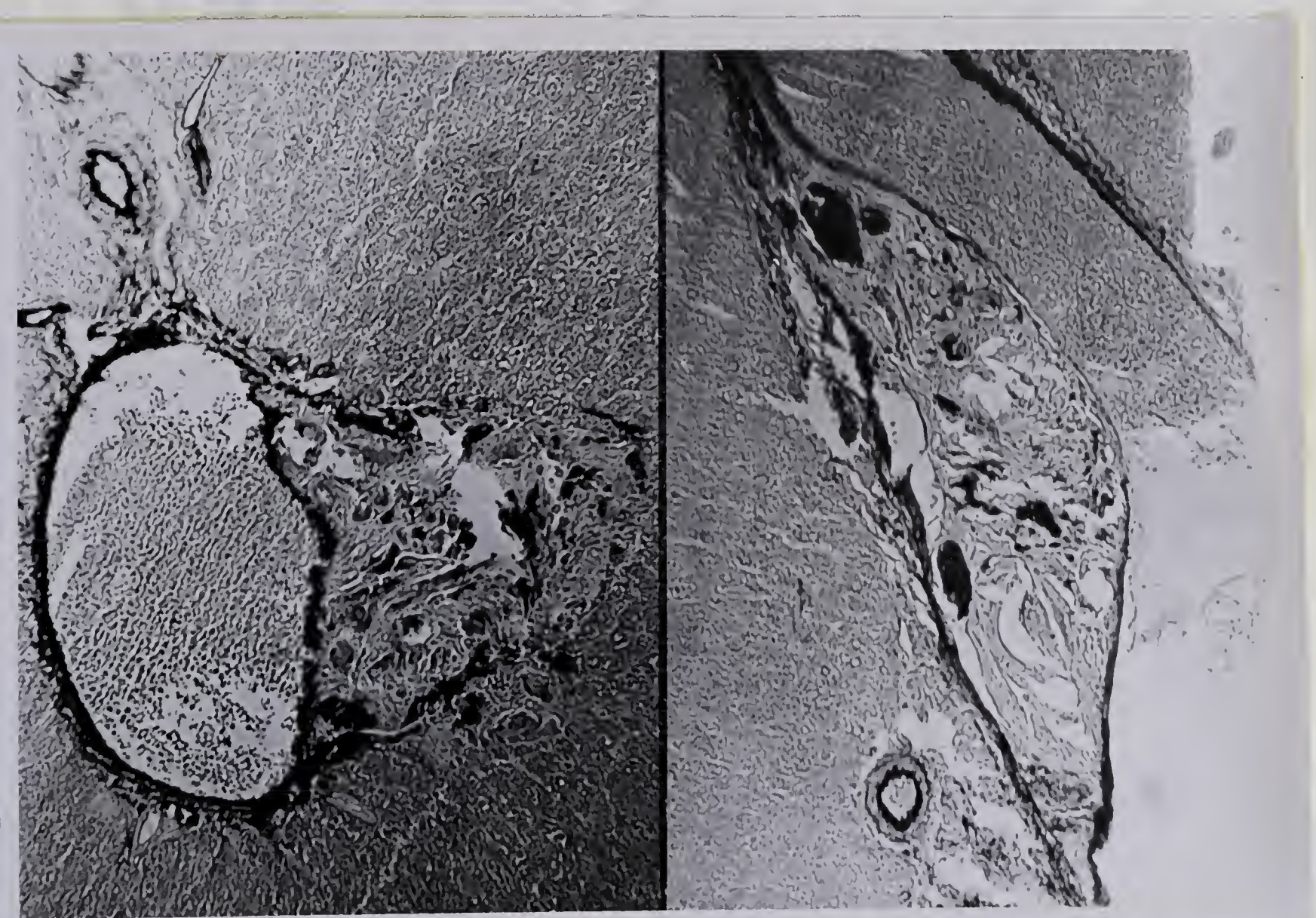

Figure 47. *Histology of transplanted islets.* The transplanted islets can be seen lodged on the side of a blood vessel. Secretory granules can be seen stained dark blue (Aldhyde Fuschsin) X 100 mag.

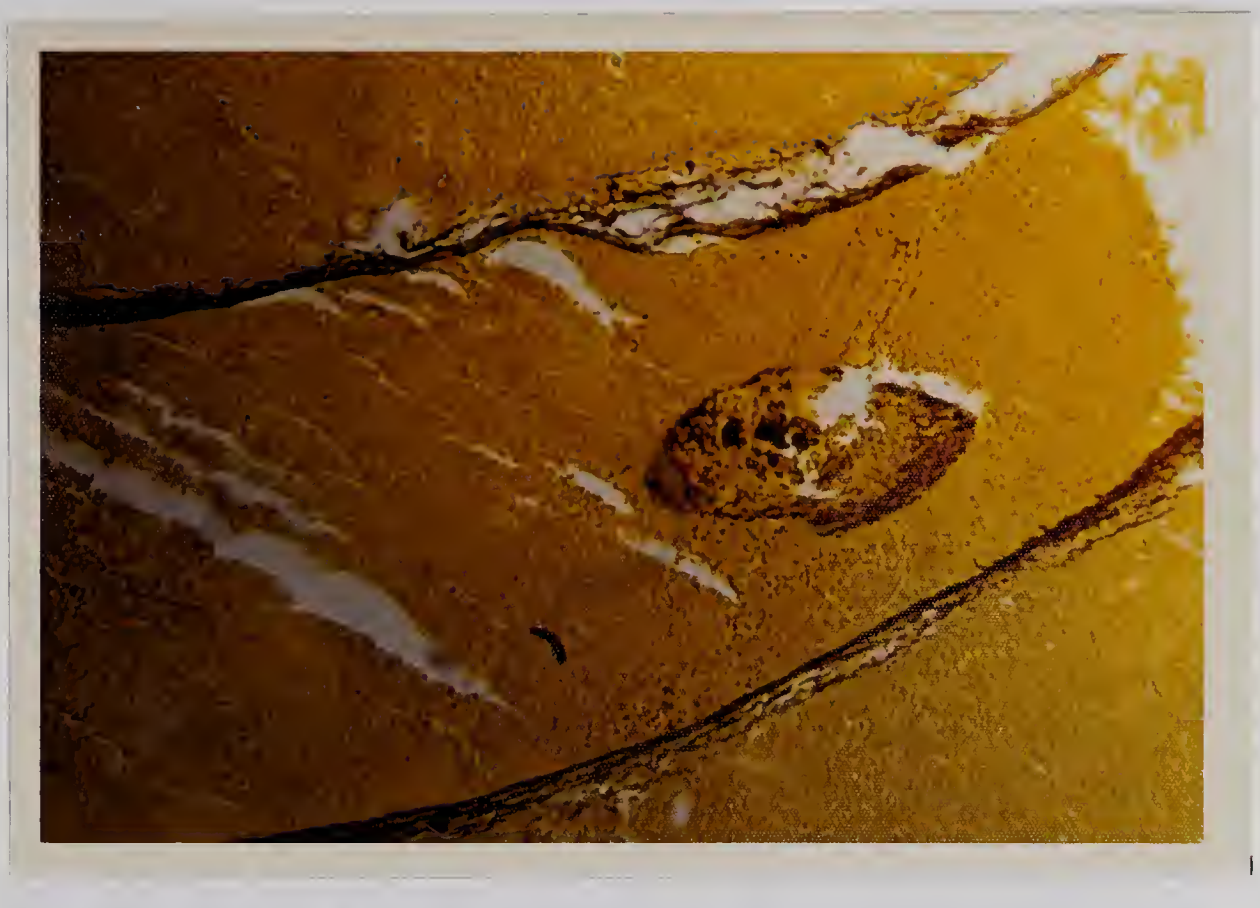

Figure 48. *Color histology of transplanted islets.* The darkly stained secretory granules can be seen in the tranplanted islets. X 100. (Aldhyde Fuschsin).

Serial sections were also taken of the pancreas to see if the islets' secretory granules had stained purple (Figure 49).

Figure 49. *Islet from pancreas of transplanted rat.* A faint outline of the islet can be seen with no staining of the secretory granules. X 400. (Aldhyde Fuschsin).

A normal islet is shown in Figure 50.

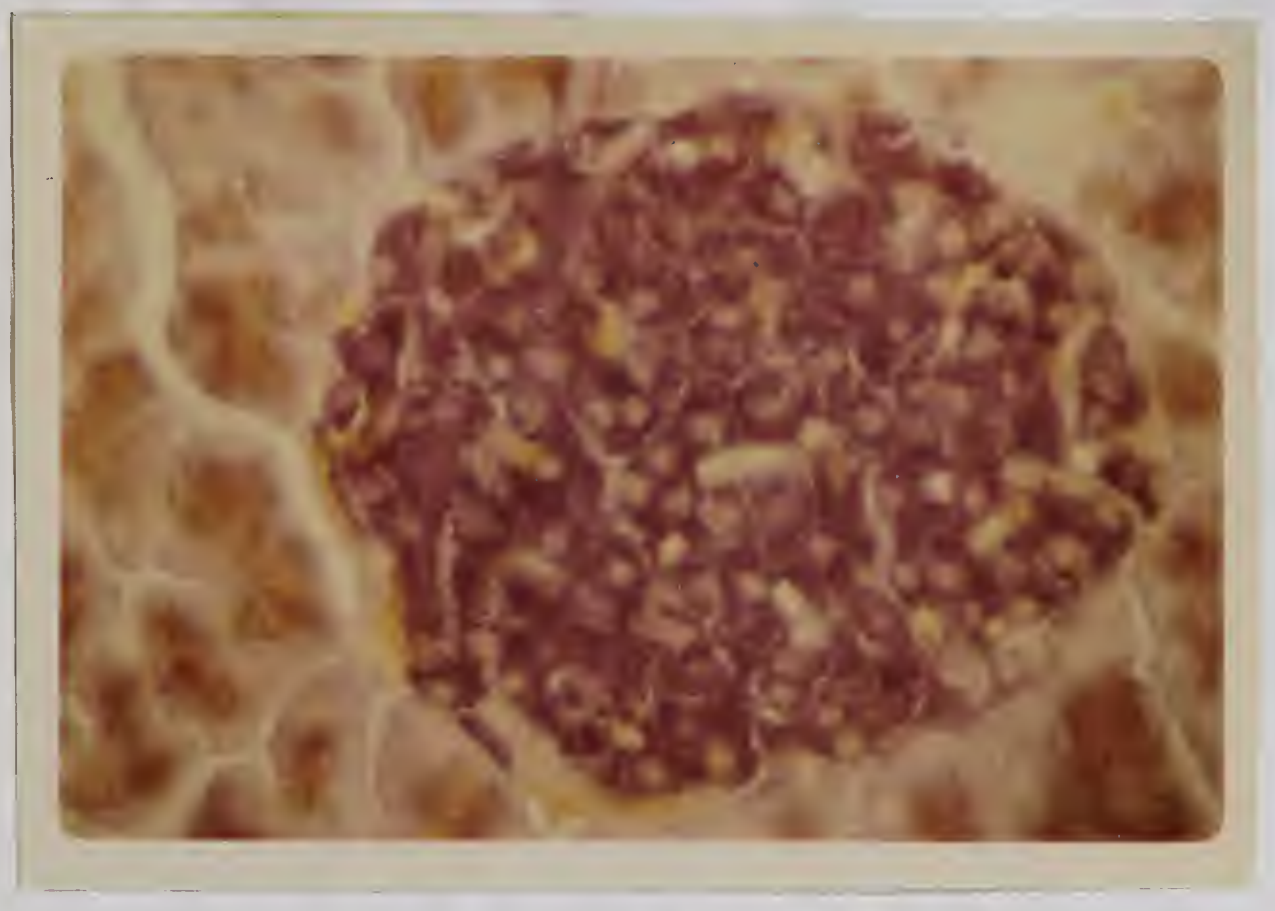

Figure 50. *Islet in pancreas.* A normal stained islet with the secretory granules stained dark purple. X 400. (Aldhyde Fuschsin).

The normal islet shows staining of the secretory granules, indicating function of the beta cells. The islet in the pancreas of the transplanted rat shows a faint outline, with no staining of the granules, indicating beta cells are non-functioning.

DISCUSSION

In the perifusion studies done, the control group in double stimulation studies responds to each glucose challenge. Frozen-thawed and cultured islets follow controls very closely, but the non-cultured group seem to have decreased insulin production during the 2nd glucose challenge. Killed islets had an initial wash out of insulin and then almost zero insulin production. Perifusion

with a single stimulation by hypertonic glucose show that both the 7.5% and 10% groups of frozen-thawed-cultured islets follow the control group. The non-cultured group of islets secreted 50% less insulin than the controls. The killed group had no insulin secretion.

The electron microscopic findings compare with those of Hultquist and Pontin (105). However, islets which have been isolated using the same protocol show a spectrum of ultrastructural changes, both in relation to different cells within a single islet and between different islets. In controls (with 7.5% DMSO added and then removed) some of the beta cells display excellent preservation of fine structure, while other beta cells show mild patchy retrogressive changes including mild swelling of mitochondria and cisternae rough ER, and commencing lysis of the central cores of some beta cell granules, with an increase in the number of cores with a rod-like or rhomboidal outline. Generally, plasma membranes remain intact.

Islets which were frozen in 7.5% DMSO, thawed and then had this protective agent removed, show moderate patchy ultrastructural changes in beta cells, including moderate swelling of mitochondria or rough ER, and hyaloplasm, moderate clumping and peripheral margination of nuclear chromatin, and lysis of the central cores of approximately 50% of beta cell granules in the most severely damaged cells. The above changes may be reversible, and with transplantation as with culture of islets (105), severely damaged beta cells likely disappear through autolysis, with marginally damaged

cells likely to recover and resume secretory function.

To date, transplantation of frozen-thawed cryoprotected islets have been given to only one syngeneic Lewis rat, rendered diabetic by streptozotocin. There was ≃ 200 islets injected into the portal vein following thawing and DMSO removal. In view of the small number of islets transplanted, there is a surprising degree of amelioration of the diabetic state. There is no comparable report on the medical literature for cure of diabetes with frozen-thawed islets, though cure by syngeneic freshly harvested islets is widely reported and accepted.

The mean daily urine volume in the diabetic animal was 70 ml/day and was always 4^+ for urine glucose, with the simple qualitative test; this fell to a mean of 12 ml in the 14, 15, and 16 week and the qualitative values for urine glucose were negative. The hyperglycemic state before transplant was cured; the blood glucose becoming normal. There was steady weight gain and improved vitality.

The results of these *in vitro* experiments support the conclusion that cryoprotected rat islets appear to survive and indeed this does extend the range on an adult organ that can be cryo-preserved.

SUMMARY

Islets removed from adult rats were frozen in 7.5 and 10% DMSO to -196°C, thawed, and in some cases cultured for two days prior to the perifusion. The culture for 2 days seems to allow metabolic recovery as this group of cells responded similarly to the control

group. The electron microscopic examination showed that DMSO added and removed is well tolerated by the cells and that some damage occurred in the frozen group, but not irreversible damage. The one transplanted rat returned from a hyperglycemic state to a normal glycemic state following transplantation.

CONCLUSION

Organized tissue, starting with the simple heart valve, the more complex fetal heart and finally the small organ, the islet of Langerhans can all be preserved in the deep frozen state. This extends the range of organized tissue that can be cryopreserved. With low temperature storage of islets, banking of these small organs may be possible, which could eventually lead to the cure of diabetes.

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