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EX-VIVO CANINE HEART PRESERVATION: METABOLIC STUDIES

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- Introduction:

1a) Clinical cardiac transplantation - historical perspective.

1b) Current status of heart transplantation.

2. Methods of cardiac preservation.

3. Viability assays for cardiac preservation.

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- Purpose of the study and description of the model.
- ^{31} Phosphorous nuclear magnetic resonance analysis of canine heart preservation = 4 hour hypothermic immersion versus 24 hour hypothermic perfusion.

ABSTRACT

The field of cardiac surgery has rapidly expanded in recent years. the development of the current techniques has followed a better understanding of cardiac metabolism. Even though the current hypothermia-based preservation techniques are not ideal, they permit a wide range of surgical procedures to be successfully performed. Where the limitations of the preservation technique are extreme is in the field of cardiac transplantation. Currently, donor organs are immersed in a bath of ice cold saline solution and can be preserved for a maximum of 6 hours.

A few years ago, a new method of graft preservation was developed by many investigators; it was based upon the assumption that the heart could be preserved better and for longer periods of time if it was supplied with a constant source of 0_2 and nutrients. Adequate functional preservation was then achieved after 24 hours of perfusion of the organ with an oxygenated physiological solution.

It is clear that if better cardiac preservation for transplantation purposes is to be achieved, it will be only through a better understanding of hypothermic metabolism. In the first part of this thesis, the development of cardiac preservation and transplantation, aerobic and anaerobic metabolism as well as parameters to assess graft viability will be briefly reviewed.

The recently developed method of nuclear magnetic resonance spectroscopy allows for continuous monitoring of high energy phosphate compound (ATP and Phosphocreatine) and intracellular pH of tissues or whole organs. In order to better understand why ex-vivo hearts can be preserved for longer periods when perfused with oxygenated crystalloid solutions under hypothermic

conditions, two groups of canine hearts were studied with nuclear magnetic resonance spectroscopy.

GROUP I = Canine hearts preserved for 4 hours by immersion into a 4°C saline solution.

GROUP II = Canine hearts preserved for 24 hours by continuous coronary perfusion with a modified oxygenated Krebs solution at 4°C.

The longer preservation of ATP and phosphocreatine, as well as the slower decrease of intracellular pH in Group II hearts are hypothesized to be the reasons why perfused hearts can be preserved for longer periods of time.

RESUME

La chirurgie cardiaque a subi d'importants developpements au cours des dernieres années. Ces progres n'ont éte rendus possibles que gráce a une meilleure compréhension du métabolisme cardiaque permettant une preservation prolongee basée sur l'hypothermie. Presentement, les greffons sont immerges dans une solution de salin physiologique a 5°C; cependant, apres 6 heures de preservation, les coeurs sont irrémediablement endommages.

Dans le but d'ameliorer et de prolonger la preservation du myocarde, une methode de perfusion continue du coeur à l'aide d'une solution de Krebs oxygenee a eté mise au point il y a quelques annees. Par cette methode, des coeurs ont pu être experimentalement preserves pour au-dela de 24 heures.

Il est clair que si la preservation doit être améliorée pour repondre aux strictes contraintes de la transplantation cardiaque, cela doit être accompli selon une meilleure compehension du metabolisme sous conditions hypothermiques.

Afin de comparer le metabolisme du coeur hypothermique sous conditions totalement anaerobiques versus perfusion oxygénée continue, la methode de spectroscopie par resonnance nucleaire magnetique fut utilisee. Le metabolisme des éléments phosphatés a haute energie (Adenosine tri-phosphates-ATP, Phosphocréatine - Pcr) ainsi que le pH intracellulaire et l'accumulation d'éléments phosphastes inorganiques furent mesurés dans deux groupes de coeurs canins ex-vivo.

GROUPE I = Coeurs soumis à 4 heures d'hypothermie isolée dans un bain de solution saline a 4°C.

GROUPE II = Coeurs perfuses par une solution de Krebs modifiée et oxygenee a 4°C pour 24 heures.

Les coeurs perfuses pour 24 heures (Groupe II) ont demontre une meilleure preservation de l'ATP, de la phosphocreatine ainsi qu'une moindre diminution du pH intracellulaire.

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LIST OF ABBREVIATIONS

ATP	=	Adenosine - 5 - Triphosphate
cc	=	Cubic centimeter
C-13	=	Carbon - 13
cm	=	Centimeter
cm H_2O	=	Centimeter of water (pressure in)
CO_2	=	Carbon Dioxide
CPK	=	Creatine Phosphokinase
dp/dt	=	Positive derivative of developed pressure overtime
gm	=	gram
gm%	=	gram per 100 milliliters of solution
H - 1	=	Hydrogen
H_2O	=	Water
Hr	=	Hour
L	=	Litre
LV	=	Left ventricle
LVEDP	=	Left ventricular end-diastolic pressure
LVEDV	=	Left ventricular end-diastolic volume
m	=	Meter
mEq	=	Milli-equivalent
mEq/L	=	Milli-equivalent/litre
mg	=	Milligram
mmHg	=	Millimeter of mercury
MHz	=	Megahertz
min	=	Minute
ml	=	Millilitre
mOsm	=	Milliosmole
mOsm/L	=	Milliosmole/litre
MRI	=	Magnetic Resonance Imaging
N_2	=	Nitrogen
NMR	=	Nuclear Magnetic Resonance
O_2	=	Oxygen
^{31}P	=	Phosphorus - 31
pCO_2	=	Tension of carbon dioxide

LIST OF ABBREVIATIONS (cont'd)

PCr	=	Phosphocreatine
pH	=	pH
pH_e	=	Perfusate pH
pH_i	=	Intracellular pH
P_i	=	Inorganic phosphate
pO_2	=	Tension of Oxygen
psi	=	Pound per square inch
S.P.	=	Sugar phosphate
T	=	Testa
U	=	Unit
N	=	Micron
"	=	Inch

INTRODUCTION

1) Clinical Cardiac Transplantation - historical perspective.

2) Current status of heart transplantation.

3) Methods of cardiac preservation.
   a) Normothermic preservation.
   b) Cryopreservation.
   c) Metabolic reduction.
      i) Chemical
      ii) Hypothermic
      iii) Hyperbaric
   d) Perfusion.
      i) Biological perfusate
      ii) Artificial perfusate
      iii) Summary

4) Metabolic considerations.

5) Viability assays for cardiac preservation.

6) Nuclear Magnetic Resonance Spectroscopy.

CLINICAL CARDIAC TRANSPLANTATION

HISTORICAL PERSPECTIVES

1a) CLINICAL CARDIAC TRANSPLANTATION - HISTORICAL PERSPECTIVE

For more than 40 years, diseases of the heart and blood vessels have been the major cause of death in North America. Heart and blood vessel diseases affect an estimated 42,750,000 Americans (1). In 1981, 984,610 people died of cardiovascular disease, more than 20% of them before they reached the age of 65 (2). It is assumed that many of these people would benefit from heart transplantation. Heart transplantation has become an accepted therapeutic modality for treatment of end-stage cardiac disease.

Such a modality has been attractive to surgeons and cardiologists for many years. It is important to realize that the development of successful clinical heart transplantation depended on the achievement of several major milestones:

1) Advent of safe cardiopulmonary bypass.
2) Development of proper surgical technique.
3) Understanding of myocardial preservation.
4) Acquisition of means for diagnosing and treating rejection.

A good discussion and description of the major steps in the development of heart transplantation is available in any good textbook dealing with the subject. This is not, by any means, an exhaustive historical review. Following, are the major landmarks in the development of cardiac transplantation.

1905 - A Carrel	- First successful canine heterotopic cardiac transplantation.
1950 - Demikhov	- Experimentation with heterotopic transplantation in the chest with circulation exclusion of recipient heart.
1953- Gibbon	- Development of safe cardiopulmonary bypass.
1960 - Lower & Shumway	- First successful series of canine orthotopic transplantation. - Surgical technique of donor heart excision and implantation at mid-atrial level. - Preservation of graft using topical hypothermia at 4°C.
1964 - Hardy	- First human orthotopic transplantation using a chimpanzee heart.
1967 - Barnard	- First successful human homo-transplantation.
1968 - Shumway	- Implementation of first clinical program (Stanford University).

Early widespread enthusiasm for the operation led to the performance of more than 100 cardiac transplants in the year 1968, but dismal clinical results soon dampened the interest. The initial overwhelming expansion of transplant programs around the world rapidly slowed down when the tremendous problems associated with uncontrollable rejection, side-effects from immunosuppressive agents and post-op management created an unacceptable morbidity and mortality. The concern was so great that, in the early 1970's only four programs around the world were still performing clinical heart transplantation: Stanford (Shumway), Richmond (Lower), France (Cabrol) and South Africa (Barnard). A major breakthrough occurred in the late 1970's, when cyclosporine-A, a very potent fungal-derived immunosuppressant, became widely accepted as a substitute or adjunct to previous immuno-suppressive agents.

The advent of cyclosporine-A renewed the interest in cardiac transplantation and a slower but more cautious implementation of major transplant centers around the world was observed.

CURRENT STATUS OF HEART TRANSPLANTATION

1b) CURRENT STATUS OF HEART TRANSPLANTATION

As discussed previously, there has been many advances in the field of organ transplantation in the past decade. Heart transplantation has followed this evolution. Even though the surgical technique has not changed very much, improvements in anesthesia and post-operative care, as well as a better understanding of the patho-immunology of organ rejection, have made heart transplantation to become a well-accepted therapeutic modality for treatment of end-stage cardiac disease or of mortally-ill cardiac patients (4,5).

Between 1968 and December 31st, 1984, 55 centers in 9 countries performed 1,644 transplants in 1,599 recipients (6). One thousand of these patients were in the USA, 53 in Canada and 546 outside of North America. In 1983, 284 heart transplants or about 25% of the total number ever performed, were reported worldwide. In 1985, there were only 10 American medical centers with active heart transplantation programs but 15 more were establishing so that by the end of 1986, 39 centers were active in the USA. According to the International Heart Transplant Registry (7), in 1986, 55 centers worldwide were performing heart plus or minus heart-lung transplantation (6) (39 in the USA, 9 in Canada, 14 outside North America). In 1990, 211 centers have reported heart transplant procedure (9).

The International Society for Heart Transplantation (7), since its inception in 1980, has maintained a voluntary registry of data on heart transplantation. Its members have been providing accurate information on almost all the transplants performed internationally.

An exponential growth in the number of heart transplants has been noticed since 1976 (32 in 1976, 117 in 1981, 284 in 1983, 962 in 1985 and 3054 in 1990).

Up to 1991, 16 687 hearts have been transplanted. These patients, with a mean age of 44 years old (range: 4 days to 70 y.o), constitute a very active branch of the society.

The survival rate has progressively increased since the first human transplant in 1967 (Table 1).

Table 1 - SURVIVAL POST CARDIAC HOMOTRANSPLANTATION

	1968-73	1974-81	1985-90
1 year	44%	63%	85%
2 years	35%	55%	
3 years	27%	51%	78%
4 years	21%	44%	
5 years	18%	39%	70%

The cost in the USA is estimated at 100,000$ (US) for the entire first year of care for each cardiac allograft patient (4). Eighty-two per cent of patients in the Stanford experience returned to employment or activity of choice (8) (defined as restoration of overall functional capacity sufficient to provide the patient an unrestricted option). Ninety-seven per cent of these patients reached NYHA Class I cardiac disability (virtually all patients had Class IV clinical status with a predicted survival of less than 6 months before transplantation).

A further restriction to the expansion of cardiac transplantation will be the limited availability of donor organs. The number of transplants needed annually in the USA, as evaluated by the Stanford group is between 1,000 and 5,000 (8). In Canada, the morbidity and mortality of heart failure is 51,250/year (6). Considering only the patients who are between 10-54 years old,

who are dying with an isolated cardiac disease in hospital, 1,400 only would be amenable to heart replacement. The actual rate of transplantation in Canada is in excess of 100/year.

The gradually increasing number of patients with irreversible brain death who are identified as suitable renal allograft donors is currently approximately 2,000 in the USA (8). The potential heart donor pool is approximately 20-30% less. If the indications for heart transplantation are considerably expanded in the future, a major donor shortage can be anticipated. In the Stanford experience, the survival rate of patients selected for transplantation and for whom no donor organ became available is less than 5% at 6 months. Even though, Lower in the late 1970's proposed a technique for prolonged graft preservation permitting long distance procurement, the technique allows for only 4-6 hours of ischemia. This limits the potential organ donor pool to only 1,000 - 1,500 miles from the transplant center, depending on the mode of transportation. This fact accounts for most of the interest in cardiac preservation research. If more of the rare cardiac allograft donors is not to be lost, the preservation period has to be extended.

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METHODS OF CARDIAC PRESERVATION

2) METHODS OF CARDIAC PRESERVATION

A) Normothermic preservation.

B) Cryopreservation.

C) Metabolic reduction.
- i) Chemical
- ii) Hypothermic
- iii) Hyperbaric

D) Perfusion.
- i) Biological perfusate
- ii) Artificial perfusates
- iii) Summary

E) Bibliography.

3) METHODS OF CARDIAC PRESERVATION

Clinical open heart surgery began in 1953, when Lewis and Taufic (1), in Chicago, closed an atrial septal defect using inflow occlusion and moderate systemic hypothermia with systemic cooling. As early as 1939, Gibbon (2) was working on the development of a heart-lung machine with which he closed an atrial septal defect clinically in 1953 (3). It was, however, the corection of Fallot's tetralogy by Lillehei (4) in 1954, using controlled cross-circulation from a donor, that led to the explosive expansion of open heart surgery.

The earliest open heart surgery provided the surgeons with a still, bloodless field by simply occluding the venae cavae and aorta. The viability of the brain and other tissues was then maintained by reducing their energy demand with moderate (30°C) systemic hypothermia. The major problem with this technique was the time: only 10 minutes of interruption of the circulation was possible before irreversible cerebral damage occurred. Systemic hypothermia below 30°C was accompanied with an unacceptably high rate of ventricular fibrillation. With the patient's circulation supported by a pump-oxygenator lower temperatures could be used and the tolerance to complete myocardial ischemia could be extended to 1 hour (5) before myocardial damage occurred.

Melrose and Bentall (6) introduced in 1955 the concept of "elective cardiac arrest": after aortic occlusion, rapid injection of a 2.5% solution of potassium citrate in blood arrested the heart. The heart was shown to "washout" the potassium upon reperfusion with damage-free recovery of a normal beat. After many papers demonstrated areas of subendocardial necrosis following potassium-citrate arrest (7,8), there was a worldwide swing away from the use of such solutions. The problem was later proved to be related to the extremely high potassium concentration of these early solutions. (9)

With the abandonment of cardioplegia, coronary perfusion became the technique of choice for myocardial preservation. Because of improvements in design of oxygenators, filters, surgical expertise and post-operative management, the mortality of open heart surgery fell steadily throughout the 1960's and early 1970's. The perfusion technique with a beating heart was felt by the surgeons to be, conceptually, the ideal method of preserving myocardial integrity but at the expense of producing poor surgical access.

Shumway (10,11) became the pionneer of topical hypothermia of the heart with an aortic occlusion. As early as 1963, he reported a series of open heart operations for congenital defects with a 3% operative mortality!! He achieved these results by making a well of the pericardium and running 4°C saline into it after occluding the aorta. This technique allowed safe periods of aortic occlusion of up to 150 minutes. The preservation period was extended to 4 hours for the isolated donor heart for transplantation using this method.

In an attempt to protect the myocardium and at the same time produce a clear operative field for the surgeon, the Europeans kept the concept of cardioplegia alive. The St. Thomas solution was developed in England (12) and used routinely by Braimbridge in 1975. By 1978, coronary perfusion (fibrillatory arrest without aortic clamping) as a mean of preserving the myocardium had virtualy disappeared from clinical practice in the world. Cardioplegia is currently the standard method but controversy still exists about the ideal composition of the solution. In Europe, east of the Rhine, a sodium-poor, calcium-free, magnesium and procaine solution is preferred. Great-Britain and Western Europe use solutions similar to the St. Thomas solution (extracellular ionic concentration). The USA favor potassium-enriched solutions containing little or no magnesium.

With the development of organ transplantation, the problem of prolonged extracorporeal graft preservation appeared. Although some types of prosthetic ogans may be useful in the future, the full panoply of physiological function which a biological organ fulfills cannot be matched by these devices. With the recognition of cost-effectiveness of organ transplantation and the scarce supply of donor organs, better ways have to be found for prolonged graft preservation. Following is a brief discussion of the various methods for donor heart preservation with a view to transplantation.

A) NORMOTHERMIC PRESERVATION

Myocardial metabolic support under normothermic conditions has been done in two ways. The first method consists in symbiotic perfusion of the organ with an intact organism of the same species or of a closely related species (13). Angell (14,15) showed, in 1968, that resuscitated hearts connected to neck vessels and then stored in a pouch in the neck of recipient dogs for up to 4 days were capable of supporting the circulation when subsequently transplanted orthotopically in a third animal. The main advantage of such a method is that the heart is stored in an ideal environment with almost normal physiological and biochemical conditions. Cardiac xenobanking for human recipients could be considered only if the intermediate host is chosen from a closely related species. Dupree et al (16) stored isolated hearts that were obtained from stumptail monkeys in the abdomen of baboons. If no immunosuppression was given, the hearts would die on day 5. When immunosuppression was used (host irradiation), the hearts were still beating vigorously on days 9-11.

The second method consists of in vitro normothermic perfusion. Heart-lung preparations from donor dogs have been stored in a water bag at 36-37°C by Robicsek (19,20,21). Ventilation was accomplished by applying positive and negative pressure within the chamber containing the organs and coronary perfusion pressure was maintained by a stabilizing blood reservoir suspended above the preparation. If taken live from the donor animals, the hearts continued to beat effectively for 12 hours.

Viability could be extended to 24 hours by dialyzing cross-circulation with a living animal (22) but congestive heart failure occurred secondary to pulmonary edema. More recently,

Tago (23) preserved hearts for 24 hours by aortic root, moderately hypothermic, whole blood perfusion. The hearts were then reimplanted orthotopically. Fourteen of the 16 hearts successfully supported the recipients circulation. Histologic and ultrastructural studies at 24 hours and 7 days after reimplantation showed good preservation. Cells were of normal size with normal nuclei and all the other cellular components well preserved (normal mitochondria and microvasculature). Only slight interstitial edema was noted. In the same line, Solis (24), using the same system as Tago (23), measured ventricular function following transplantation, using sonomicrometric techniques. The hearts that were preserved ex-vivo for 12 hours and then implanted in the orthotopic position had left ventricular function statistically equal or better than acutely denervated or conventionally preserved transplanted hearts.

Blood perfusion offers many advantages - such perfusates may have an "endothelial supportive factor" (25) that could support microvascular integrity. Red blood cells can protect the heart in 3 ways:

a) protective effect on vascular endothelium.
b) improved oxygen transport and delivery to tissues.
c) optimization of tissue perfusion by redistribution of the capillary blood flow (13).

Whether any or all of these mechanisms are protective is controversial but there is little doubt about the beneficial effect of blood perfusion on myocardial preservation (26,27,28).

Platelets are thought to play a supportive role in endothelial cell function apart from their role in thrombosis and hemostasis. Thrombocytopenia has been shown in vivo to cause ultrastructural and permeability changes in the microvasculature

(29). In Solis (24) paper, one of the major problems encountered was marked deterioration of the hearts within a few hours - demonstrated by marked edema, increased coronary vascular resistance and decreased coronary blood flow. Light microscopy revealed platelet aggregation obstructing the micro-circulation and ischemic changes. Ibuprofen, a non-steroidal anti-inflammatory agent, was added to the blood perfusate to abolish this phenomenon. In addition to its anti-aggregating platelet effect, Ibuprofen may also have had an effect on granulocytes, inhibiting in vitro granulocyte aggregation, superoxide production, lysozyme release and chemotaxis.

Plasma proteins such as albumin, gamma globulin and hemoglobin are important because they increase the filtration coefficient across the capillary walls and play a significant role in maintaining normal capillary permeability (30).

As will be discussed in the section on metabolism, fatty acids are the preferred susbtrates in well-oxygenated hearts. In hypoxic and anoxic states, fatty acid oxidation is suppressed and glycolysis is stimulated. Under these circumstances, the heart turns to anaerobic glycolysis for energy production. Maximal glucose transport becomes important and the lack of insulin may deprive the heart of adequate substrate. Insulin, therefore, is required in normothermic, whole blood perfusates. The addition of glucose and insulin affords a choice of aerobic energy production between fatty acid oxidation (supplied by fresh blood) or glycolysis.

Therefore, blood components seem to offer some protection to myocardial integrity. On the other hand, many important problems are associated with normothermic blood perfusion systems. Having a beating heart in the system requires close and constant monitoring of different parameters (coronary vascular resistance and blood flow, arrhythmias, etc). A precise warming and

temperature monitoring system have to be added to the unit. Over long periods of time, the quantitative and qualitative equilibrium of blood components must be maintain by artificial means such as dialysis, cross-circulation or changing of perfusate. Good matching between blood and the perfused organ is also an inherent pre-requisite.

B) CRYOPRESERVATION

Suppression of myocardial metabolism for prolonged preservation by cryopreservation (deep freezing) has been very attractive because the storage methods based on this principle have the theoretical advantage of requiring little or no control or monitoring during the preservation stage; such methods should theoretically allow for very extended storage time (13).

Many problems are associated with organ preservation under cryogenic temperatures. If the organ is frozen slowly, extracellular ice crystals form and cause a marked intracellular hyperosmolarity. Cryoprotectants such as glycerol or dimethyl-sulfoxide will partially prevent the formation of extracellular crystals but they have a very slow cellular penetration. These compounds are toxic at 20°C, therefore rapid freezing is required.

Puppy hearts have been subjected to cryopreservation (31). After being cooled to -4°C, the organs were subjected to mechanical dehydration and then saturated with 70% glycerol. After a storage period of 20 hours, the hearts demonstrated a normal beat, color, tone and excellent perfusion upon rewarming. No studies have yet conclusively shown good preservation of adult mammallian hearts with this method.

C) METABOLIC REDUCTION

Cardiac preservation by reduction of myocardial metabolism is a compromise between normothermic and cryogenic methods. There are basically 3 ways to reduce metabolic rate: chemical, hypothermic, hyperbaric. The 3 methods will be briefly discussed.

i) Metabolic Reduction by Chemical Inhibition

Nakae (32) have flushed isolated rat hearts with 1% $MgSO_4$ in ringer's lactate solution at 37°C. The tolerance of the hearts to normothermic ischemia could be extended from less than 15 min to 1 hour. Dog hearts arrested with $MgSO_4$ in combination with acetylcholine or chlorpromazine and kept warm in situ were shown to resume good contraction when heterotopically transplanted 3-4 hours later. The presumed protective effect of $MgSO_4$ is thought to be related to (13):

a) Reduction of metabolic needs.
b) Preservation of transmembrane potentials in the ventricular myocardium.
c) Maintenance of integrity of the cellular membrane, prolonging the function of the ionic pump.

Chlorpromazine (34) has a stabilizing effect on membrane permeability. It reduces mitochondrial swelling, lysosomal leakage and cellular autolysis.

Combining chemical inhibitors with hypothermia (4°C), it was possible to extend the duration of rat heart preservation to twice that obtained by hypothermia alone or by metabolic inhibitors alone (33).

ii) Metabolic Reduction by Hypothermia

It has been demonstrated that oxidative metabolism is reduced by 50% for every 10°C decrease in temperature (13). Theoretically, at 2°C, the metabolic rate of most organs would be equivalent to approximately 5% of the normothermic value (35). A reduction in myocardial oxygen uptake of 50% at 27°C has been demonstrated (36). Cooling alone can preserve organ viability only for a short period. Shumway and associates (37,38) successfully carried out orthotopic transplantation of canine hearts that had been cooled under anoxic conditions in 2-4 C saline solution for 4-7 hours; hearts preserved by this method for over 12 hours failed to support the circulation of the recipient animal.

Buckley's (39) group in Boston assessed the role of hypothermia and cardioplegic arrest on prolonged preservation of canine hearts. They found that hypothermia (11°C) for 90 minutes induced by high - K+ cardioplegic arrest preserved 99% of function as measured by left ventricular stroke work index before and after arrest. If the hearts were maintained at 24°C rather than 11°C, they recovered 80% of their funtion upon reperfusion. Normothermic arrest after aortic cross-clamping without cardioplegic use showed only 39% return of function at 45 minutes. Cardioplegic arrest could increase the return of function to 78% at 60 min and 54% at 90 min. On electron microscopy, normothermic hearts demonstrated more myocytic and capillary endothelial damage than the hypothermia-cardioplegia groups.

Hess (40) studied the function of the two most important subcellular organelles involved in the excitation-contraction coupling mechanism: the sarcoplasmic reticulum (SR) and the myofibril. The SR is the intracellular network of tubules that have been incriminated as the source of coupling calcium within

the myocardial cell and, via an ATP-dependent calcium pump, responsible for actively sequestering cytosolic calcium, resulting in relaxation. Hess worked on the basis of his previous studies incriminating sarcoplasmic function as one of the first intracellular processes to break down as a result of ischemia (41,42). Currently, there is also good ultrastructural evidence for a qualitative change in myofilament function in acute ischemia (43-45). On that basis, Hess'group looked at quantitative depression of myofibrillar ATPase activity and, SR calcium uptake rates in canine myocardium subjected to global ischemia. The group of hearts that were arrested under normothermic conditions for 30 minutes showed marked decrease in SR calcium uptake velocity and ATPase activity as opposed to the hearts that were arrested at 8-12°C (septal). The normothermic hearts showed good protection of SR but no protection of myofibrillar function. The hearts that were arrested with cardioplegic solution and maintained at 8-12°C showed very good protection of both SR ATPase and myofibrillar function. It was also found that the protection obtained by hypothermia and cardioplegia was lost by 60 minutes of ischemia in the absence of reperfusion with the cardioplegic solution.

Greenberg and Edmunds (46) found that the maximum tolerable ischemic time (for the maintenance of normal ventricular work capacity) of the canine heart is 10 minutes at 37°C, 15 min at 28°C, 30 min at 18°C and 60 min below 10°C. Nevertheless, Lower and Shumway (47) showed that the canine heart may be successfully homografted orthotopically, even after 6-8 hours of storage in saline at 2-4°C. Canine hearts protected by selective cardiac hypothermia at 15-20°C will tolerate up to 2 hours of arrest. The work capacity of cold-arrested hearts may exceed 80% of normal after restoration of circulation, whereas hearts arrested at normothermia show a work capacity of only 16-29% of normal (47).

iii) Metabolic Reduction by Hyperbaria

This method was used largely on an empirical basis. It was originally thought that high oxygen pressures would cause the gas to permeate the tissues and thereby support the metabolic needs of an organ during preservation (13)) On a theoretical basis, it was calculated that 7 atmospheres would satisfy the oxygen requirements of the myocardial tissue to a core depth of 1 cm (48). Matloff (49) showed that with pressures up to 4 atmospheres, there was no significant penetration of oxygen into renal tissue. It was Lyons (50) and Feenster that then suggested that hyperbaric organ preservation was mostly due to tissue metabolism reduction. Hyperbarism inhibits oxidative enzymatic systems and decreases the oxygen uptake by mammalian tissue preparations (51,52).

D) PERFUSION

Perfusion of organs is best accomplished by maintenance of ciculatory function in the cadaver donor, and this method is physiologically superior to any available method of in vitro perfusion. However, the cardiac deterioration that occurs in brain dead patients (massive brain trauma with increased intracranial pressure, for instance) which is incompletely understood, limits the duration that in vivo organs can be maintained in this way.

The aim of ex-vivo perfusion is to support the metabolic activity of the organ by continuously supplying oxygen and nutrients with wash out of metabolic waste products. Extracorporeal perfusion, though, is limited by many factors; a constant oxygen source is necessary, structural damage to blood components can occur; and optimal perfusate composition is yet to be found. Edema and weight gain of the organ, microembolization and perfusion resistance can severely damage the organ.

In 1912, Carrel (53) demonstrated that hearts removed soon after death could continue to function ex vivo if supplied with the necessary substrates. In 1936, Kountz (47) reported post-mortem ressuscitation of 65 human cadaver hearts with restitution and maintenance of coordinated ventricular contraction by perfusion of the coronaries. Since these early experiments, numerous attempts to store hearts by the perfusion method in conjunction with hypothermia have been made. Various perfusates have been used; they can be divided into biological or artificial solutions.

i) Biological perfusates

Whole blood has been used mostly at normothermia or in conjunction with moderate hypothermia. Levy (54) in 1965, circulated whole blood through a bubble oxygenator and perfused hearts at 37°C. The hearts kept on beating for up to 11 hours with no change in sodium, potassium or calcium concentration in the perfusate. In 1967, Pitzele and Dobell (55,56) used an extracorporeal perfusion circuit primed with whole blood and incorporated a silicone rubber membrane oxygenator. Canine hearts were successfully supported for 9-11 hours at 30°C and 12-14 hours at 20°C. Compared to unstored hearts, the preserved organs showed intact left ventricular function and myocardial metabolism.

Cleveland and Lower (37) perfused canine cadaver hearts for 4 hours using dilute unfiltered blood. They observed a gradual rise in perfusion pressure, decrease in coronary flow rate and increase in perfusate SGOT. After 3 hours, microscopic examination revealed many areas of subendocardial infarction with microthrombotic occlusion of small arterioles with interstitial edema. After orthotopic transplantation, performance of the hearts perfused with whole blood was poor whereas hearts perfused with dilute filtered blood performed well. Feenster and Lillehei

(35) perfused hearts for 24 hours with pulsatile flow at pressures of 100 mmHg. Oxygenated dilute blood to which low molecular weight dextran had been added was used. The system was maintained at 8-10°C under 3-4 atmospheres of hyperbaric oxygen. Upon homotransplantation, the resuscitated hearts showed forceful contraction, normal left ventricular function curves, minimal weight gain and normal histology.

There are many problems associated with whole blood perfusion:

a) Production of hemolysis (57,58,59).
b) White blood cell and platelet destruction-aggregation (60-63).
c) Plasma protein denaturation (64-67).

Perfusion with plasma is possible only at temperatures of 26-28°C or above because of its low oxygen-carrying capacity, and as a result, depending on the level of hypothermia, relatively higher perfusion flows may be required when compared to those using whole blood (13). Successful storage of canine hearts for up to 24 hours by hypothermic (18-24°C) plasma perfusion combined with hyperbaric oxygenation (3 atmospheres) has been reported by Feenster (67). These hearts were evaluated by orthotopic transplantation. Good functional recovery lasting up to 13 days was obtained with hearts that had been stored for 4-12 hours. However, the duration of survival of the recipients became progressively shorter as the length of the preservation period was increased to 16-24 hours.

In all the experiments mentionned, the severity of morphological damage increased as the preservation period was lengthened. It was found by Ferrans and Levitsky (68), in an attempt to decrease the incidence of interstitial edema, that when the osmolarity of the plasma perfusate was raised from 256 to 316 mOsm/L, the post-preservation interstitial edema was

absent and the evaluation of these hearts by ex-vivo perfusion and iso-volumic left ventricular pressure balloon showed a normal level of myocardial contractility. In 1970, Levitsky perfused hearts with filtered plasma for 18 hours and compared these with control hearts immediately after excision. The active length-tension curves were unchanged for up to 18 hours of preservation and was depressed at 24 hours. The dp/dt of preserved hearts was 1301 mmHg/sec after 18 hours and 1310 mmHg/sec in the non-preserved group. The dp/dt fell to 900 mmHg/sec after 24 hours of preservation ($p < 0.05$). Interstitial edema was present in all preserved hearts. Alterations in cellular glycogen content, in the morphologic appearance of the sarcoplasmic reticulum and mitochondria correlated well with the hemodynamic values. During the period of preservation, Levitsky observed that depressed myocardial function could be predicted by increases in perfusion pressure above 70 mmHg and increasing acidosis.

ii) Artificial Perfusates

The advantages of balanced salt solutions for organ perfusion are that they are easy to prepare and their formulation can be varied in many ways. On the other hand, these solutions will always lack the characteristics and useful properties of blood or plasma such as effective oncotic pressure and viscosity. Only a fraction of the metabolites normally present in plasma or blood can be incorporated.

Hypothermic cardiac perfusion with a chemically defined medium resembling plasma (electrolytes) has repeatedly yielded edematous organs in 1-6 hours and gross evidence of cardiac failure following in vitro evaluation (69,70,71). The addition of colloid (69,71) to the perfusate and of a filter to the perfusion system (72) in order to prevent embolization of

particulate matter seemed to benefit survival of the stored hearts.

Proctor and Parker (73) used a modified Kreb's solution with a low calcium concentration and also containing 9.6% dextran 70, chlorpromazine, hydrocortisone, insulin and procaine. The hearts were stored successfully for 72 hours at 4°C with continuous perfusate filtration (3-8 um size) under a perfusion pressure of 20-30 cm H_2O. After orthotopic transplantation, the hearts were able to assume control of the circulation with a mean systolic pressure of 70-100 mmHg without inotropic support. The overall rate of viability with this method was 75%.

In 1980, Guerraty (75) perfused hearts for 24 hours using a modified Kreb's solution. The hearts were then orthotopically transplanted and compared to a group of freshly excised hearts. He found no correlation between coronary resistance and recipient survival (all the transplanted hearts reported functioned well). A rapid rise of perfusate CPK, on the other hand, correlated well with cardiac failure after transplantation.

Ex vivo preservation by continuous perfusion for 24 hours or longer has also been studied by Copeland (76). Using hypothermic, asanguineous, low-pressure perfusion with a modified Kreb's solution and pre-treatment of the donor animal with phenoxy-benzamine and glyceryl trinitrate, they successfully transplanted 30 hearts.

Guerraty (75) had found a positive correlation between graft weight gain and coronary resistance during preservation. Interstitial edema, he thought, caused external compression of the vascular bed. He concluded that a weight gain less than 25% and a coronary resistance increase less than 30% after 24 hours of preservation constituted reliable criteria of graft viability. The difference observed in these 2 studies in perfusate CPK (less

in Guerraty's than in Copeland's series) might be explained by the use of less calcium in the perfusate and pre-treatment of the donor animals with diltiazem which may have decreased the cellular damage by reducing membrane permeability to calcium. Guerraty (75) recommended to observe the following factors for adequate prolonged preservation:

1) Donor heart coronary vasodilation prior to cardiectomy with diltiazem or nitroglycerin.

2) Rapid electromechanical arrest by cardioplegia to reduce depletion of energy substrates.

3) Avoidance of air embolism.

4) Filtration to avoid microembolization.

In 1981, Warner (77) studied subcellular function after 24 hours of preservation using Guerraty's (75) method. Sarcoplasmic reticulum (SR) calcium-stimulated, magnesium-dependent ATPase activity was not different when comparing hearts perfused in vitro for 24 hours to hearts preserved in 4°C saline for 3 hours. Contractile protein function measured as myofibrillar calcium-ATPase curves demonstrated a depression of maximal ATPase activity in the non-perfused hearts but not in the perfused hearts. He concluded that myocardial preservation in 4°C saline for only 3 hours resulted in a significant degree of ischemic-reperfusion injury to the excitation-contraction coupling system. In contrast, the intact heart could be maintained for 24 hours by continuous non-pulsatile, hypothermic perfusion with a minimum of ischemic-reperfusion injury to the excitation-contraction coupling system.

In 1986, Burt and Copeland (78) did a very complete study of hearts preserved for prolonged periods. They evaluated isolated

rabbit hearts in a Langendorf preparation. They had 5 different groups of hearts and studied them functionally:

	GROUP	RESULTS
I	Cooling + immediate reperfusion	No loss of function 25 min to recover
II	Cardioplegic arrest + cooling + immediate reperfusion	Full and immediate recovery of function
III	Cardioplegic arrest + cooling + reperfusion after ischemia at 5°C	95% recovery of function
	and 25°C	89% recovery of function
IV	Cardioplegic arrest + cooling + 24 hour perfusion + reperfusion	84% recovery of function
V	Cardioplegic arrest + cooling + 24 hour perfusion + ischemia 25°C + reperfusion	75% recovery of function

Their data clearly indicated that most of the damage occur during the preservation period. Both compliance and contractility declined in hearts preserved for 24 hours. The loss of compliance was thought to be secondary to contracture and edema. They also clearly demonstrated a clear correlation between edema (weight gain) and depression of contractility and compliance. This was reinforced by histologic evaluation which found many areas in which contracture bands had formed. This data also indicated that the effects of the preservation and ischemic periods were at least in part additive. The authors concluded that the mechanism involved in tissue injury during

preservation and ischemia are not yet known. The most frequently cited damage mechanisms are:

a) energy supply depletion
b) alteration of ionic gradients
c) waste products accumulation
d) cellular edema
e) membrane leakiness

iii) Summary

If perfusion above 30°C is to be used, perfusates of high oxygen-carrying capacity (whole or diluted blood) are required if excessively high coronary perfusion flows are to be avoided in order to meet the high oxygen demands of normothermic tissues. At temperatures less than 28°C, non-cellular perfusates, such as plasma or balanced salt solutions can be used since the decrease in tissue oxygen uptake coincides with an increase in oxygen dissolution in these fluids. The use of whole blood presents the disadvantages of hemolysis, cellular aggregation and denaturatiion of labile plasma proteins.

At a constant coronary flow, increases in blood viscosity, obtained by varying the hematocrit from 7% to 68%, result in corresponding increases in left ventricular pressures under isovolumic conditions (13). The role played by viscosity is also pointed up by the fact that when the viscosity of cryoprecipitated plasma is increased by the addition of starch, a linear relationship between viscosity and left ventricular pressure comparable to that of blood is obtained (13). Also, compared to pure plasma, perfusion with viscous plasma significantly retards the development of myocardial edema (13). It is therefore conceivable that perfusion with a non-viscous perfusate such as cryoprecipitated plasma results in an uneven distribution of flow and localized foci of hypoxia, even though the perfusion pressure may be adequate. The disadvantages of

whole blood perfusion, therefore, seem to be avoidable by the use of viscous non-cellular solution.

Under hypothermia, the depression of membrane activity leads to intracellular influx of sodium and water. At 10°C, perfusion of the isolated canine heart with high flow and high perfusion pressure leads to 50-100% weight gain over 24 hours. If a low flow-low pressure system is used, a 30% weight gain is observed (79). Myocardial edema consists of edematous myocytes, damaged capillary membranes, narrow vascular lumens, lysis of myofilaments, dilatation of SR and mitochondrial swelling.

In conclusion, adequate myocardial preservation can be obtained by simple perfusion techniques. Although blood carries advantages as well as disadvantages, some beneficial properties of blood perfusates can be relatively easily added to non-cellular perfusates.

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METABOLIC CONSIDERATIONS

3) METABOLIC CONSIDERATIONS

Exhaustive review of cellular metabolism can be obtained from any good biochemistry textbook. The following is only a brief review of energy production pathways occurring in myocardial tissue. This information was basically derived from Katz(1) and Lehninger's (2) textbooks.

Energy generation in the heart occurs through two major metabolic pathways:

1. Glycolysis.
2. Oxidative phosphorylation.

Glycolysis is essential for aerobic breakdown of carbohydrates to CO_2 and H_2O. It may also provide ATP under anaerobic conditions. Oxidative phosphorylation is the major energy-producing reaction.

a) Glycolysis

In the glycolytic pathway, each glucose molecule is metabolized to either Lactate or Acetyl CoA (Fig 1). When glucose is converted to lactate, the net synthesis of ATP is 2 moles of ATP/mole of glucose (4 moles of ATP are synthesized but 2 moles of ATP are required to phosphorylate glucose to fructose 1,6-biphosphate).

The rate of glycolysis can be modulated at various points (see fig 1):

1. Glucose transport.
2. Hexokinase reaction.
3. Phosphofructokinase reaction.
4. Glyceraldehyde - 3 - P Dehydrogenase reaction.
5. Lactate Dehydrogenase reaction.

Glucose entry into the myocardial cell is carrier-mediated but does not require energy. It simply moves down a very high concentration gradient. Factors that accelerate this carrier-mediated transport are:

- Insulin
- Epinephrine
- Hypoxia

The rate of the hexokinase reaction is deeply influenced by variations in the intracellular levels of G-6-P, ATP, ADP, AMP and P_1. The most important control at this level is an increased concentration of G-6-P which inhibits hexokinase phosphorylation of glucose. The structure of the enzyme is altered by increased G-6-P, reducing its ability to phosphorylate glucose. Therefore, any accelerated flux of substrates through the subsequent reactions of glycolysis, by consuming G-6-P, increases the rate at which the phosphosugar is produced from glucose. ATP has an inhibitory effect while ADP, AMP and P_1 stimulate glucose phosphorylation.

The phosphofructokinase reaction is the major control point of glycolysis. Various substrates or conditions affect this enzyme's activity as following:

Increased Enzyme Activity	Decreased Enzyme Activity
ADP	Acidosis
AMP	ATP
P_1	Phosphocreatine
F-1,6-DP	Citrate
cAMP	
NH_4+	

As hexokinase, phosphofructokinase (PFK) is also modulated by high and low-energy phosphate compounds. Acidosis exerts a powerful inhibitory effect on this enzyme. Citrate, one of the intermediates of oxidative phosphorylation, has a well-documented and important ability to inhibit PFK. In this way, increased aerobic metabolism, which produces citrate, can slow glycolysis.

The glyceraldehyde-3-P dehydrogenase reaction is not of major importance in regulating glycolysis. Under hypoxic or ischemic conditions, where PFK becomes strongly activated in the heart, glycolysis control shifts to this reaction. This enzyme is regulated largely by product inhibition in that it is extremely sensitive to the inhibitory effects of 1,3 - diP glycerate and NADH accumulation. Under hypoxic conditions, NADH is not oxidyzed and its accumulation slows glycolysis. Glycolysis in the ischemic heart is therefore regulated by a different set of metabolites than those in the well-oxygenated heart. Glycolytic control in the ischemic heart shifts from a step that is governed by energy needs (ATP) to one in which control is exerted by the ability of the cell to oxidize substrates and coenzymes.

In the well-oxygenated hearts, the lactate dehydrogenase (LDH) reaction is not utilized. Instead NAD is regenerated from NADH in the mitochondria. In the myocardium, as opposed to white skeletal muscle, which depends heavily on anaerobic glycolysis for energy production, the reaction catalyzed by LDH plays litle role in determining the metabolic fate of pyruvate; instead pyruvate metabolism is controlled by pyruvate dehydrogenase, which catalyzes the formation of Acetyl-CoA.

The final step in aerobic glycolysis is the conversion of pyruvate to Acetate. This 2 carbon fragment which enters the tricarbonylic acid (TCA) cycle does so as a complex bound with coenzyme A (CoA-SH). The acetate is attached to the sulfhydryl

group of CoA by a high-energy thio ester bond, much as the terminal phosphate group is attached to ATP by a high-energy phosphoester bond. Acetyl-CoA can be formed in 2 ways:

1) Carbohydrate metabolism during glycolysis.
2) Beta - oxidation of fatty acids.

The formation of Acetyl-CoA can take place only under aerobic conditions in the mitochodria. The conversion of pyruvate to Acetyl-CoA is catalyzed by the enzyme pyruvate dehydrogenase (Fig 1). This reaction which liberates CO_2 is through a huge multi-enzyme compound. "The extreme complexity of the regulation of this step in carbohydrate metabolism is not surprising when it is recognized that the metabolic fate of pyruvate stands at the crossroads between anaerobic and aerobic metabolism. Pyruvate can be converted to lactate, thereby regenerating oxidyzed NAD for continuing glycolysis as in the heart under anaerobic conditions. On the other hand, in the well-oxygenated heart, pyruvate enters into oxidative metabolism by conversion to Acetyl-CoA"(1).

Glycogen is an important source of carbohydrate for glycolysis. The synthesis and breakdown of glycogen are regulated by enzymes which can exist physiologically in active or inactive forms and whose interconversion is regulated by a series of hormonally determined reactions (Fig. 2).

b) Oxidative phosphorylation

Oxidative phosphorylation takes place almost entirely within the mitochondria. All of the substrates, metabolites and cofactors must traverse the membrane barrier between the cytosol and the mitochondiral matrix. Fat and carbohydrates must first be converted into 2 carbon fragments before entering the oxidative metabolic pathways.

Fat + CHO -> 2 carbon fragments -> oxidative metabolisme

Fats are found in 2 forms - free fatty acids (FFA) are bound to plasma proteins, mainly albumin. Triglycerides (TGL) are complexes of fatty acids (FA) and phospholipids esterified to glycerol, a 3 carbon sugar.

Uptake of FFA by the cell is due to passive diffusion across the cell membrane. TGL enter the cell after hydrolysis during which the esterified FA is released from glycerol by the lipoprotein lipase present on the capillary endothelium. This enzyme is also activated by epinephrine. The intracellular FA are then activated by forming a complex with coenzyme A (CoA). Acyl-CoA is a precursor for the acyl-carinitine derivatives which can cross the mitochondrial membrane. Such activation requires ATP. Once acylcarnitine is formed, transfer occurs and acylCoA is retransformed inside the mitochondria (carinitine acts solely as a carrier). Breakdown of acyl-CoA to Acetyl-CoA then occurs in the mitochondria producing a substrate for the TCA cycle. Fat metabolism is absolutely dependent on a continuing supply of oxygen. When coronary flow is interrupted, ATP can no longer be produced from fats.

The TCA cycle (Fig 3) is catalyzed by enzymes which are free within the mitochondrial matrix. Only 1 ATP molecule is directly formed by the cycle. The greatest portion of the chemical energy released by the TCA cycle is in the 4 oxidative reactions forming 3 NADH and 1 $FADH_2$. ATP can then be produced from NADH and $FADH_2$ by the respiratory chain-linked phosphorylation. All the NADH produced from the TCA cycle is readily accessible to oxidative phosphorylation in the mitochondria. On the other hand, in the case of NADH produced during glycolysis, reduction of the coenzyme occurs in the cytosol and so must be returned to the mitochondria before it can be converted into ATP. Also, under aerobic conditions, where lactate is not formed, the NAD

essential for glycolysis must be derived from intramitochondrial supplies. The mitochondrial membrane being not permeable to NAD or NADH, the membrane-transport mechanism is the malate-aspartate cycle (see Katz (1), pp 35-72). The malate-aspartate cycle resupplies the cytosol with NAD which can be used for glycolysis while it makes NADH available in the mitochondria for oxidative phosphorylation and production of ATP.

As mentioned previously, almost all the ATP produced by aerobic metabolism is obtained by a series of oxidative reactions. The enzymes catalyzing these reactions are tightly bound to the mitochondrial membrane. Each mole of NADH produces 3 moles of ATP while each mole of $FADH_2$ produces 2 moles of ATP.

ENERGY BALANCE - SUMMARY

Reaction	Substrate-level Phosphorylation ATP	Resp. Chain Phosphorylation ATP	Total ATP
I - ANAEROBIC			
Glucose -> Lactate	2	0	2
II - AEROBIC			
Glucose -> Pyruvate	2	0	2
2NADH -> 2NAD	0	4	4
2Pyruvate -> 2Acetyl CoA	0	0	0
2NADH -> 2NAD	0	6	6
2Acetyl CoA -> $4CO_2$	2	0	2
6NADH -> 6NAD	0	18	18
$2FADH_2$ -> 2FAD	0	4	4
Net ATP Production	4	32	36

Anoxia or ischemia halts the pathways responsible for producing almost 95% of the ATP derived from carbohydrate metabolism. In addition, ischemia inhibits completely the oxidation of fatty acids, which normally comprise a more important energy source than carbohydrates for the heart. The devastating effects of ischemia on cardiac function are therefore clearly understandable.

The enzymatic transfer of high-energy phosphate bonds to ADP occurs through 2 general classes of high-energy phosphate compounds that can serve as phosphate donors. In the first group, 3-phosphoglyceroyl phosphate and phosphoenolpyruvate are generated by catabolism of fuel molecules or during glycolysis. In the second group, phosphocreatine is the major storage reservoir of chemical energy in muscles. The phosphate group in phosphocreatine is enzymatically transferred to ADP by the enzyme creatine kinase.

PCr + ADP ---> Creatine + ATP

It is very important to emphasize that ATP does not function primarily as a reservoir of chemical energy, instead, it acts as a transmitter or carrier of energy. The amount of ATP in the cell at any given time, is sufficient for only a short period. Phosphocreatine in the cardiac cell, is the major reservoir of energy. Phosphocreatine is formed whenever ATP is at a high concentration. Since there is no other pathway for the formation or dephosphorylation of phosphocreatine, as soon as ATP falls, it is replenished from phosphocreatine.

LIST OF FIGURES:

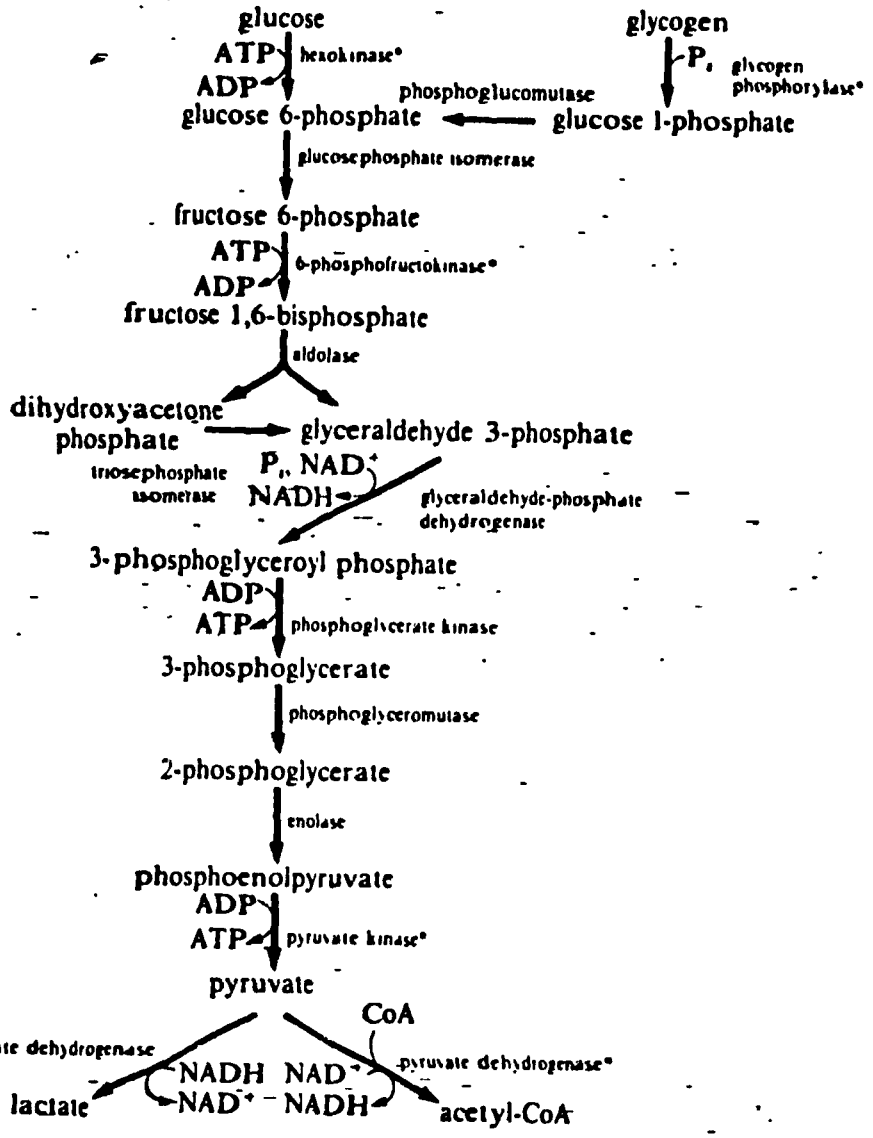

Figure 1: The Glycolytic Pathway.

All enzymes can function physiologically in the reverse direction except those marked with an asterisk.

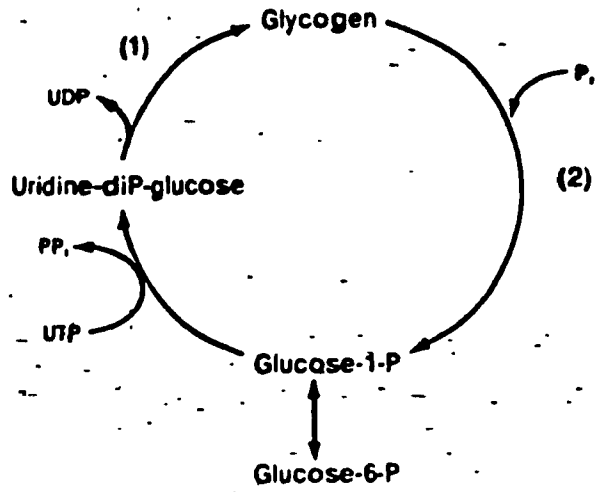

Figure 2: Glycogen Formation and Breakdown.
Pathways of glycogen synthesis(left,reading upward) differ from those of glycogenolysis(right,reading downward). Glycogen synthesis is controlled mainly by glycogen synthesase,which catalyzes reaction 1; and glycogenolysis is regulated by phosphorylase, which catalyzes reaction 2.

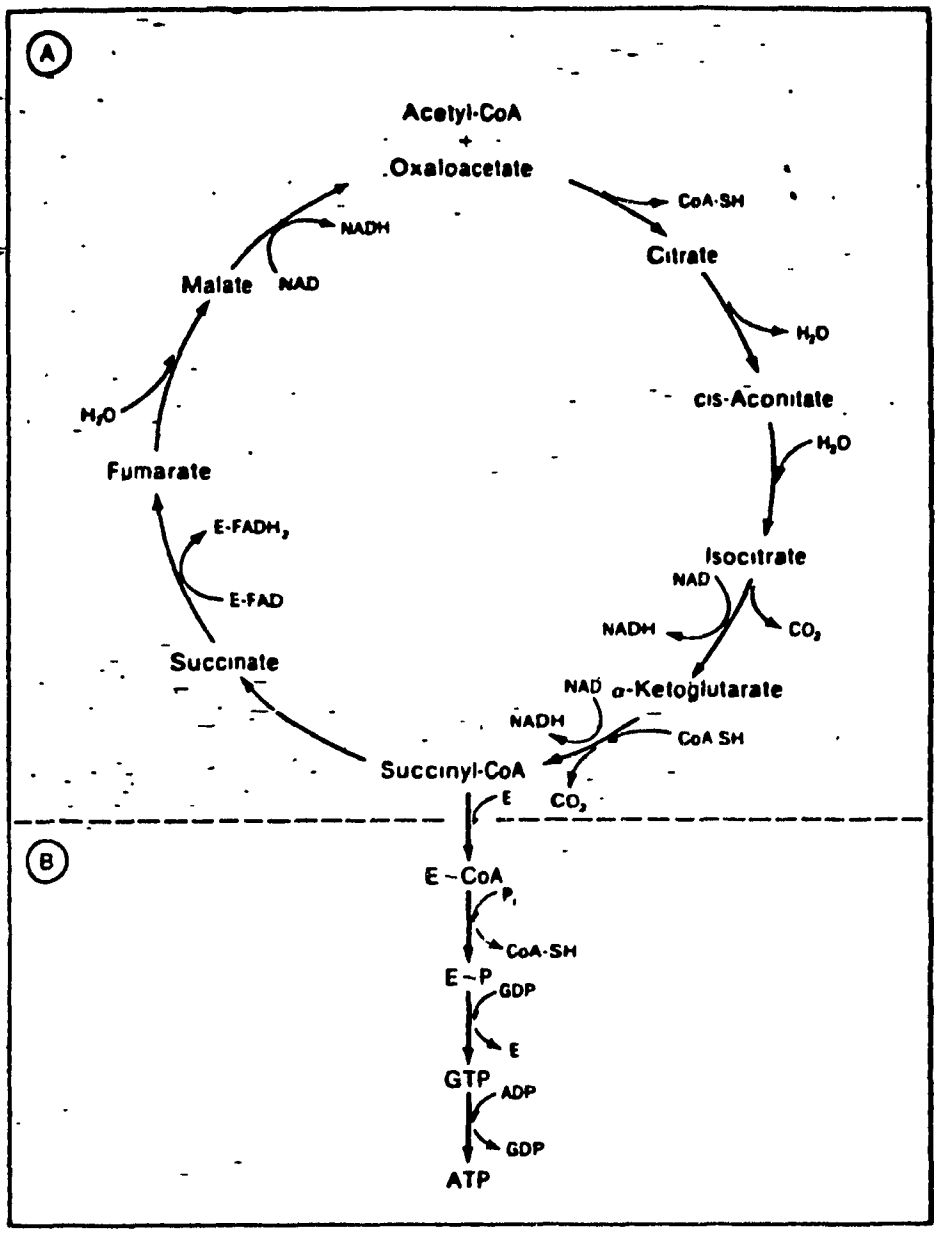

Figure 3: Pathways of acetyl-CoA oxidation.

A: Tricarboxylic acid cycle.

B: Substrate level phosphorylation.
Each mole of enzyme-bound CoA released from succinyl-CoA provides for generation of a single mole of ATP by substrate level phosphorylation.

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VIABILITY ASSAYS FOR CARDIAC PRESERVATION

4) VIABILITY ASSAYS FOR CARDIAC PRESERVATION

In cardiac transplantation, as opposed to kidney or pancreatic transplants, the heart must be able to support recipient's circulation immediately after reimplantation. As opposed to other organs, there is no good mechanical support device that can replace the failing graft for long periods with no harm. The support of adequate body perfusion per se, consists in the best viability test for a heart that has been preserved for a certain period of time. This method has the disadvantage to be very impractical as a pre-transplant predictor of function.

As the duration of the preservation period is increased, the chances for progressive imbalances between the numerous factors influencing viability become greater, and the necesity for accurate prediction of functional integrity of the stored heart prior to transplantation assumes a particular significance.

As mentioned in an earlier section, the graft is usually maintained either in a 4-5°C saline bath prior to clinical transplantation, or continuously perfused for extended periods in the experimental setting. It is accepted that a release of SGOT, LDH, or CPK or a rise in calcium, potassium or inorganic phosphates (1) in the saline solution or perfusate as well as a rise in perfusion pressure or coronary vascular resistance (2,3) can all predict varying degrees of graft dysfunction upon reimplantation. These evaluation tests though, are largely negative in the sense that significant changes involving the parameters indicate a deterioration of the stored heart; the absence of such changes does not necessarily reflect preservation of the ability of the organ to support a physiological work load following rewarming and orthotopic implantation.

In 1981, Bethencourt and Laks (4) studied myocardial edema and compliance during preservation of canine hearts using hypothermic, low pressure, continuous perfusion with a modified Kreb's solution. They demonstrated a statistically significant rise in gravimetric heart water content from 78 ± 0.8 ml/100 gm to 84.2 ± 0.3 ml/100 gm after 24 hours of perfusion ($p < 0.01$); the passive compliance of the hearts, measured by inflatable intraventricular balloon-tipped catheter, decreased from 1.49 ± 0.03 ml/mmHg to 0.65 ± 0.14 ml/mmHg ($p < 0.05$) in the same period of time. Light and electron microscopy after 24 hours of preservation showed marked pericapillary edema but excellent preservation of intracellular structure. By measuring the coronary resistance during preservation, they found a positive correlation between this parameter and the change in passive compliance. The passive compliance after 24 hours of preservation also correlated well ($r = 0.73$) with the myocardial compliance post-transplantation. The authors concluded that a change in passive compliance provides an additional index of heart viability.

Microscopic methods can be very useful to assess structural integrity of the heart post-preservation. Light microscopy permits mostly an assessment of interstitial and myocytic edema while electron microscopy will assess sarcoplasmic reticulum and mitochondrial integrity. These methods are valid in the experimental laboratory but do not permit an immediate assessment of graft viability as rapidly as required in the clinical setting.

Myocardial content of Lactate, glycogen, ATP and phosphocreatine can be obtained from biopsy specimens. Complex biochemical assays are required for these tests and significant damage can be inflicted on the hearts by taking the biopsies. Valuable ischemic time is also required for processing and analysis of the samples. Myocardial ATP content so far seems to

be the best index of heart functional preservation. Jennings et al (5) in 1978, studied canine ischemic myocardium. Sudden occlusion of a coronary artery results in rapid conversion to anaerobic metabolism with consequent loss of high energy phosphates from the ischemic myocardium. Initially, phospho-creatine forms further ATP (PCr + ADP --> ATP + Cr) but the tissue content of this very important reservoir of high-energy phosphate bonds was found by the authors to be reduced to 30% of its initial level by 60 seconds of ischemia (warm). Synthesis of high-energy phosphate continues in the ischemic tissue but at a much reduced rate compared with control aerobic conditions because anaerobic glycolysis is the only source of new high-energy bonds (as described in the section on metabolism, only 2 moles of ATP per mole of glucose are derived from the breakdown of glycogen to lactate). In severe ischemia, little glucose being available, glycolysis is limited by a low cellular supply of glycogen, a progressive intracellular acidosis and by destruction of nucleosides and nucleotides by nucleotidases.

Jennings (5) cites that depletion of high energy phosphate is associated with failure of the cells to recover contractile function. This author tried to determine the relationship between ATP depletion and cellular death as evaluated by characteristic changes in ultrastructure, cell volume regulation and reperfusion studies. After acutely occluding the circumflex coronary artery, Jennings demonstrated that after 15 minutes of warm ischemia, only 30% of the initial ATP remained; this amount was associated with reversible ultrastructural changes. After 30 minutes, 9% of ATP was present. At 40 mminutes, when only 7% of ATP remained and the cells showed completely irreversible ultrastructural changes and markedly altered cell volume regulation mechanisms. The author concluded that a close relationship exists between the marked depletion of high energy phosphate and lethal injury. Again it must be emphasized that

such biochemical assays for ATP determination are long and complex to perform and, therefore do not apply well to clinical heart transplantation. At present, there is no good non-invasive method to assess the quality of the graft before repertusion. Quantitative birefringence measurements on myocardial tissue give a good indirect assessment of the ability of the muscle fibres to respond to ATP and calcium.

The method is rapid and simple and has been shown to be a reliable monitor of myocardial protection during cardiopulmonary bypass and to give an indication of the immediate post-operative condition of the patient (8,9,10). In 1987, Darracott-Cankovic (7) applied the method to clinical heart transplantation. After taking Tru-cut needle biopsies of 50 hearts, the birefringence ratio was measured before excision and during transport of the graft. Fifteen hearts showed poor birefringence ratios before excision, 67% of the recipients of these hearts required post-op inotropic support. 22 grafts deteriorated during transport; 50% of the recipients received post-op inotropic support. Thirteen hearts showed good ratios before excision and during transport; none of the recipients had to receive inotropes in the recovery period. No harmful damage was inflicted to these hearts and results were available within 15 minutes of sampling.

Intracellular pH (see next section on NMR spectroscopy) is important for glycolytic metabolism. All the methods so far used to measure it are inaccurate and reflect mostly interstitial pH rather than intracellular pH. Only Nuclear Magnetic Resonance permits very accurate measurements of intracellular pH.

In conclusion, it is very hard to predict if a preserved heart will regain full functional integrity after reimplantation. Crude measurements can be done but they all have the disadvantage of either being long or cumbersome to perform. These methods are also usually destructive by the fact that biopsy samples are

required. It is clear, however, that energy is necessary for the muscle fibres to contract. Metabolic pathways under normothermic, physiological conditions are well known; however, a method has to be described to non-invasively assess cardiac metabolism under hypothermic conditions. The method has to be accurate, flexible, rapid and reliable. If hypothermic myocardial metabolism can be better defined, then viability of preserved hearts will be better predicted; this would also allow intelligent and better planned improvements in the preservation technique.

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NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

5) NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

31-Phosphorus nuclear magnetic resonance (^{31}P-NMR) spectroscopy allows non-invasive study of the metabolism of ATP, creatine phosphate (PCr) and inorganic phosphates (Pi) in isolated, intact, functioning organs. It permits quantitation of dynamic aspects of the metabolism of these compounds. The method is non-invasive, can be repeated at will and allows physiological function in intact organs.

Historical Perspective(1)

1945 - Purcell (Harvard) Black (Stanford)	-	Detection of magnetic absorption properties of protons.
1960 - Cohn and Hughes	-	First high resolution spectrum of a solution of ATP.
1973 - Moon and Richards	-	Measurement of intracellular pH of human red blood cells.
1974 - Henderson	-	First ATP spectrum from cells.
- Hoult & Radda	-	Full spectrum of intact muscle.
- Lanterbur	-	Definition of spatial distribution of protons in a sample - the basis of NMR imaging.

NMR spectroscopy was initially used by physicists to measure magnetic properties of various nuclei (atoms). The chemists then recognized the power of NMR in elucidating the structure of compounds. In the early experiments, proton spectroscopy (H-1) was used, then carbon-13 (C-13). Isotopes of phosphorus, nitrogen, fluorine, sodium, magnesium and lithium are now widely used.

NMR spectroscopy is based on the magnetic properties of nuclei while ultraviolet and visible spectroscopies are based on properties of electronic states in molecules. Nuclei with odd mass and/or odd atomic number behave as spinning electrical charges and therefore have a magnetic moment. 31-Phosporus (31-P) has an odd atomic mass and number.

In their usual state, 31-P nuclei have their magnetic moments aligned in a random fashion (Fig 1). If the nuclei are placed in a strong magnetic field (H_0), the magnetic moment of each nucleus will align either parallel (low energy state) or anti-parallel (high energy state) to the external magnetic field (H_0). If energy is provided (Fig 2) at the proper frequency in the radio frequency range, energy will be absorbed, and a transition from the lower to higher energy state can take place.

If the radio frequency signal is interrupted, nuclei will spontaneously return to their initial low energy state. A signal with a certain frequency will then be emitted (fig 2).

NMR spectroscopy therefore requires a strong homogeneous magnetic field (produced by a superconducting magnet), a radio frequency transmitter and a receiver. A computer collects and stores the received signals; it then performs Fourier calculations to obtain a spectrum (Fig 3).

Radio frequency pulses of 1-100 second duration are applied every 0.5 - 5 seconds. The receiver is turned on after each pulse and the radio frequency signal induced by the decay in magnetization as nuclei return to the lower energy state is measured. Results of each pulsed signal are stored in the computer and then averaged over pre-programmed blocks of time.

This decay in the radio frequency signal, called the free-induction decay, contains information about all the nucleus-specific transitions that occurred in the sample. The free-induction decay, which relates signal intensity to time, is virtually uninterpretable to the average NMR user. Using the Fourier transform, the relationship of signal intensity to time is mathematically manipulated to produce a spectrum relating intensity to frequency (Fig 3). In such a spectrum, each phosphorus compound is associated with a well-defined frequency. The absolute or relative concentration of each compound can be deducted by the surface area under each resonance peak.

Most nuclei of biological interest have isotopes observable by NMR. Ease of observation depends both on natural abundance and inherent NMR sensitivity. Stable nuclei with high natural abundance are hydrogen (H-1), fluorine-19 (F-19), sodium-23 (Na-23) and phosphorus-31 (P-31).

Not all nuclei are equally NMR-sensitive. A proton is the most sensitive (and most abundant) nucleus. Each nucleus is NMR-sensitive over a relatively narrow range of radio frequency radiation, and its characteristic radio frequency range is easily separable from those of other nuclei. For a magnetic field strength of 8.46 Testa (T), the field strength of the earth being 10^{-5}T, H-1 signals are observed at a radio frequency of 360 MHz and those of P-31 at 145.75 MHz. Frequency is usually expressed numerically as parts per million (ppm), which is the ratio of the difference between frequency of a specific nucleus and a

reference resonance in hertz divided by the spectrometer operating frequency in megahertz.

NMR spectroscopy is now very sensitive. Technological advances in both instrumentation and computers have so progressed that, for example, 5 umoles of ATP in a 1 gm heart can be detected in a few seconds.

The ^{31} P-NMR Spectrum

Typical heart spectra (Fig 3) consist of a series of peaks each occuring at a defined frequency (resonance). Identity of the compounds corresponding to these seven resonances has been deduced from:

1. Knowledge of the composition of hearts based on traditional biochemical measurements.

2. P-31 spectra obtained from solutions of standard compounds.

3. Characteristic changes in the position of the resonances, i.e., the chemical shift, when pH, ionic strength or anion concentration is changed.

cAMP, ADP and NADH are not present in high enough concentrations to be detected by NMR in reasonable time. As shown on Fig 3, the beta-ATP (B-ATP) peak is composed only of ATP, the gamma peak (Y-ATP) consists of both Y-ATP and Y-ADP while the alpha peak contains Alpha-ATP, alpha-ADP and NAD.

NMR can be used to quantitate the concentration of the substances observed. The intensities or surface areas of the resonances are proportional to the total number of phosphate nuclei. This means, for example, that all ATP molecules in all regions of the heart (on the surface or in the endocardium, in

the left atrium or in the right ventricle, in the intracellular water or the extracellular space, etc) contribute equally to the areas of the 3 ATP resonances. Relative concentrations can be deduced by comparing the surface areas of ATP, for example, from one spectrum to the other (the Beta and gamma peaks are used for ATP). NMR spectroscopy permits non-invasive, non-destructive measurement of all the phosphorus compounds simultaneously. It also provides information about the sugar phosphate pool. Of great practical value, NMR spectroscopy can be used to assess sequential changes in any of the NMR-observable compounds due to alterations in substrate availability or changes in chemical or gaseous environment in the same heart.

NMR provides a very accurate methods to measure absolute concentrations of phosphorous compounds. It gives more accurate, and possibly higher, ATP and phosphocreatine values than those obtained by traditional biochemical assays using freeze-clamped tissue. Absolute concentrations can be obtained in 2 ways. A capillary containing a known concentration of a NMR-observable compound can be inserted in the sample tube to serve as an internal standard. The area of this resonance peak can then be compared to the area of the resonances corresponding to the compounds to be measured. A second method consists in measuring the absolute concentration of ATP, for instance, by conventional biochemical methods and use this as a standard to which the resonance peak areas can be related.

Some of the resonances shown on heart spectra undergo predictable shifts when pH, ionic strength, dielectric constant, temperature or counterion is changed. The sensitivity of some of these compounds to changes in pH has proven to be useful in developing NMR as a very precise non-invasive pH-meter. Since most of the ATP in muscle is bound to Mg^{2+} and the resonance position of Mg-ATP and phosphocreatine are not sensitive to changes in pH near neutrality, the resonance positions of these

phosphates are not useful indicators of intracellular pH (pH_i) in biological samples. In contrast, the position of the inorganic phosphate resonance peak is sensitive to changes in pH near neutrality.

$$H_2PO_4^- + H_2O \text{ ---> } H_3O^+ + H\,PO_4^{2-} \quad pK = 7$$

The dominant determinant of the position of the P_i peak in a tissue spectrum is pH. Absolute assignment of pH and changes in pH can be calculated from the position of the P_i peak in relation to the pH-stable phosphocreatine peak.

The computer can measure precisely the distance between the 2 peaks. This distance can then be converted into pH units. NMR is uniquely suited to assess the existence of compartments and metabolic gradients formed by compartmentation. A pH gradient of 0.5 - 1 pH unit between mitochondria and cytoplasm has been demonstrated for hepatocytes (2) and E. Coli (3).

In summary, NMR spectroscopy provides an ideal means to study high-energy phosphate compounds metabolism non-invasively in global hearts. The method is precise, repetitive and permits dynamic measurements of ATP, PCr, P_i and sugar phosphate compounds. The intracellular pH environment can also be assessed. Whole intact hearts can be studied and various preservation methods can be compared in a reliable, reproducible way.

Figure 1

^{31} P nuclei (arrows) have their magnetic moment aligned in random fashion. When subjected to a high magnetic field (H_0), the magnetic moment of each nuclei align either parallel (low-energy state) or anti-parallel (high-energy state) to the external magnetic field (H^0).

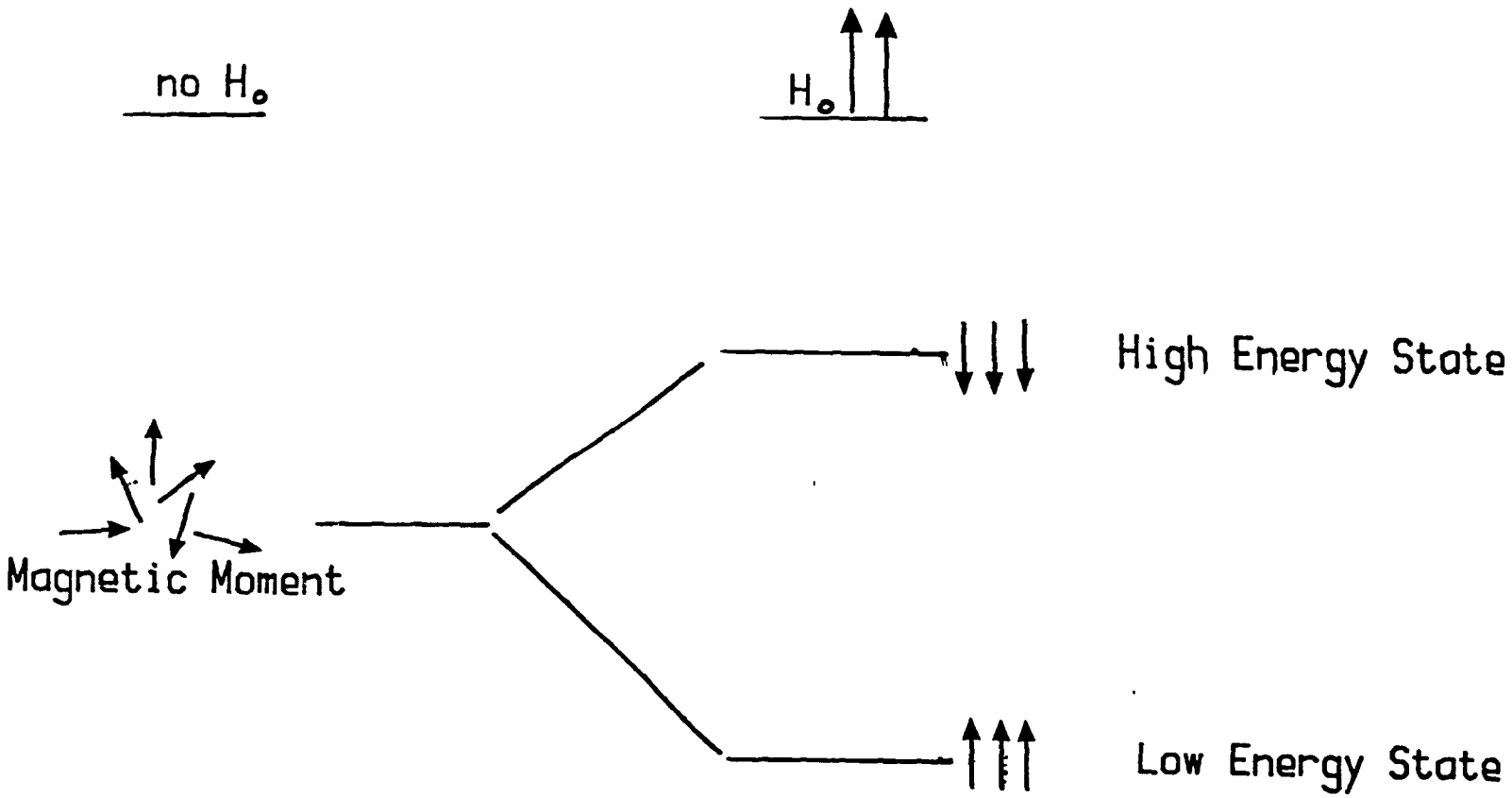

no H_o
H_o
High Energy State
Magnetic Moment
Low Energy State

Figure 2

Once the magnetic moments (arrows) are aligned, energy is provided at the proper frequency. The energy is absorbed and a transition from the lower to higher energy state takes place. When the radio frequency signal is interrupted, nuclei spontaneously return to the initial low energy state. A signal is emitted and picked up by a receiver.

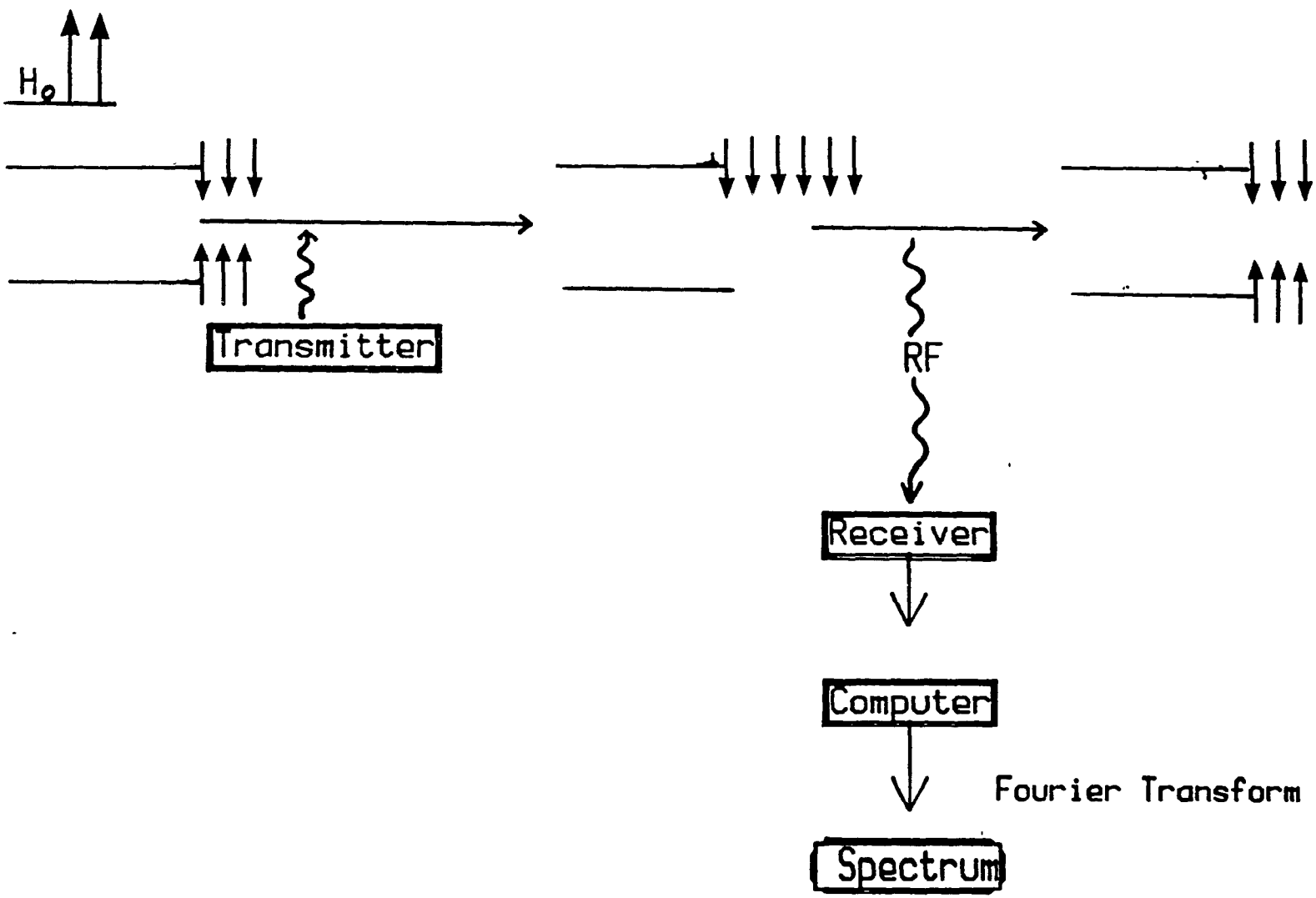

H_o
Transmitter
RF
Receiver
Computer
Fourier Transform
Spectrum

Figure 3a

Typical ^{31} P-NMR spectrum obtained during spectroscopy of whole hearts. (ATP= Adenosine -5' - triphosphate)> The spectrum is composed by fourier transformation of the signal emitted by the nuclei returning from the high energy to the low energy state (see figure 1-2).

^{31}P-nmr spectroscopy : typical heart spectrum

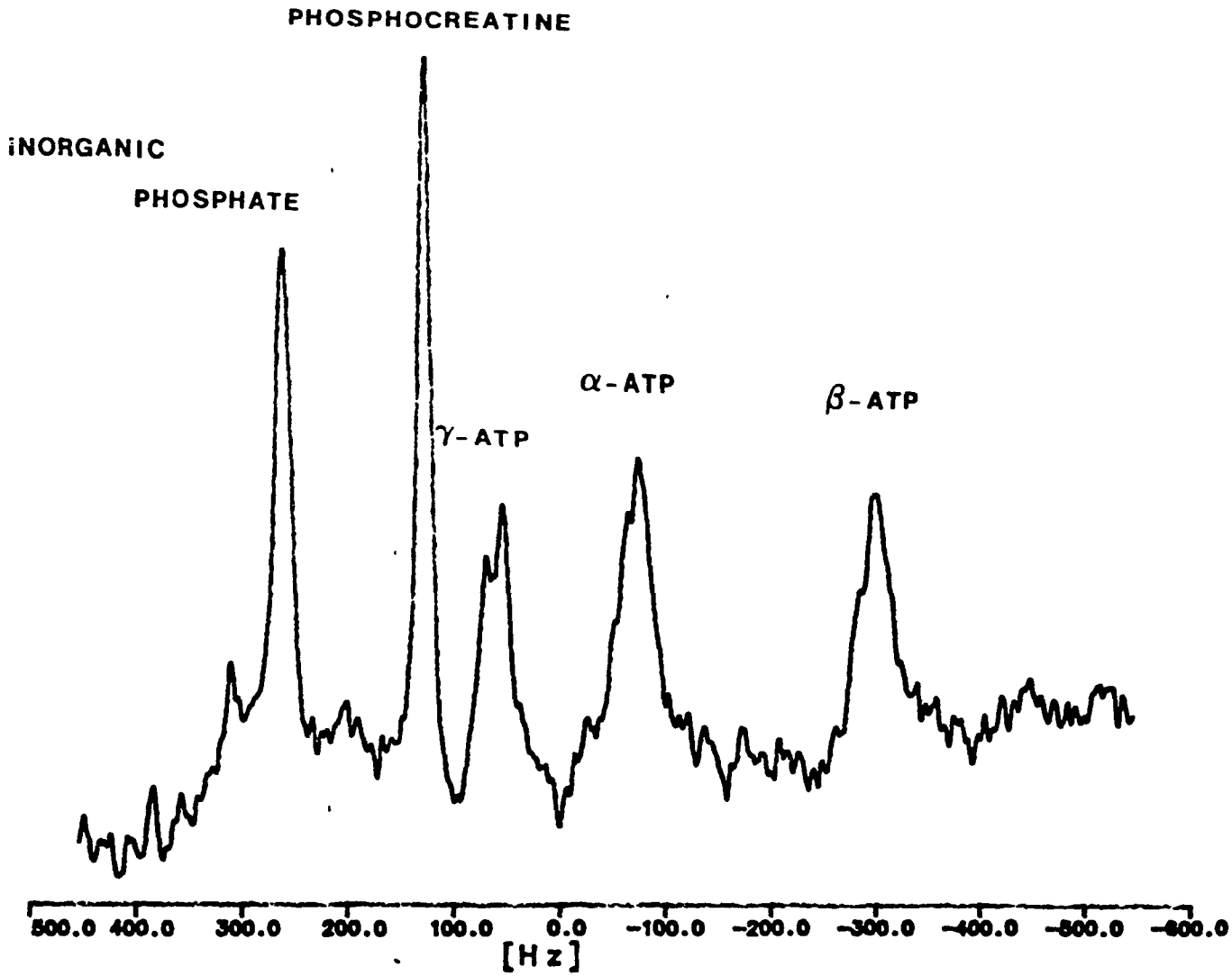


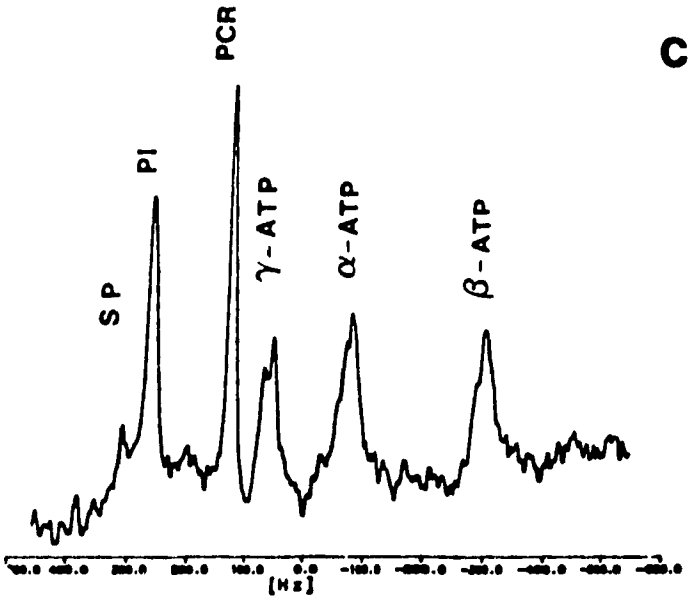
Figure 3b

Progression of ^{31} P- NMR spectra during group I experiments. The relative surface area of each peak is used to determine relative changes in concentrations.

cardiac Preservation

conventional method

(EXP. 1)



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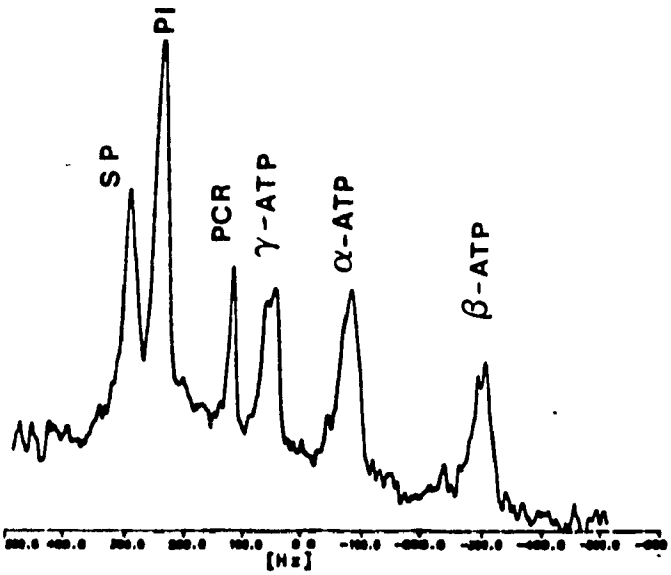

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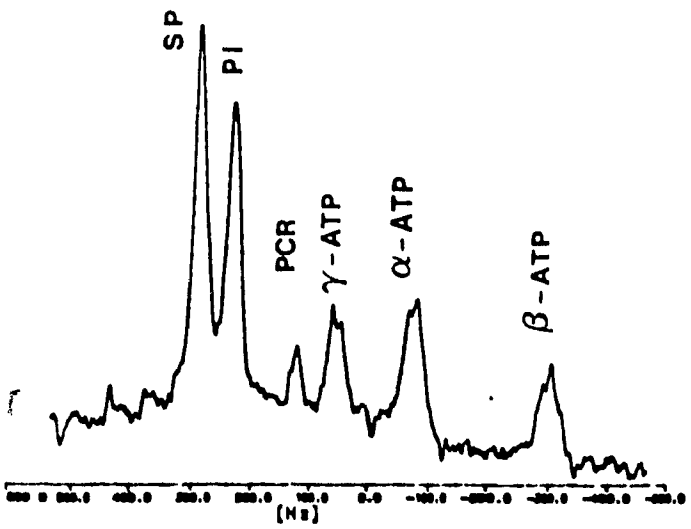

4 HOURS

SP = SUGAR PHOSPHATE

PI = INORGANIC PHOSPHATE

PCR = PHOSPHOCREATINE

ATP = ADENOSINE TRIPHOSPHATE

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PURPOSE OF THE STUDY AND DESCRIPTION OF THE MODEL

As mentionned in the previous sections, cardiac transplantation has become a well-accepted modality of treatment for end-stage cardiac disease. The number of cardiac transplantations performed annually has risen exponentially in the past few years.

The cardiac transplantation era can be divided into 2 well-differentiated episodes. First, in the mid to late sixties, major technical progress was achieved by the teams led by Shumway, Lower and Barnard. Lower for the first time described a preservation method by which the heart could be maintained viable for up to 4 hours. It consisted of immersing the graft in a 5°C saline bath. The successes of these pioneers prompted a multitude of centers around the world to start major transplantation programs. Improved techniques for post-operative hemodynamic and respiratory management reduced the early post-operative loss and led to optimism in the new field of cardiac transplantation.

Despite all this, the brutal reality of cardiac rejection led to unacceptable long term survival in this initial era. Poor understanding of the immune mechanisms of rejection and a limited armantarium of immune suppressants were the major reasons for early and late graft dysfunction. Most of the transplantation programs stopped except for a few (Stanford, Richmond in the USA, Paris and South Africa).

After many years of intense laboratory and clinical research, new immunosuppressants, particularly the fungal-derived Cyclosporine-A, started being used mainly in renal transplantation programs. It was soon realized that these very potent new agents were to open a new episode in the cardiac transplantation era.

The Stanford group which had maintained its cardiac transplantation program throughout the difficult years started using Cyclosporin-A in the early 1980's. The success was immediate.

Soon but cautiously, new programs started active heart transplantation with a better understanding of the mechanisms involved in cardiac rejection as well as a more extensive battery of new immunosuppressants.

Despite all these new advances, the heart preservation technique described by Lower 20 years ago is still used in all transplantation centers. It is well accepted that this method is safe for only 4-6 hours (the Stanford group has performed a transplant after a 7 hour cold ischemic period). After that time, it is known that the heart has a very poor ventricular function, most of the time not sufficient to sustain the recipient's circulation after transplantation.

Even though it is thought that most of the metabolism of the organ is deeply altered and slowed down under hypothermic ischemia, the fact remains that the heart still deteriorates under such conditions. It has always been assumed that aerobic glycolysis is completely inhibited by hypothermia but the mechanisms have never been well understod. A better preservation method has therefore to be sought.

Isolated kidney perfusion has been successfully used in transplantation for many years. The safe preservation period has been extended to 36-48 hours. This leaves plenty of time for precise matching of the donor and recipient, therefore minimizing the risk of late rejection.

The perfusion method also offers the advantage of vastly extending the potential organ donor pool. If such a method could be applied to cardiac transplantation, it is possible to postulate that hearts could be transported across continents when a transplantation is urgently needed. As opposed to chronic renal failure, during which patients can be maintained alive by chronic hemodialysis, end-stage cardiac failure patients very often die while on the transplant waiting list because of relatively limited extracorporeal life support devices. The long distance often separating donor from recipient sometimes is the cause for non-use of potentially good grafts.

A lot of progress in isolated heart perfusion has been achieved in recent years but major questions still remain. Is cardiac metabolism under hypothermic perfusion conditions aerobic or anaerobic? What are the metabolic factors determining the short time limit associated with the current clinical preservation technique as described by Lower? How can these factors be accurately measured? What viability assay would permit rapid, non-invasive assessment of the preserved hearts?

Many other unanswered questions remain in the field of cardiac preservation. In order to attempt answering the above questions, it was decided to use the hypothermic (4[C), low-pressure (20 cm H_2O) perfusion model as described by Dr. Guerraty (1), and compare the high energy phosphate metabolism of hearts preserved for 24hrs by this method to hearts preserved by 4 hour hypothermic saline immersion.

As mentionned previously, the current preservation technique used in clinical heart transplantation is the same that Dr. Lower described 20 years ago. The method consists of rapid immersion of the donor organ in a 5-7°C saline bath. It is well known

that hearts preserved in this fashion will have a very poor ventricular performance if the preservation period is extended to more than 6 hours. The fact that the metabolism occuring under hypothermic ischemia is mostly unknown, explains why the exact metabolic factors limiting the preservation period have never been well defined. The heart being a four-chamber muscle continuously pumping against varying resistances, must rely on a constant reserve of energy to fulfill its function. If there is no energy, the heart cannot contract. There are very few viability assays able to predict the performance of a preserved heart. The more valid assay is energy content, or ATP. As discussed in the previous sections, it has often been postulated that intracellular pH is also important in regulating enzymatic pathways necessary for ATP formation and muscle contraction.

Biochemical assays for ATP content on muscle tissues are relatively easy to do but multiple biopsies on the organ have to be done. The assay for phosphocreatine, the precursor necessary for ATP productions is also reliable but tissues have to be handled in a very specific fashion to allow for precise measurements, leaving much room for error. There are no good methods for biochemical measurement of intracellular pH.

^{31} Phosphorous Nuclear Magnetic Resonance (31P-NMR) is a relatively new method to study high-energy phosphorous compound metabolism in isolated organ preparations. It is non-invasive and very accurate; it also permits repetitive, dynamic measurements, therefore allowing a precise assessment of the time-course of the metabolic parameters studied. ATP, phosphocreatine, inorganic phosphate and sugar phosphate compounds can be easily measured; it is also the only method allowing precise measurements of intracellular pH. One of the disadvantages of biopsies is that they do not reflect what is happening metabolically in the whole organ. If for example, a

compromised area of the heart is biopsied and biochemical assays reveal very low levels of ATP, the heart performance will be completely unpredictable depending on the extent of damage in relation to the whole organ. ^{31}P-NMR measures the average concentration of high-energy phosphorous compounds of the organ in a global fashion.

It was decided first to study canine hearts high-energy phosphorous compound metabolism during 4 hours of hypothermic ischemia. As per Lower's method, the hearts were immersed in a 5°C bath of Ringer's lactate solution. The hearts were maintained at that temperature and studied by ^{31}P-NMR spctroscopy for 4 hours.

A special circuit was then devised and canine hearts underwent continuous, hypothermic coronary perfusion with a modified Kreb's solution for 24 hours, as per Guerraty's method. The 2 experiments were then compared in terms of maintenance of intracellular phosphate, ATP, phosphocreatine, inorganic and sugar phosphate compounds.

By using the NMR technology and comparing the topical hypothermia method to the perfusion method, we attempted to clarify the metabolic advantages of the latter method and to better define the factors limiting preservation periods.

^{31} Phosphorous nuclear magnetic resonance analysis of canine heart preservation: 4 hour hypothermic immersion versus 24 hour hypothermic perfusion.

31p-NMR Analysis of canine heart perservation: 4 hour hypothermic immersion versus 24 hour hypothermic perfusion.

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Since 1968, several groups (3,7), have developed an experimental method of continuous hypothermic perfusion of the isolated heart which allows acceptable ventricular fonction after 36-48 hours of perservation. In our own laboratory, 90% of the canine hearts preserved for 24 hours by continuous, low pressure perfusion with an oxygenated modified Krebs solution at 5-7°C regained ventricular furction when the non-working hearts were studied after neck implantation.(7).

At present, no reliable assay exists which would predict ventricular performance after in vitro heart preservation. It is known that myocardial ischemia results in rapid depletion of tissue phosphocreatine (PCr) and adenosine 5 triphosphate (ATP) (8). Quantitative birefringence to indirectly assess the ability of cardiac muscle biopsies to respond to ATP and calcium has been applied to clinical heart transplantation (9). It was demonstrated that a good correlation existed between birefringence ratio before or after preservation and ventricular function after transplantation. High energy phosphate compound stores may help to predict ventricular performance after ex-vivo heart preservation (10, 12).

Although Hollie (11) demonstrated in warm-ischemic hearts that intracellular acidosis was not responsible for the rapid functional decline observed early in the anoxic period, a low intracellular pH (pHi) has been suggested as a cause for inhibition of glycolytic and enzymatic activity, e.g. phosphofructokinase (12).

In order to better define the metabolic advantages of low pressure, continuous, oxygenated hypothermic perfusion versus simple hypothermic immersion of canine hearts as currently used clinically, 31-Phosphorous Nuclear Magnetic Resonance (^{31} p-NMR) spectroscopy was used to measure ATP, Phosphocreatine (PCr) and intracellular pH changes and compare the 2 methods of cardiac preservation mentioned above.

METHODS

Nine mongrel dogs (10-12 kg) were anesthetized with sodium pentobarbital (30 mg/kg) and then given positive-pressure ventilation. Through a left thoracotomy, the brachiocephalic trunk and left subclavian artery were ligated. Heparin (2mg/kg) was given intravenously. After cross-clamping the aorta, 500 cc of high potassium cardioplegia (table 1) was infused into the aortic root and pericardial topical cooling applied. After cardiectomy was performed, a 5 cm long cannula (1/4" 3/8" tygon tubing) was inserted and secured into the aortic cuff. All excess fatty tissue was excised. The hearts were rapidly transferred to an adjacent NMR unit in a 5°C Ringer's Lactate (RL) bath and they were suspended by the aortic cannula into a polyvinyl bag. The bag was positioned in the center of the magnetic field and the NMR coil was adjusted in close apposition to the heart which was entirely contained within its circumference (figure 1). The hearts wre weighed immediately after cardiectomy and at the completion of the preservation periods. The hearts were separated into 2 groups.

In group I (n=5), the hearts were preserved for 4 hours in a 4°C RL bath (table I and figure 1). Since no metal part could be used within 30 feet from the magnetic field, a circuit of 30 feet of tygon tubing had to be devised through which 3 L of 4°C RL solution were recirculated around the heart (figure 2). A closed circuit was maintained with a Sarns roller pump and cooling coils immersed in an alcohol-ice solution. The heart septal temperature was maintained at 5-7°C for the duration of the experiments.

In group II (n=4) the hearts were suspended in the polyvinyl bag as described in group I (see figure 2). Instead of being continuously topically irrigated with cold RL solution, these hearts underwent continuous coronary perfusion with a modified Kreb's solution for 24 hours. A second circuit (figure 3) for perfusion was added in parallel to the cooling circuit described for group I.

The 2 circuits wre primed with 4 liters of modified Kreb's solution (table 2) which was recirculated through a $0.4m^2$ membrane oxygenator (1 Sci-Med Life Systems Inc, Minneapolis, Minnesota.), cooling coils and a 0.5 micron pre-bypass filter (Shiley Canada Ltd, Model SFBFS, Montreal, Quebec). Perfusion pressure was maintained at 20cm H_2O for 24 hours (flow = 30-60 cc/min). Oxygen (O_2) and Carbon Dioxyde (CO_2) flow rates through the membrane oxygenator were adjusted hourly to maintain perfusate pH at 7.40 (temperature - corrected) and PO_2 at 120 mm Hg. The heart septal temperatures wre maintained at 5-7° for the duration fo the experiments.

^{31} P-NMR spectra wre obtained at 25.8 MHz at 5-7°C in a 1.5 Tesla whole body spectrometer (Philips). The spectra were collected in blocks of 64 averages under fully relaxed conditions using a 90° pulse. (30 second interpulse delay). Relative

concentrations were determined fromn the peak integrals of each compound as described below.

Adenosine - 5^1 - triphosphate (ATP) contains 3 high-energy phosphate bonds which are all identified separately on the NMR spectrum (1I). They correspond to the Gamma, Alpha and Beta peaks.
The Gamma peak corresponds to ATP and ADP; the Beta peak corresponds to ATP only and the Alpha peak corresponds to ADP and NADH (mostly NADH). Only the Beta and Gamma peaks wre therefore considered for calculations of ATP.

Measurements of relative concentrations of phosphorous compounds (ATP + PCr) were done in the following way. During preservation, phosphorous compounds within the hearts are transformed from one form to another (see previous sections on metabolism) but the TOTAL phosphate pool of each heart remains constant throughout the preservation period. If the surface area of each peak obtained during spectroscopy (as in figure 4) is added, the sum equals the total phosphate pool of the heart studied at this particular time and that sum remains constant throughout the experiments. Only the relative surface area of each peak within each spectrum changes from hour to hour as the phosphorous compounds within the heart are metabolized from one form to another. Since the surface area of each peak is very difficult to accurately measure, the baseline of each spectra was determined and each peak was cut out from the sheet of paper on which the spectra were printed.

For every spectrum obtained, the peaks were cut out and weighed on a highly sensitive electronic scale. Once the sum of all peaks for a single spectrum was obtained, each peak was then separately weighed in order to obtain their weight in relation to the sum (representing the TOTAL phosphate pool). Scanning was started within 8 minutes of cardiectomy and the first spectra

were used for subsequent comparison of relative ATP and PCr concentrations. It was expected that ATP and PCr peak weight over the total weight of the first obtained spectrum on each heart (within 8 minutes of cardiectomy) were arbitrarily used as 100%.

The subsequent measurements were therefore expressed as percentages from these initial values.

Since ^{31} P-NMR spectroscopy was done for these experiments and knowing that the total phosphorous compound pool remains constant within hearts. All measurements of ATP and PCr obtained in this fashion completely eliminate the accumulation of tissue edema as a potential source of error in measurement. These are in fact, equivalent to biochemical assays based on "dry weight" as opposed to "wet weight"

Intracellular pH (pHi) measurements were obtained by computer measurements of the chemical shift between the inorganic phosphate peak and the pH-stable PCr peak (see previous section).

RESULTS

All values quoted in the next sections will be referred as means ± standard deviations. Weight gain was 15 ± 8% in group I hearts after 4 hours and 33 ± 11% in group II after 24 hours ($p < 0.01$). Coronary vascular resistance was measured in group II hearts by dividing the perfusion pressure (maintained at 20cm H_2O) by the perfusion flow (ml/min/gm) required to maintain a constant pressure. The resistance augmented from 44 ± 16 cm H_2O/ ml^{-1}. min^{-1}. gm^{-1} to 56 ± 19 cm H_2O/ ml. min^{-1}. gm^{-1} after 24 hours of continuous perfusion.

In group I, the post-cardiectomy intracellular pH (pHi) was 7.39 ± 0.04. It gradually decreased to 6.44 ± 0.08 at 4 hours (figure 5 and Annex I).

In group II, the post-cardiectomy intracellular pH (pHi) was 7.41 ± 0.05. As mentionned previously, computer measurements of the chemical shift between the inorganic phosphate peak (pH-dependent) and the Phosphocreatine (PCr) peak (pH-stable) was used to measure the pHi of the hearts. Within 1-2 hours, it was observed that the inorganic phosphate peaks, which had been monophasic in all group I spectra, became biphasic and eventually triphasic. An example of a typical spectrum obtained in group II is shown in figure 6. The inorganic phosphate peak is biphasic and the 2 peaks when related to the pH-stable position of the PCr peak correspond to pHi's of 6.49 and 5.92 respectively. In order to rule out intramyocardial accumlation of 2,3 - DPG or sugar phosphate compounds that might alter the shape of the inorganic phosphate peak, resolution enhancement scanning of the spectra was performed. This technique allowed precise separation of the peaks corresponding to these 2 phosphate compounds from the inorganic phosphate peak (The peak corresponding to perfusate-contained phosphate compounds could also be clearly delineated (see figure 6) which remained biphasic and sometimes triphasic. The multiphasic peaks therefore obtained truly corresponded to "compartmentalization" of perfused hearts into 2 or more different pHi compartments. Figure 7 illustrates this phenomenon in one of group II hearts over 24 hours of perfusion.

In order to evaluate if this phenomenon was due to non-uniform distribution of intracellular pH within group II hearts, Hydrogen-Magnetic Resonance Imaging (H-MRI) was perfomed on each heart at the completion of the experiments in this group. Figure 8 demonstrates one of the images obtained. The darker colors correspond to lower water content of the tissues being imaged. As seen in this particular heart (sagittal section), there was very inhomogenous distribution of water density across the muscle wall of the heart at the end of 24 hours of perfusion ex-vivo. Darker areas were scatterred mostly in the subendocardial layer

as well as some intramyocardial areas, indicating lower water contents.

Phosphocreatine stores were depleted very rapidly in both groups (see figure 9 and annex III). In group I, PCr stores were 8 ± 8% of initial after 4 hours. A significantly higher relative concentration was observed after 4 hours in group II (40 ± 16%; $p<0.005$). On the other hand, PCr stores were nearly completely depleted after 24 hours of perfusion in group II (figure 9 and annex III).

ATP was also depleted in both groups (fig 10 and annex II) but at a much slower rate than PCr. In group I, 61 ± 14% of initial ATP concentration remained after 4 hours compared with 90 ± 6% in Group II ($P<0.005$). After 24 hours of perfusion, only 33 ± 8% of initial ATP stores remained in group II hearts.

DISCUSSION

^{31} P-NMR spectroscopy appears to be a safe, reliable, non-invasive method to accurately assess high-energy phosphate compounds metabolism and intracellular pH of whole hearts preserved for transplantation. The other advantage of this method, compared with conventional biochemical assays, is that very precise measurements of the above mentionned parameters can be obtained regardless of the total water content of ex-vivo organs. Since phosphorus compounds are used for these determinations (as opposed to Hydrogen - NMR spectroscopy) and the total pool of these compounds in the closed circuit used in this model remains constant throughout the experiments, the measurements of ATP, PCr and intracellular pH remain precise and are not affected at all by tissue edema accumulation. The results obtained continuously can therefore be compared to "dry results" as usually referred to in conventional biochemical

assays. The hypothesis is that the intracellular metabolic status may be used as an indicator of the morphological and functional integrity of the isolated heart after preservation and before reimplantation in the recipient animal.

In both groups of hearts, severe intracellular acidosis developed (Group I: pH = 6.44 ± 0.08 at 4 hrs; Group II: ph = 5.86 ± 0.06 at 24 hrs) The difference in pH was significant ($p<0.005$) with the Group II hearts being much more acidotic at 24 hrs than Group I hearts at 4 hrs. As previously discussed, intracellular pH probably plays an important role in post-reimplantation function of preserved heart. A clear advantage in intracellular pH was demonstrated when this parameter was compared at 4 hours in both groups (GroupI = 6.44 ± 0.08; Group II = 6.75 ± 0.15; $p< 0.005$)

The phenomenon of pHi myocardial "compartmentalizatoin" observed in group II suggested non-homogenous distribution of various pHi milieux within the same heart.

H-MRI imaging of these hearts performed at the end of the preservation periods, demonstrated a non-uniform distribution of water content across the muscle wall with less water in the subdendocardial layers and some intra-myocardial areas. It is postulated that such areas were underperfused secondary to either coronary vasoconstriction or low perfusion pressure reaching the subendocardial layer. The relative hypoperfusion would have caused relative ischemia of these areas causing accumulation of lactic acid from anaerobic metabolism and impaired washout of metabolites. In order to test the hypothesis that subendocardial areas that contained less water correspond to the more acidic compartments observed by ^{31} P=NMR spectroscopy, a method to determine H^+ ion concentration in relation to H- MRI pictures would have to be designed. At the current time, this is technically not possible.

It was a consistent finding in our previous experiments of heterotopic heart transplantation after 24 hours of perfusion using this same model that the transplanted hearts required a longer warm reperfusion time and more defibrillation than non perfused hearts (after 4 hours) to return to sinus rhytm (4,7). It is postulated that such electrical disturbances may be in part related to the uneven distribution of intracellular pH at the time of warm reperfusion.

Therefore, the metabolic advantage of continuous perfusion over simple hypothermic ischemia (demonstrated by the delayed depletion of ATP and PCr stores) could be further potentiated by techniques leading to a more even distribution of pHi environments within perfused hearts. Such techniques might include addtion of coronary vasodilators such as nitrates or calcium-channel blocker to the perfusate or higher perfusion pressures.

It is unlikely that the development of myocardial acidosis over 24 hours of perfusion is due to depletion of the buffer contained in the solution (THAM) which is continuously recirculated through the circuit. The very large volume of perfusate (4 litres) contained much more THAM (0.25gm/L) than what would be required to buffer out the 24-hour production of H^+ ions by hearts weighing on average. 68 ± 12 gms.

Phosphocreatine stores were almost completely depleted in both group I and group II. This process was very rapid as shown in figure 9. The exact significance of such a phenomenon is difficult to assess. Group I hearts represent the current method of myocardial preservation used in clinical transplantation; if we assume that currently transplanted hearts have nearly no phosphocreatine remaining as a storage form of high-energy phosphate bonds upon reimplantation as shown in group I experiments (Annex), it is postulated that it is either not

required for immediate ventricular function upon reimplantation or that it is very rapidly resynthesized upon graft reperfusion with warm blood. Previous work done by Keibel and Rovetto (10) in the warm ischemic rat heart model suggested very rapid resynthesis of phosphocreatine stores during the reperfusion period. Such a rapid and complete replenishment of phosphocreatine stores implies that cellular energy production pathways are inhibited during ischemia but become functional immediately upon warm, oxygenated reperfusion of the graft.

Whether phosphocreatine stores could be used as an index of preservation remains to be proved. The above observations suggest that very low amounts of this storage form of energy are sufficient to allow for good function of transplanted hearts shortly after reimplantation (the time necessary to replenish stores). If group II hearts are compared with Group I at 4 hours (Figure 9 and annex III), the latter hearts contained only 8 ± 8% of the initial phosphocreatine stores versus 40 ± 16% in Group II ($p<0.005$). The difference is very significant but experiments on the funtional recovery of such hearts (ie. dp/pt and developed pressure) should be done in order to demonstrate an advantage of containing high stores of high-energy phosphates upon reimplantation. If in fact, a better and more rapid functional recovery could be demonstrated in perfused hearts after 4 hours of preservation for example, then such an advantage (higher PCr stores at the end of the preservation period) would be clinically more relevant.

ATP stores were depleted in both groups. As shown in figure 10 and Annex II, group I hearts contained 61 ± 14% of the initial ATP stores as compared to 90 ± 6% in group II after 4 hours ($p<0.005$). Again, in order to determine the clinical relevance of such a significant difference, hearts of both groups would have to be subjected to functional testing (ie dp/dt and developed pressure) after 4 hours of preservation. Therefore,

from these observations alone, no clear conclusion can be drawn. On the other hand, studies by NovicK et al (13) on the preservation of hypertrophied pig myocardium with oxygenated solutions demonstrated an advantage of high ATP stores at the end of preservation.

If functional recovery of perfused hearts (group II) could be demonstrated to be better, this would probably be of great clinical relevance for example in the setting of heart transplantation with elevated pulmonary vascular resistance where the right ventricle may require a certain period of time to adopt to the suddenly increased afterload to which it is subjected.

After 24 hours of perfusion, Group II hearts contained 33 ± 8% of their inital ATP stores. Other experiments done in our laboratory (7) showed that such a significant depletion of ATP stores did not preclude from an adequate ventricular function after reimplantation. On the other hand, those hearts were not subjected to high afterloads and only left ventricular function was measured, therefore more studies are needed to correlate ATP content and functional recovery of the grafts. On the other hand, our model was exactly similar to the one used by Guerraty (4) who demonstrated successful orthotopic transplantation in dogs after 24 hours of in-vitro preservation. If we accept that the 2 sets of experiments were similar, then we can postulate that 33 ± 8% of initial ATP stores after 24 hours of preservation by continuous, cold oxygenated perfusion is sufficient to allow for functional recovery of both right and left ventricles when subjected to normal afterloads.

These are only assumptions made for the sake of discussion and in order to prove these, more experiments will have to be performed.

In summary, these experiments have clearly demonstrated that ^{31}P-NMR spectroscopy is a very precise, non-invasive method for continuous assessment of high-energy phosphate metabolism and intracellular pH of hearts preserved under 2 very different sets of conditions. The method is reproducible and reliable.

The results obtained were pure biochemical observations and no functional correlation was performed during these experiments.

This in fact, was the goal of this work. Further studies will have to be done to fully assess the clinical relevance of the results obtained.

Even though Group II hearts were less acidotic and contained more ATP and PCr than Group I hearts after 4 hours of preservation, no functional studies have ever demonstrated an advantage in ventricular recovery upon reimplantation of one method compared with the other. It the metabolic advantage observed in group II hearts after 4 hours of preservation is ever proven to correlate with a significantly better and /or faster functional recovery, this could have major implications in future heart preservation methods not only in the field of transplantation but also in most major cardiac operations.

The fact that experiments previously performed in our laboratory (7) and others (3,4,5,6) demonstrated clearly that hearts preserved ex-vivo for up to 72 hours (3) could regain functional recovery upon reimplantation is postulated to be explained mostly by the preservation of ATP stores allowed by continuous perfusion of these organs. The very severe intracellular acidosis (5.86 ± 0.06) and near-complete exhaustion of PCr stores after 24 hours as observed in group II hearts must be very rapidly reversible since other experiments using an exactly similar model (4,7) have shown functional recovery despite such severe metabolic changes.

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LIST OF FIGURES

FIGURE 1

The polyvinyl chloride bag into which the hearts were suspended.

It allowed the organs to be continuously immersed in the solution and very close apposition of the NMR coil.

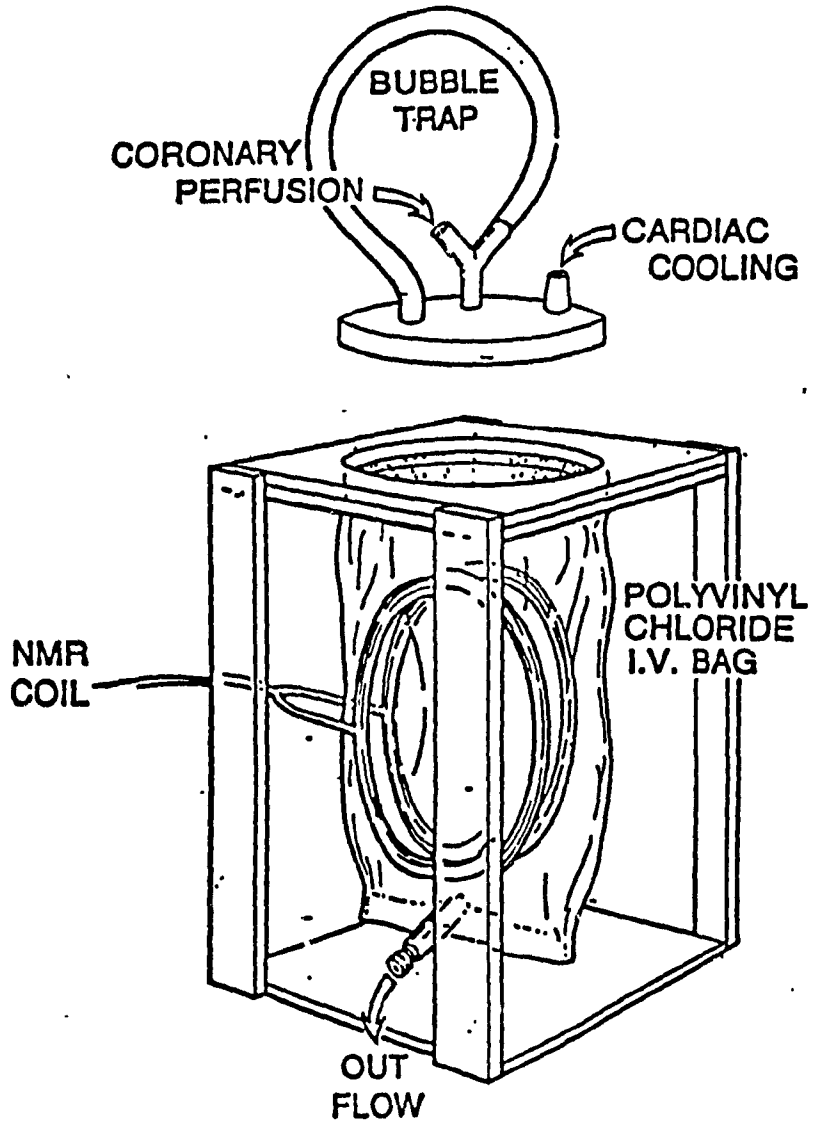

BUBBLE
TRAP
CORONARY
PERFUSION
CARDIAC
COOLING
POLYVINYL
CHLORIDE
I.V. BAG
NMR
COIL
OUT
FLOW

FIGURE 2

The cooling circuit used in Group I experiments.

The Sarns roller pump was located 30 feet from the NMR magnet. Cooling was achieved by circulating the solution through tygon coils immersed within an absolute alcohol-ice mixture.

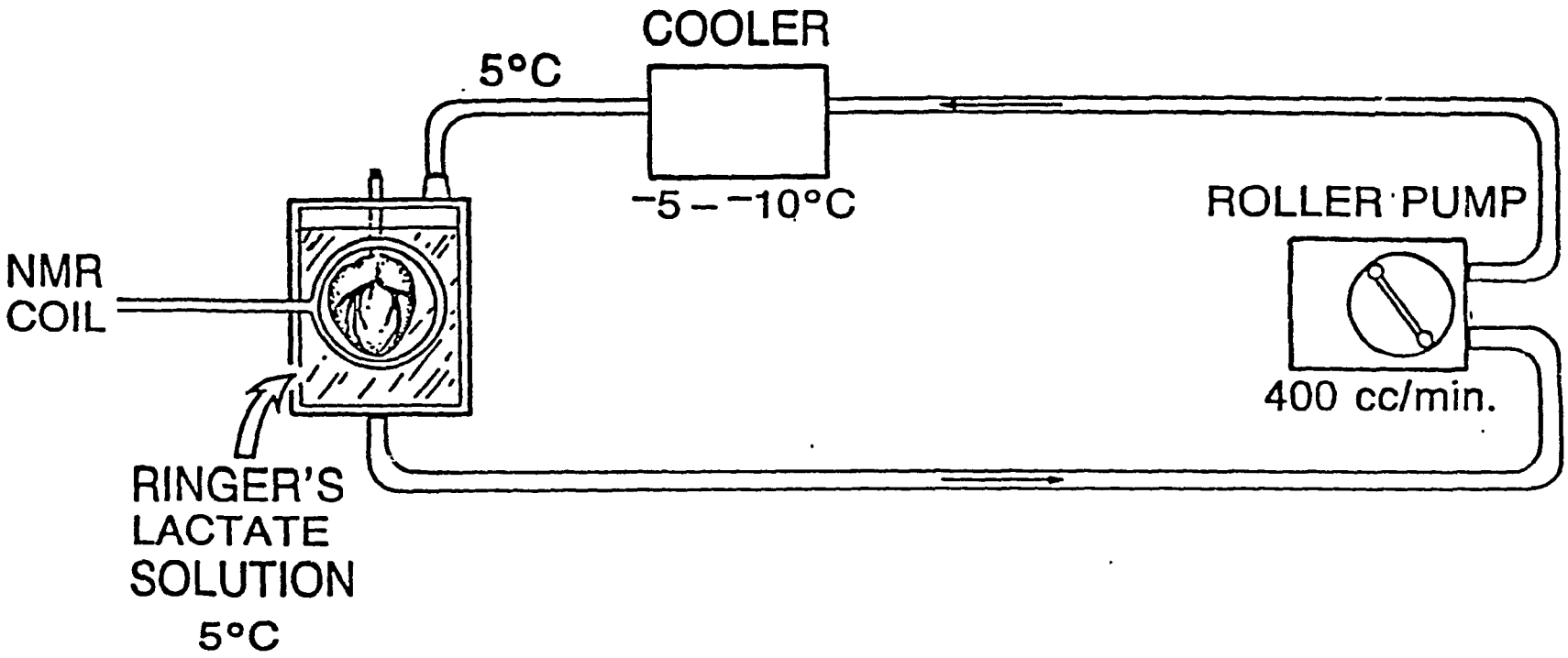

COOLER
5°C
−5 – −10°C
ROLLER PUMP
NMR
COIL
400 cc/min.
RINGER'S
LACTATE
SOLUTION
5°C

FIGURE 3

The circuit used for group II experiments.

The second roller pump was added in parallel to group I circuit. It permitted coronary perfusion of the hearts with modified Kreb's solution. Ccompressed air (CA = 4-5 L/min) and carbon dioxide (CO_2) flows were adjusted to maintain perfusate pO_2 at 120 mmHg and pH at 7.40 respectively.

The solution was filtered just before entering the coronary circulation and was recirculated into the circuit after exiting the coronary sinus and the opened right atrial cavity.

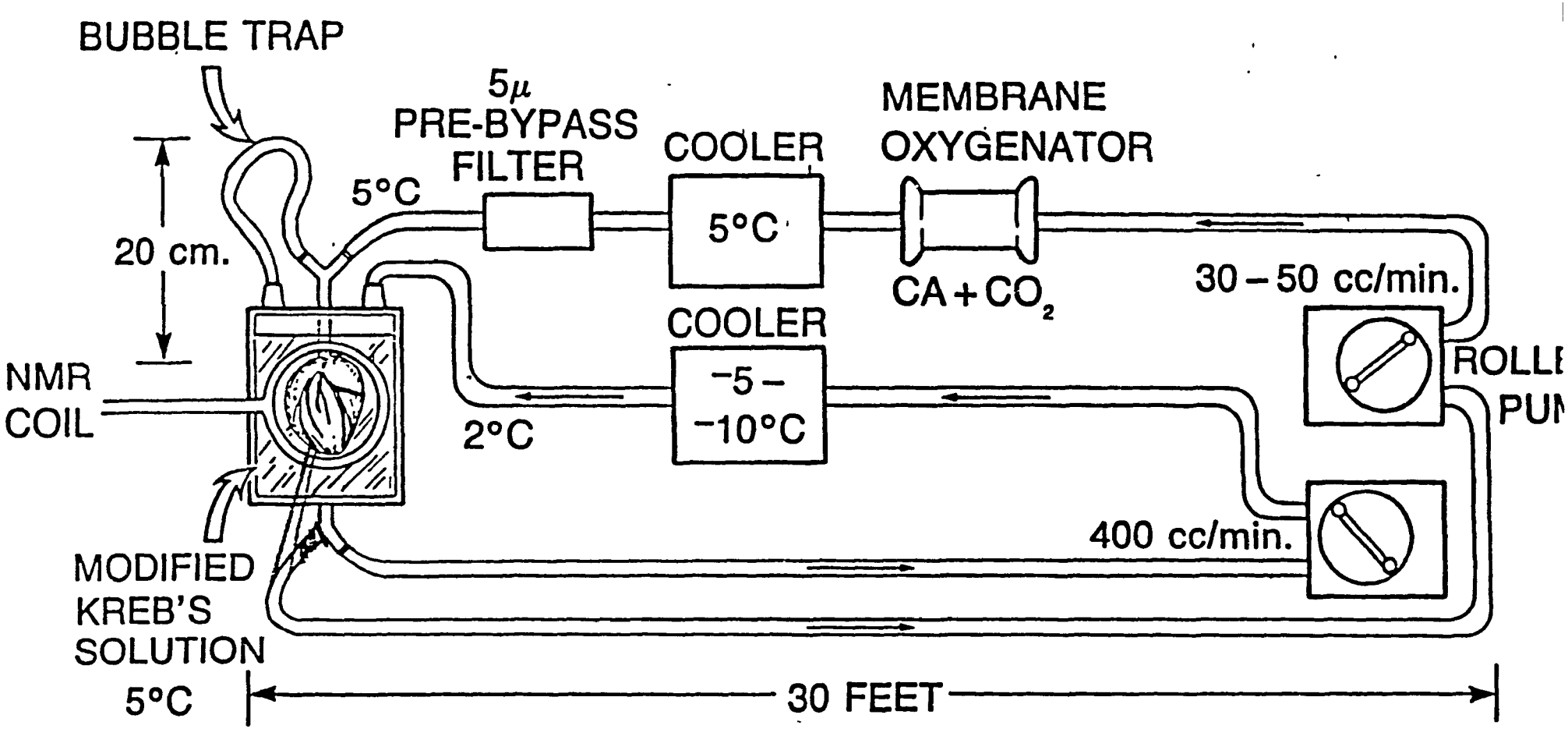

BUBBLE TRAP
5μ
PRE-BYPASS
FILTER
COOLER
MEMBRANE
OXYGENATOR
5°C
5°C
20 cm.
CA + CO2
30 – 50 cc/min.
COOLER
NMR
COIL
–5 –
–10°C
2°C
ROLLE
PUI
400 cc/min.
MODIFIED
KREB'S
SOLUTION
5°C
30 FEET

FIGURE 4

Typical NMR spectrum obtained during the experiments.

Gamma-ATP = Gamma ATP + ADP;
alpha ATP = alpha-ATP + ADP + NAD;
beta ATP = beta-ATP only (only this latter peak was used to measure relative ATP concentrations).

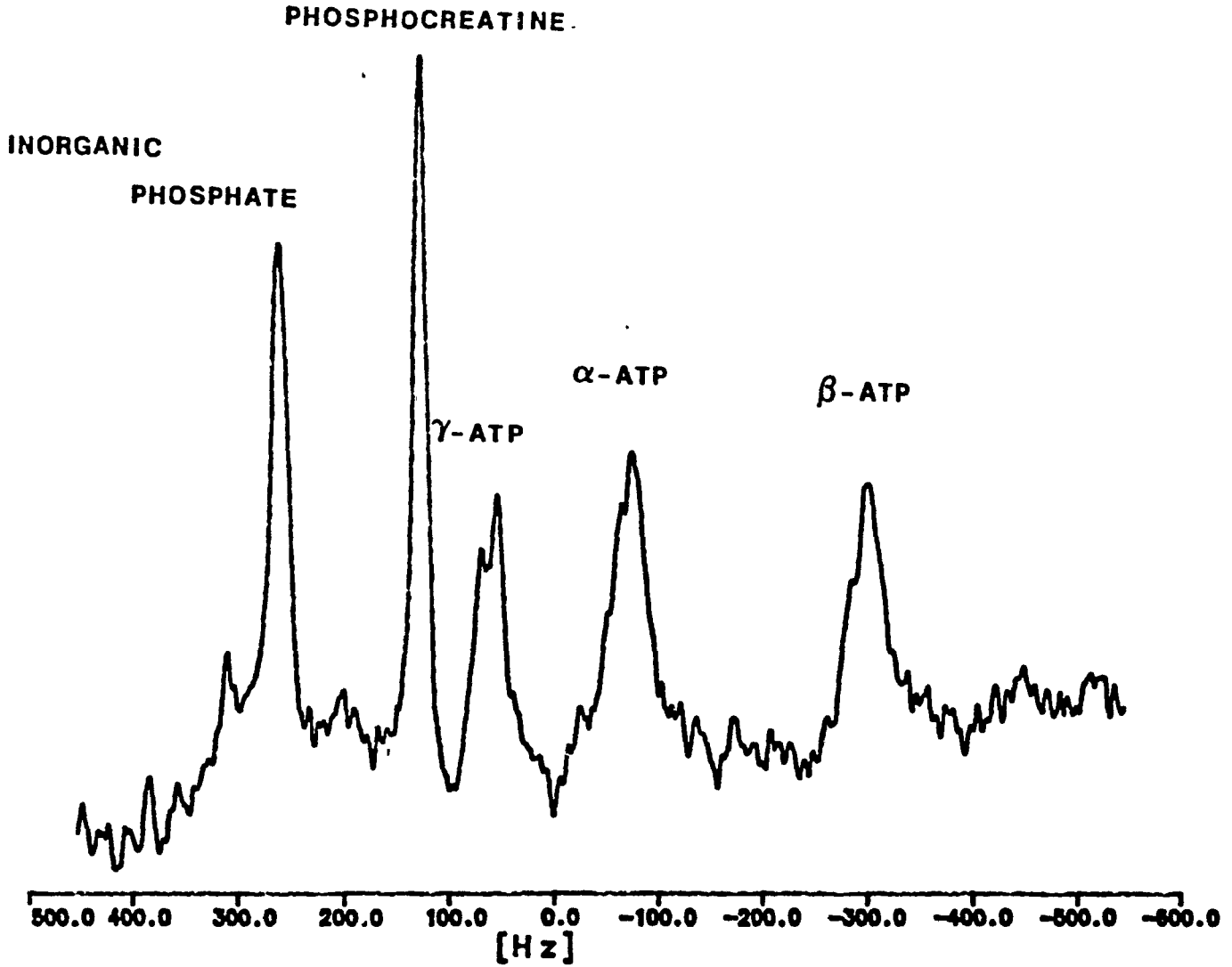

PHOSPHOCREATINE
INORGANIC
PHOSPHATE
γ-ATP
α-ATP
β-ATP
500.0 400.0 300.0 200.0 100.0 0.0 -100.0 -200.0 -300.0 -400.0 -500.0 -600.0
[Hz]

FIGURE 5

The intracellular pH.

Statistically significant differences (student's t test) were observed at 2, 3 and 4 hours.

pH

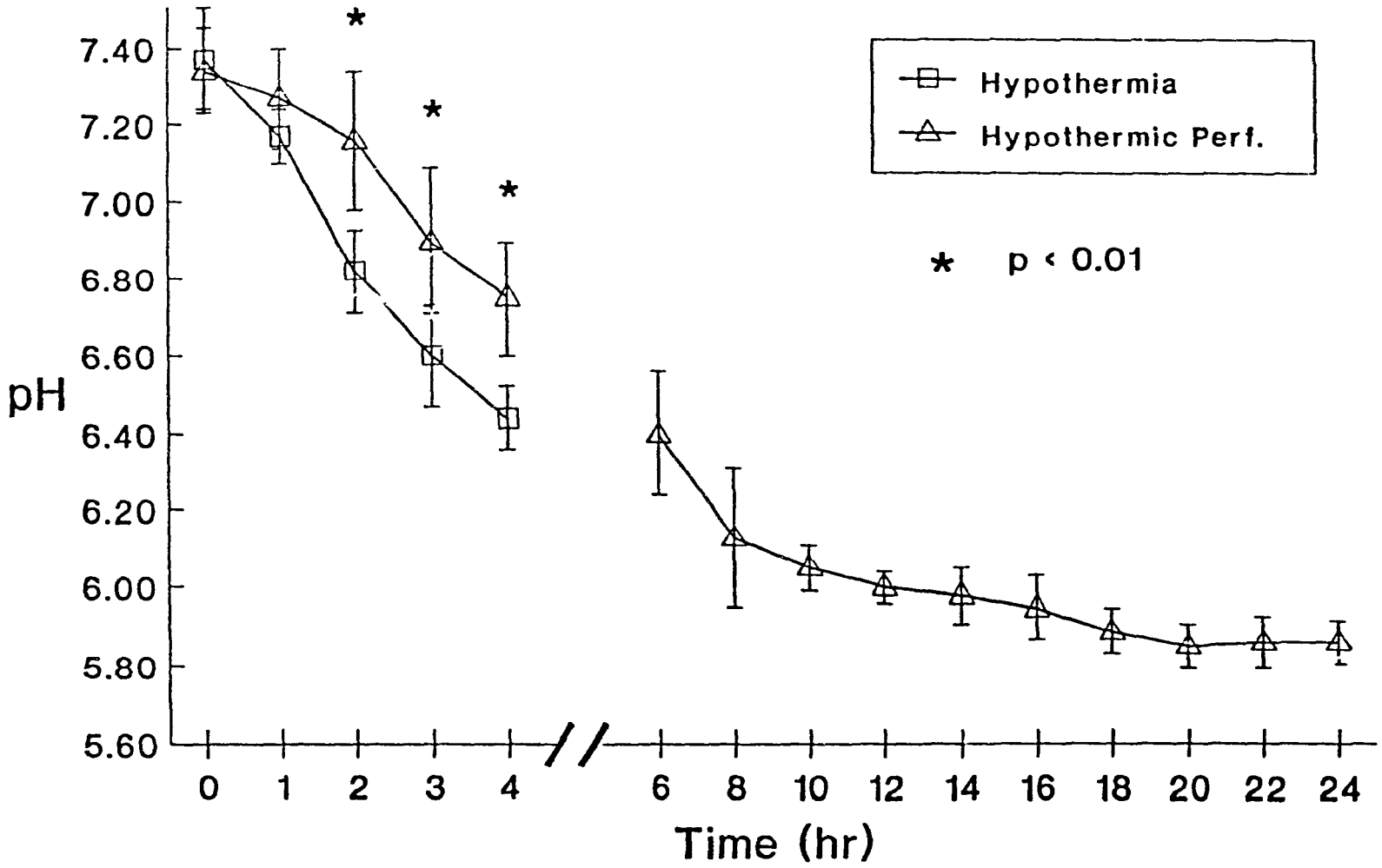

7.40
7.20
7.00
6.80
6.60
6.40
6.20
6.00
5.80
5.60
pH
*
*
*
Hypothermia
Hypothermic Perf.
* p < 0.01
0
1
2
3
4
6
8
10
12
14
16
18
20
22
24
Time (hr)

FIGURE 6

Resolution enhanced spectrum of experiment 3 (group II) after 24 hours of hypothermic perfusion.

The inorganic phosphate peak has separated into two different components each corresponding to different intracellular pH "compartments".

(P_i = Inorganic Phosphate compound, PCr = Phosphocreatine).

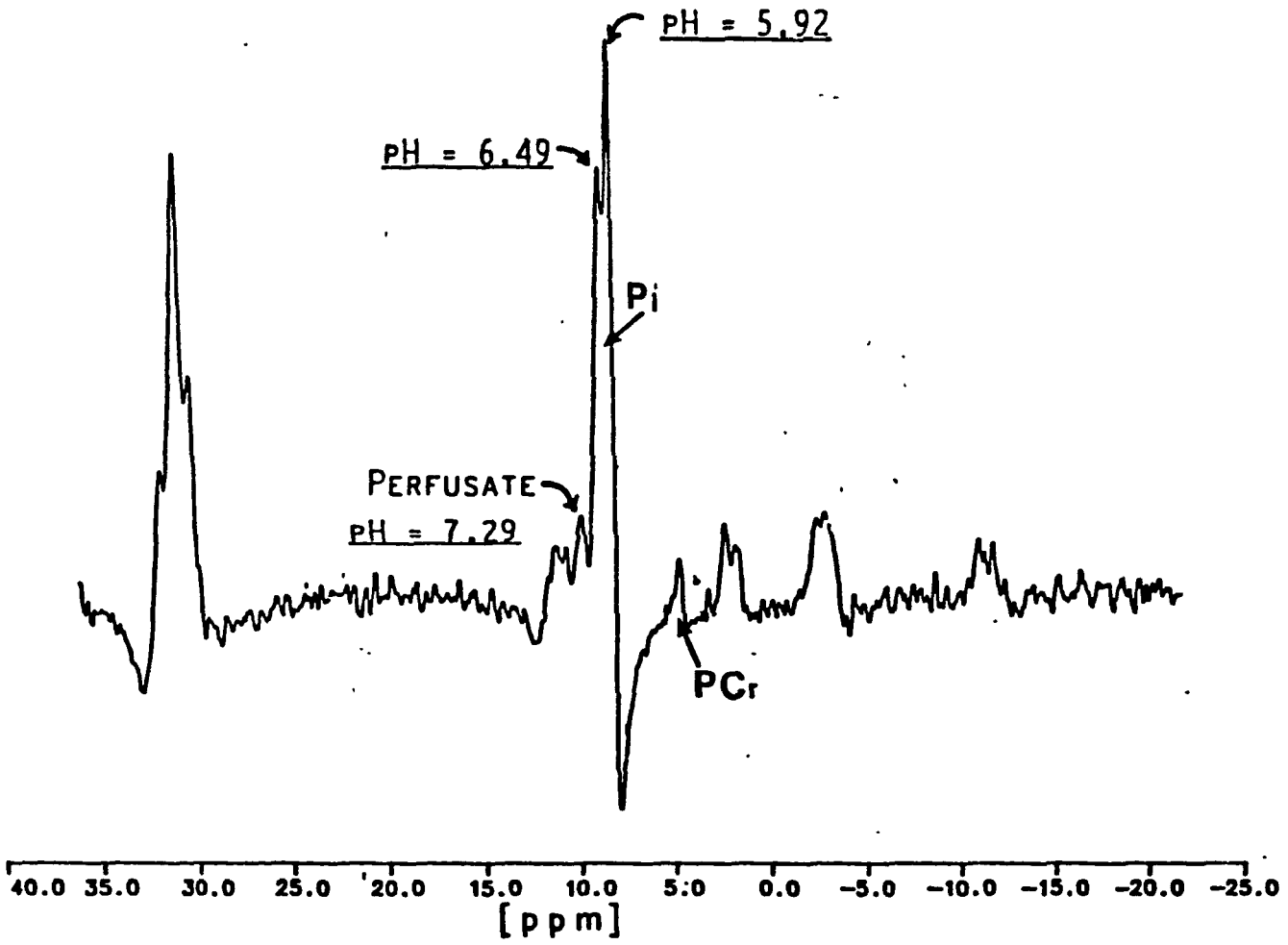
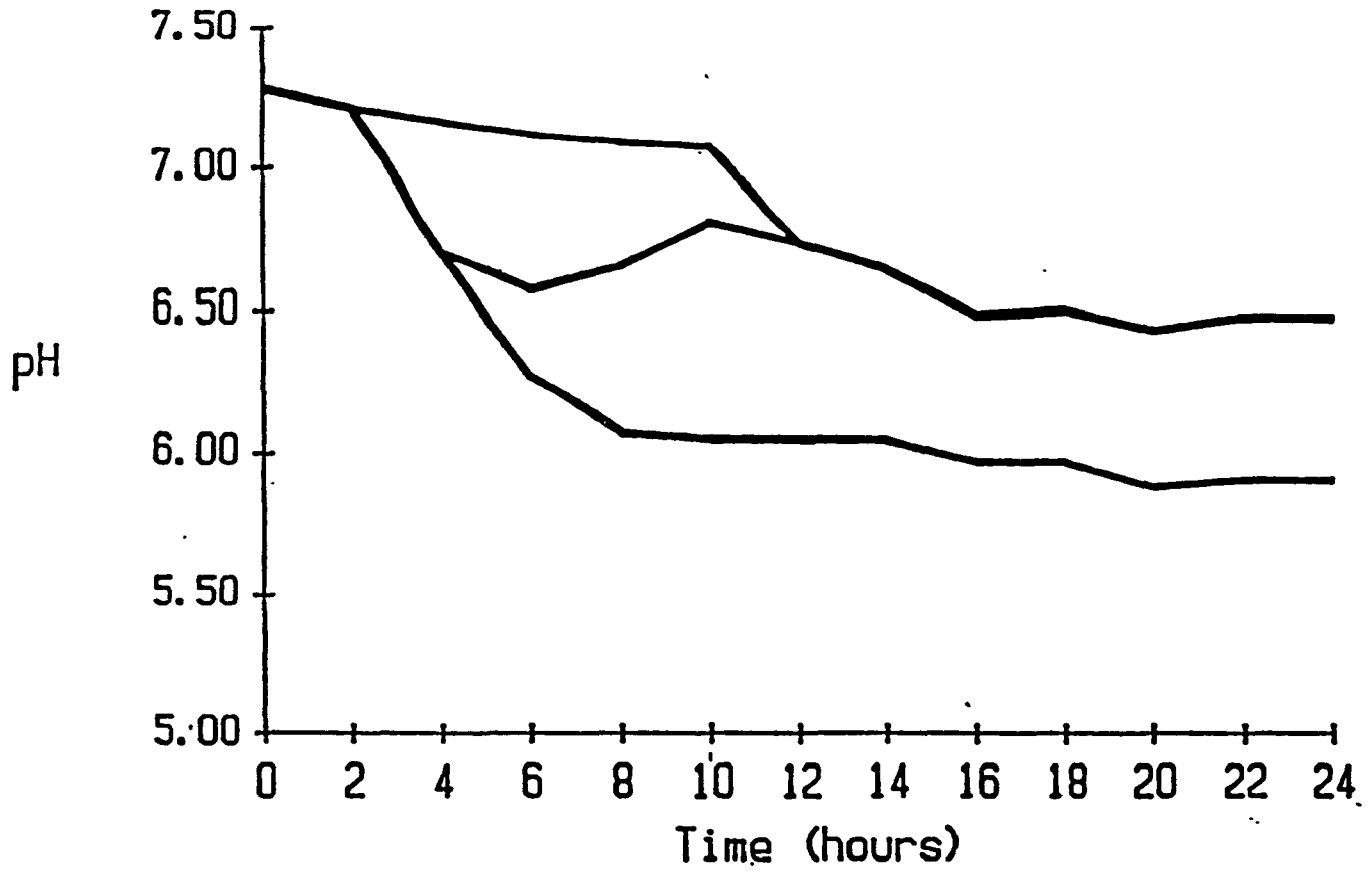

pH = 5.92
pH = 6.49
Pi
PERFUSATE
pH = 7.29
PCr
40.0 35.0 30.0 25.0 20.0 15.0 10.0 5.0 0.0 -5.0 -10.0 -15.0 -20.0 -25.0
[ppm]

FIGURE 7

Intracellular pH. Experiment 3 (group II) demonstrating the various pH_i compartments.


7.50
7.00
6.50
6.00
5.50
5.00
pH
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)

77

FIGURE 8

Proton magnetic imaging (MRI) of heart # 3 (group II) obtained after 24 hours of hypothermic perfusion. The darker areas in the subendocardium indicate a lower water content (arrows).

FIGURE 9

Phosphocreatine (PCr).

Statistically significant differences observed at 1, 2, 3 and 4 hours. (student's t test).

Phosphocreatine

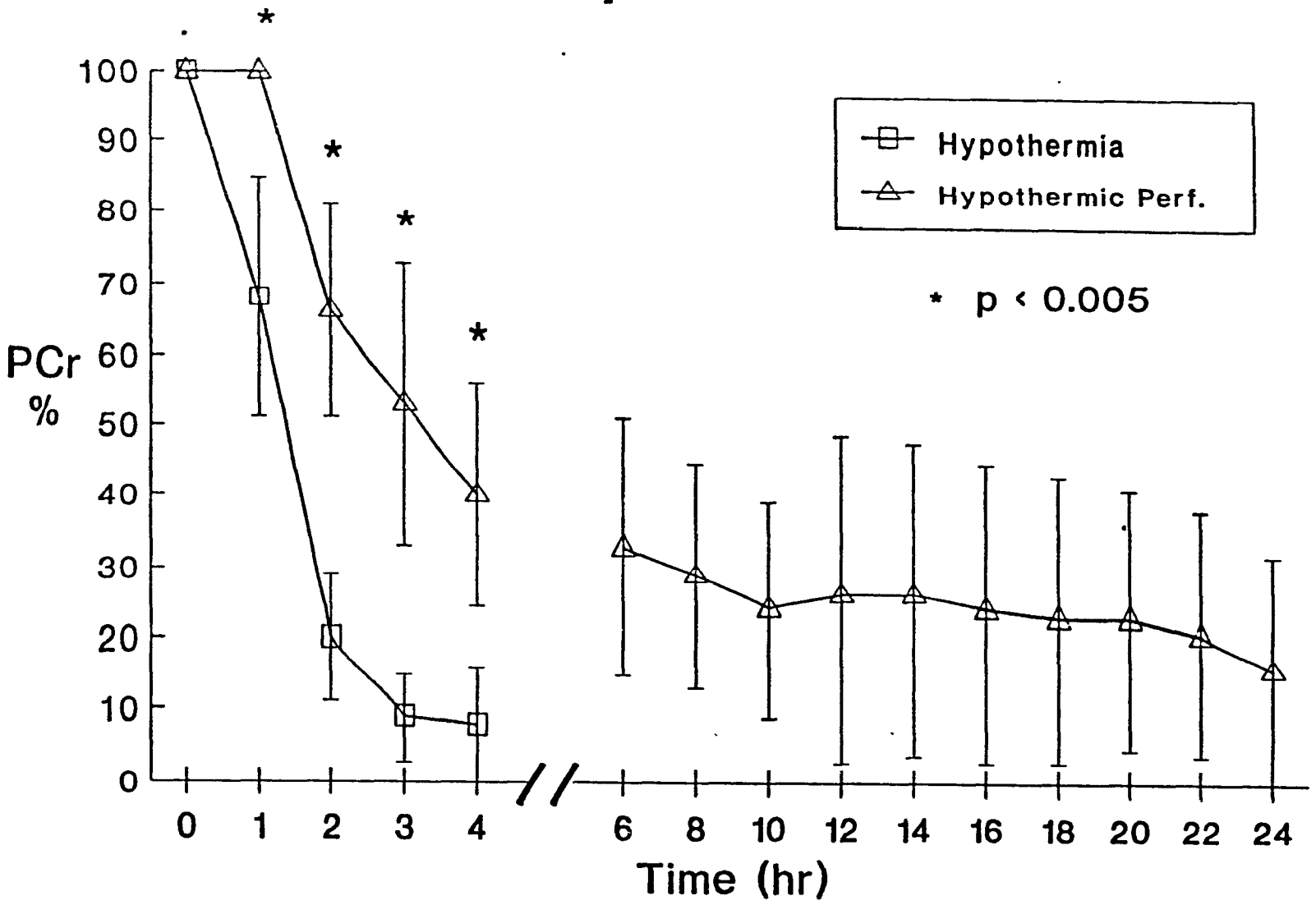
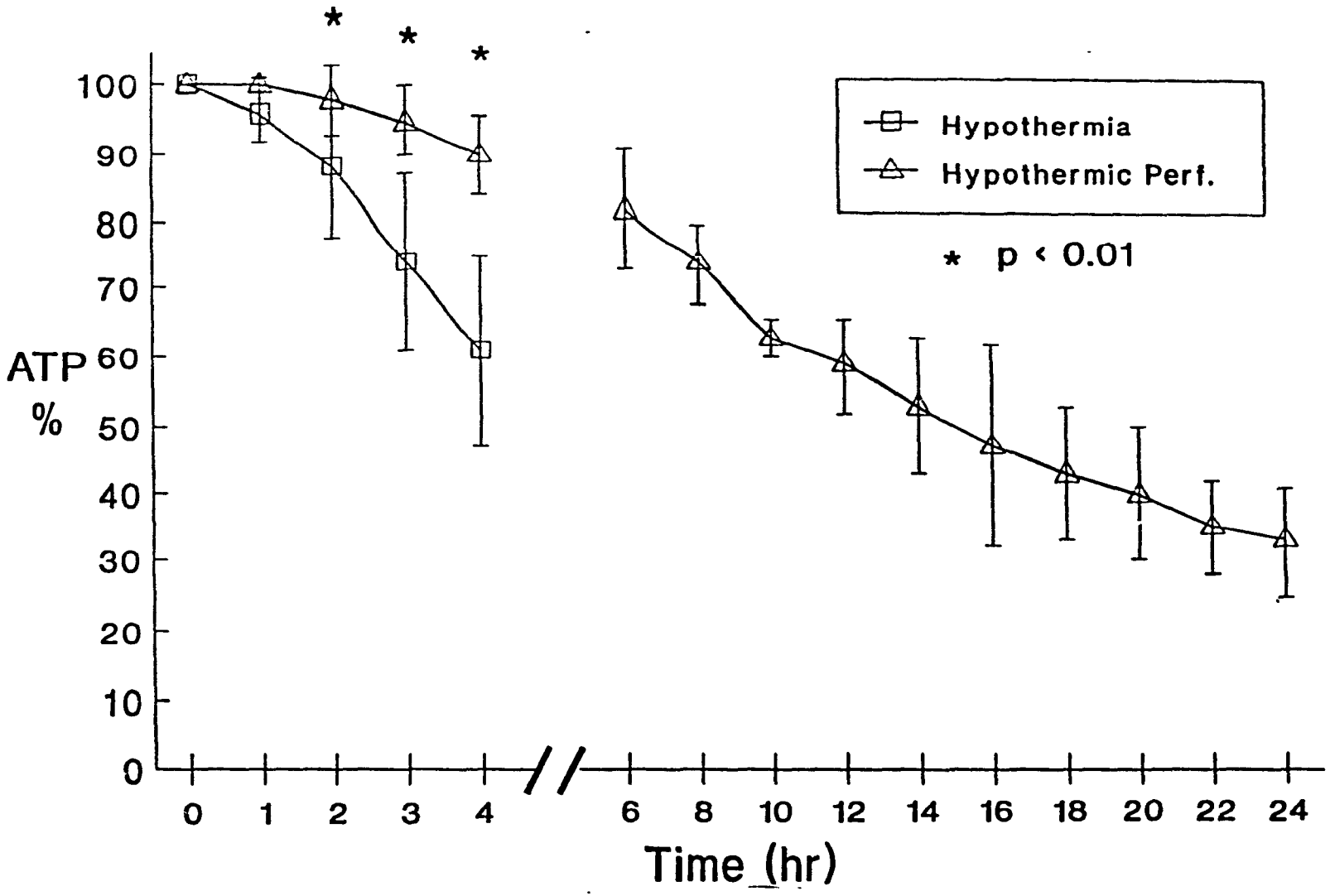


FIGURE 10

Adenosine 5′ - triphosphate (ATP).

Significant differences at 2, 3 and 4 hours.

ATP


*
*
*
100
90
80
70
60
50
40
30
20
10
0
ATP
%
Hypothermia
Hypothermic Perf.
* p < 0.01
0
1
2
3
4
6
8
10
12
14
16
18
20
22
24
Time (hr)

ANNEX I INTRACELLULAR pH

time (hrs)	group I pH_i	group II pH_i*	Student t-test#
1	7.17±0.07	7.27±0.13	not significant
2	6.82±0.11	7.16±0.18	p<0.01
3	6.60±0.13	6.90±0.19	p<0.01
4	6.44±0.08	6.75±0.15	p<0.005
5		6.66±0.21	
6		6.40±0.16	
7		6.22±0.18	
8		6.13±0.18	
9		6.06±0.10	
10		6.05±0.06	
11		6.06±0.05	
12		6.00±0.04	
13		5.99±0.05	
14		5.98±0.07	
15		5.98±0.06	
16		5.95±0.08	
17		5.94±0.07	
18		5.89±0.06	
19		5.87±0.06	
20		5.85±0.06	
21		5.86±0.07	
22		5.86±0.07	
23		5.84±0.07	
24		5.86±0.06	

pH_i = Intracellular pH.

* =In group II,hearts eventually divided into 2 or more pH compartments; the more acidic compartment was used for this table.

\# = Student t-test 1 tail (unpaired).

ANNEX II RELATIVE CONCENTRATION OF ATP

time (hrs)	ATP-group I*	ATP-group II*	t-test#
1	96±4	100±1	not significant
2	88±10	98±5	p<0.05
3	74±13	95±5	p<0.01
4	61±14	90±6	p<0.005
5		87±5	
6		82±9	
7		75±4	
8		74±6	
9		70±9	
10		63±3	
11		60±9	
12		59±7	
13		51±13	
14		53±10	
15		47±12	
16		47±15	
17		42±9	
18		43±10	
19		36±12	
20		40±10	
21		35±15	
22		35±7	
23		38±6	
24		33±8	

* = Expressed as percentage (%) of initial value.

= Student t-test 1 tail (unpaired).

ANNEX III PHOSPHOCREATINE RELATIVE CONCENTRATION

time (hrs)	PCr-group I*	PCr-group II*	t-test#
1	68±17	100±0.00	p<0.005
2	20±9	66±15	p<0.0005
3	9±6	53±20	p<0.005
4	8±8	40±16	p<0.005
5		34±12	
6		33±18	
7		30±13	
8		29±16	
9		30±18	
10		24±15	
11		25±18	
12		26±23	
13		26±24	
14		26±22	
15		24±19	
16		24±21	
17		27±24	
18		23±20	
19		27±20	
20		23±18	
21		19±17	
22		21±17	
23		20±20	
24		16±16	

* = Phosphocreatine expressed as percentage (%) of initial value.

\# = Student t=test 1 tail (unpaired).

TABLE I

Cardioplegic and Hypothermic Immersion Solutions (RL)

	Cardioplegic Solution mEq/L	Ringer's Lactate solution mEq/L
K^+	20	4
Na^+	140	130
Ca^{2+}	2.7	1.5
Cl^-	116	109
HCO_3	21	
Dextrose	14 gm/L	
Lactate	25	28
Lidocaine	200 mg/L	

TABLE II

Hypothermic Perfusion Solution (Modified Kreb's)

	mMol/L
Nacl	118.33
KcL	4.7
KH_2PO_4	1.2
MgSO4	0.6
NaHCO4	25
$CaCl_2$	0.167
Glucose	2 qm/L
Albumin	25 qm/L
Tham	0.25 qm/L
Osmolarity=	313.2 mOsm/L