THE DEVELOPMENT OF AN IN VITRO PERFUSION SYSTEM TO ASSESS THE FUNCTION OF RABBIT KIDNEYS AND ITS APPLICATION TOWARDS THE TREATMENT OF KIDNEYS WITH THE CRYOPROTECTANT DIMETHYLSULFOXIDE

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ABSTRACT

Neil Segal Ph.D Animal Science

THE DEVELOPMENT OF AN IN VITRO PERFUSION SYSTEM TO ASSESS THE FUNCTION OF RABBIT KIDNEYS AND ITS APPLICATION TOWARDS THE TREATMENT OF KIDNEYS WITH THE CRYOPROTECTANT DIMETHYLSULFOXIDE

A colloid-free, reduced pressure in vitro normothermic perfusion system was developed to assess rabbit kidney function following cryobiological manipulation. Rabbit kidneys retained significant levels of function with stable or increasing GFR, sodium reabsorption of 40 to 60%, and glucose reabsorption of greater than 80% of the filtered load.

Including dextran in the perfusate as a source of colloid osmotic pressure was deleterious to kidney function. The kidney demonstrated sensitivity to exogenous energy substrate; supplementation of lactate and glucose resulted in better function over butyrate with glucose or glucose alone. The system proved sensitive to ischemia and to stimulation with an antidiuretic preparation.

This system was utilized to examine the effects of treatment of rabbit kidneys with dimethylsulfoxide (Me_2SO). A recirculating constant pressure hypothermic (10°C) perfusion apparatus was assembled and kidneys were perfused with potassium-rich, sodium-poor solution with elevated glucose and mannitol. The pharmacokinetics of 3 M Me_2SO equilibration was studied through estimation of the inulin and Me_2SO spaces using radiochemical markers. Medullary tissue equilibration was achieved prior to cortical equilibration, which was complete after 35 minutes of exposure to 3 M Me_2SO. Me_2SO perfusion resulted in increased fluid retention by the kidney.

Function of rabbit kidneys was assessed at normothermia following Me_2SO introduction at two different rates, 35 minute equilibration at 3 M Me_2SO, and removal by an osmotic counterbalance system commencing with either 800 or 600 mOs/kg washout solution made hypertonic with mannitol.

Kidneys exhibited excellent in vitro function following perfusion with Me_2SO. Washout with solutions of hyperosmolarity commencing at 600 mOs/kg with gradual and stepwise reduction to 400 mOs/kg allowed immediate removal of Me_2SO without damage; initiation of washout at 800mOs/kg resulted in reduced function at 37°C.

RESUME

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LE DEVELOPPEMENT D'UNE METHODE DE LA PERFUSION EXTRACORPORELLE POUR DETERMINER LA FONCTION DES REINS DU LAPIN ET L'APPLICATION POUR LE TRAITEMENT DES REINS AVEC DIMETHYLSULFOXIDE

Une méthode de perfusion sans colloide à pression diminuée et à 37°C a été developpée pour estimer la fonction des reins après les manipulations cryobiologiques. Les reins ont démontré de bonnes fonctions avec un taux de filtration glomérulaire stable croissante une réabsorption du sodium entre 40 et 60% et une réabsorption de glucose de plus de 80% de la charge filtrée.

Le dextran s'est avéré dommageable aux fonctions rénales quand ajouté à la solution de perfusion comme agent osmotique. Les reins ont demontré une sensibilité à l'énergie disponible, préférant l'acide lactique avec le glucose plutôt que l'acide butyrique et le glucose ou le glucose seul. Ils ont démontré aussi une sensibilité à l'ischémie et à un agent antidiurétique.

Une méthode de perfusion hypothermique a été mise au point pour perfuser les reins avec une solution intracellulaire, à forte teneur en potassium et à faible teneur en sodium à laquelle on a ajouté du glucose et du mannitol pour la rendre hypertonique. Ce système était utilisé pour examiner l'action du diméthylsulfoxide (Me_2SO), sur les reins de lapins. Les cinétiques de l'équilibration avec Me_2SO de 3 M à 10°C ont été étudiées. L'équilibration était complète après 35 minutes de perfusion avec Me_2SO à 10°C. Le tissu médullaire s'est équilibré plus rapidement que le tissu cortical.

La fonction des reins après l'introduction, l'équilibration et l'enlèvement du Me_2SO a été étudiée. L'enlèvement a été accompli en utilisant une méthode nouvelle qui a permis l'enlèvement du Me_2SO immédiatement avec le contrôle du volume cellulaire avec une solution hypertonique. Les reins ont démontré de bonnes fonctions à 37°C après le traitement avec Me_2SO. Commençer l'enlèvement du Me_2SO avec une solution à 600 mOs/kg était mieux que le faire avec une solution à 800 mOs/kg.

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Introduction

Renal transplantation is rapidly replacing dialysis as the treatment of choice for terminal renal failure. The primary source of organs is newly deceased cadavers. Surgical techniques for transplantation have become highly successful; the major problems to overcome are immunological.

With the growing utilization of cadaver kidneys, the need for development of better immunological matching and regional organ sharing is increasing. Development in these areas are hampered by the relatively short interval of safe organ storage available using current techniques. Longterm preservation is needed.

Although successful longterm cryopreservation is routinely used for cell suspensions and thin tissues (Ashwood-Smith, 1980), the organ bank has not become reality. Although sporadic success in freezing whole kidneys has been reported, no repeatable method is available.

As the principles underlying successful cryopreservation emerge, the distinction between the characteristics of the single cell system and the complex organ widens. Recent evidence suggests that given the present technology, successful kidney cryopreservation will most probably be achieved by slow cooling in the presence of high concentrations of cryoprotectant. Earlier work has not helped elucidate the acceptable cryoprotectant, the maximal concentration tolerance, or the appropriate method of addition and removal. Recent work has shown that rabbit kidneys will survive following treatment with high concentrations of glycerol. Dimethylsulfoxide (Me_2SO), the

cryoprotectant which has shown promise in kidney freezing (Guttman et. al. , 1977) has not been subject to similar investigation.

One obstacle in kidney preservation research is the lack of a single suitable viability test to describe kidney status following preservation treatment. Historically, researchers relied on autotransplantation- an expensive, technically difficult and time-consuming process requiring highly skilled surgical techniques and revealing little information concerning localization of damage. Measurement of integrated kidney function by in vitro perfusion is an attractive alternative which has been utilized by few workers in the field of kidney preservation.

The purpose of this study was to develop a system of in vitro rabbit kidney perfusion which allowed sufficient integrated function and sensitivity to assess the functional integrity of the kidney. This system was utilized to examine the kinetics of permeation of multimolar concentrations of Me_2SO during hypothermic perfusion, and to test the hypothesis that kidney function could be preserved if addition and removal of Me_2SO were accomplished in an osmotically-controlled manner.

LITERATURE REVIEW

I. Introduction to the Freezing of Water and Solutions

Living cells, or groups of cells, exposed to temperatures below the normal body temperature, are subject to an alien environment. This adverse environment contrasts with the normal milieu which has evolved as optimal for maximal survival and productivity. In order to effect long term preservation of cells or organisms by prolonged storage at temperatures inhibiting biological function, a myriad of potentially lethal obstacles must be surmounted.

At 0^{0}C, and atmospheric pressure, pure water is in equilibrium with the liquid and solid phase. This point is termed the freezing point of water. Increasing the pressure will cause this point to be depressed to a lower temperature, in effect lowering the melting point of water. Even at atmospheric pressure when the temperature of pure water is lowered below 0^{0}C, the water does not begin to freeze immediately but supercools. Theoretically, it is possible to supercool water to -40^{0}C if nucleation particles are absent. The term freezing describes the actual crystallization of water. At 0^{0}C, the rate of molecular motion of water, the heat energy, is greater than of ice. To transform from water to ice, 80 calories of heat must be released from every gram of water which crystallizes. If water has supercooled below

$0^{o}C$ before freezing, the latent heat of fusion will cause the liquid-ice mixture to rebound, or rewarm towards $0^{0}C$, and as crystallization proceeds a temperature plateau, or isotherm occurs, where the temperature does not decrease. Cooling continues after crystallization is completed.

Water may solidify by vitrification or crystallization. The term vitrification applies to the formation of an amorphous solid without organized structure, exhibiting a glassy appearance. The vitrification of water is achieved by ultra rapid cooling, which quickly stops molecular motion without allowing sufficient time for a more organized structure to form. This only applies to pure water. Biological material cannot undergo vitrification. By the application of different cooling rates and pressures, intermediate ice forms can develop.

For the formation of ice crystals, a nucleating agent must be present. This agent can be water molecules clumped together via hydrogen bonds, or insoluble crystalline impurities which either appear crystallographically similar to ice or can interact with hydrogen binding sites of water. The potential of an agent to cause nucleation is dependent upon its size and the temperature. As the temperature decreases below $0^{0}C$, the size requirement to initiate ice formation decreases. At $-35^{o}C$, molecular aggregates of water can act as self nucleators. The size and number of ice crystals formed depends on the cooling rate. When the temperature of water

or a solution is decreased slowly, crystals develop around the scarcer, larger nucleators. A few large crystals develop, since it is energetically more stable to add to an already existing crystal rather than initiate a new one. With more rapid cooling rates, the temperature falls quickly into the range where smaller and more numerous nucleating particles become effective, giving rise to smaller, more numerous ice particles.

Solutions freeze at lower temperatures than pure solvents. Ideally, the freezing point of 1000 g of water at atmospheric pressure is decreased by 1.86^{0}C for each mole of soluble particles. At some point which is below the actual freezing point of a solution, the complete solidification of both the solvent and solute occurs. This point is termed the eutectic point of the solution. Thus, between the freezing point of a solution, and the eutectic point, there exists a mixture of ice, water and solute. The dissolved solutes cannot become incorporated into the crystalline structure of ice. This implies that the concentration of solutes increases in the water which remains in liquid form. As solute concentration increases with decreasing temperature, the solubilities of different solutes are altered and nonhomogeneous precipitation results, significantly altering the pH of the existing solution (van den Burg and Rose, 1959 ; van den Burg and Soliman, 1969).

II. Cell Damage: The Effects of Slow Cooling

By cooling cells below the freezing point of the supporting medium, two fundamental factors are involved in determining the survival of the cells (Farrant, 1970). The first is associated with the concentration of solutes in the extracellular liquid phase as water is withdrawn as ice. The second is the formation of intracellular ice. These factors form the basis of the two-factor theory of freezing damage proposed by Mazur (1965.b).

Mazur (1970) referred to Lovelock's theory on the concentrating solutes in the external medium when water is withdrawn from solution in the form of ice as solution effects. He suggested that these effects are functions of temperature, provided that the cell is maintained at that temperature long enough to establish vapour pressure equilibrium, i.e. a slow cooling rate. This term, solution effects, encompasses the concerted action of four discrete events during the slow freeze process: (I) The water in the extracellular medium is removed from the solution as ice with decreasing temperature. (II) This causes a gradual concentration of both high and low molecular weight solutes in the surrounding medium. (III) As a result, cell volume decreases to well below normal volume. (IV) Concurrent with these changes is the precipitation of solutes from the supportive solution. All four effects are assumed to occur simultaneously; thus it is difficult to ascribe solution effect damage to any one of these events in particular.

Lovelock (1957) proposed that the increased concentration of solutes and the removal of water has deleterious effects on the lipid protein complexes of cell membranes, weakening them, and increasing lipid and phospholipid losses. The cell is rendered permeable to cations and swells, eventually bursting. The lipovitelline membrane is subjected to pH changes towards acidic pH as buffering salts are precipitated by water removal. The molecules of lipoprotein are contracted to the point of actual physical contact. Smith (1954) suggested that as the temperature falls and water is drawn out of cells, the solution becomes saturated with respect to the solute. A further decrease in temperature causes all the solute to precipitate out at a point termed the eutectic point of that solution.

In contrast, Meryman (1967 , 1968 , 1970) proposed that slow freeze injury in most cells is not due to the concentration of any particular solute, but rather a result of decreased intracellular volume beyond a critical volume. As the cell reduces in size in response to increasing osmolarity, the compression of the cell contents enhances the resistance to further shrinking. This results in a hydrostatic pressure difference across the cell membranes, incurring cell membrane damage. Williams (1968) measured the amount of water loss in red blood cells and mollusks at minimal temperatures for survival. Each species of cell reached different minimal temperatures, but the amount of water loss, approximately 64% of cell volume was

near equal for both cell types.

Levitt (1962) theorized that loss of water from the protoplasm brings protein molecules in apposition, presenting the opportunity for the formation of new chemical bonds previously too distant and rigidly structured in hydrated form to permit combination. Thawing would have a disruptive force on the new combinations, permitting unfolding and denaturation. Klotz (1958) suggested that intracellular ice formation affects the organization of cellular bound water, and results in the irreversible denaturation of macromolecules. Kendrew (1963) showed that in myoglobin, the exterior of the protein is encased in a hexagonal water lattice, while the interior exhibits clathrate-like, pentagonal water structures. Karow and Webb (1965) explained slow freezing injury as a consequence of the extraction of this bound water from cellular structures for incorporation into ice crystals, denuding proteins of lattice-arranged bound water essential to cell integrity.

Although the precipitating factor for damage during the slow cooling of cells-the concentration of solutes in the unfrozen liquid phase-is incorporated in the theories described above, the controversy over the mechanism of action is still unresolved. Rall et. al. (1978) observed that the survival of red blood cells correlated with salt concentration during freezing. Fishbein and Winkert (1978) froze solutions of the enzyme catalase. Increased NaCl concentration was damaging to the enzyme; freezing in KCl solutions was less deleterious, suggesting

that loss of activity was due to acidification of the solution during slow cooling. Wiest and Steponkus (1979) found that red cells behave as osmometers in solutions as high as 3.5 M salt concentration without reaching a minimum cell volume. Reports on the effects of dehydration of nucleated cells do not support the minimum cell volume hypothesis (Mironescu and Seed, 1977 ; Mironescu, 1978 ; Griffiths, 1978).

Attention has been focused on the cell membrane as an important site of freeze-thaw damage during slow cooling. Meryman et. al. (1977) proposed that extracellular ice formation results in a loss of membrane material due to forces exerted on the membrane during plasmolysis. Evidence that membrane fluidity is important for freeze-thaw survival was provided by Kruuv et. al. (1978). Yeast cells grown on different sources of fatty acids, which altered membrane fluidity, showed different rates of survival after freezing and thawing. Mironescu (1978) found no correlation between cell size and survival after exposure of Chinese Hamster cells to hypertonic solutes. Close correlation was found between survival and loss of cellular K^+, assumed due to the onset of membrane leakage. Griffiths (1978) assessed freezing damage to Chinese Hamster cells using specific radiochemical markers. Cell death was linked to a loss of membrane integrity, increased permeability to cations, and loss of cytoplasm. The extracellular ice surrounding frozen cells was thought to be a mechanism of red cell haemolysis by Nei (1970).

III. Cell Damage: The Effects of Rapid Cooling

Just as the solution effects associated with slow cooling rates are potentially deleterious to the cell, the rapid freezing of the cell can be equally, if not more, destructive. Between -5 and -15^{0}C, ice forms in the external medium, whereas the cell remains unfrozen and supercooled, probably due to the fact that the plasma membrane blocks the growth of ice crystals into the cell (Mazur, 1965a). The supercooled water has a higher chemical potential than partly frozen water, so it leaves the cell. If cooling is slow enough the water leaves at a rate which maintains an equilibrium with extracellular fluid, and no intracellular ice forms. If not, the cell water continues to supercool and freezes interiorly (Bank and Mazur, 1973). Intracellular ice formation, during cooling and rewarming of cell systems, is considered to be the primary factor for cell death during freezing (Leibo et. al., 1974). Smith et. al. (1951) observed that extracellular ice crystals did not physically damage the one-celled amoeba; but intracellular ice, induced by seeding the cell with an ice-tipped pipette at 0.8^{0}C was always lethal. Rey (1960) found that mouse fibroblasts warmed slowly from -40^{o}C to -20^{0}C were disrupted by the growth of minute ice crystals. While freezing mouse hepatic parenchymal cells, Trump et. al. (1964) noted that at slow rates of cooling, ice crystals were large and primarily extracellular. In rapidly frozen tissue, small ice crystals were seen both intra-and extra cellularly.

Mazur (1970) noted that yeast cells cooled at 100°C/min contained 70% of their original water at -50°C. He proposed that the value for the critical cooling rate which will produce intracellular crystallization is a function of the volume: surface area ratio of the cell, and the cell permeability to water. Large spherical cells, and cells with low permeability to water, require a cooling rate lower than the critical rate of smaller, or more permeable cells. Mazur and Schmidt (1968) studied the interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast cells. Survival rate dropped sharply when cooling velocities above 7°C/min were used and this was correlated with the same velocities that the cellular water is calculated to become increasingly supercooled and thus more liable to freeze.

Mazur (1977) presented a series of equations which allow one to calculate the extent of supercooling in cells as a function of cooling rate, given cell permeability to water, its temperature coefficient, the initial osmolarity of the cell and the ratio of cell surface to volume. To prevent intracellular freezing the water content of the cell must approach an equilibrium before intracellular nucleation occurs. In this way the probability of intracellular ice formation with cooling rate was calculated for yeast and human red cells. When the calculated survivals were compared with actual experimental survival curves, the drop in survival coincided with an increase in the probability of intracellular freezing. Leibo et. al. (1978) observed

this same pattern in unfertilized mouse ova using a cryomicroscope which allows visualization of the ovum during controlled freezing and thawing.

Farrant et. al. (1977) froze Chinese Hamster cells using a two - step cooling technique. Cells cooled rapidly to, and held at, a temperature just above that at which intracellular nucleation occurs tolerate a second rapid cooling step to -196°C and rewarming. This suggested that the cells were sufficiently dehydrated at the holding temperature and contained few ice nuclei. Cells cooled to and held at a higher temperature in the first step contained ice nuclei, killing the cells. It was concluded that cell damage correlated with the total amount of ice formed per cell, rather then the size of the individual ice crystals.

Fujikawa (1980) studied membrane damage of human erythrocytes by freeze - fracture and freeze - etching techniques. The results were consistent with a direct damaging effect of intracellular ice crystals. He suggested that ice formation in direct contact with a membrane causes bilayer membrane molecular disruption which results in post-thaw haemolysis.

IV. The Effect of Cooling and Warming Rates on Cell Survival

There are different optimal rates for each type of cell (see Figurel), and for identical types under different freezing conditions. Mazur et. al. (1970) compared the effects of different cooling velocities, warming velocities and cryoprotective agents on two different cell lines; mouse marrow stem cells, and Chinese hamster tissue culture cells. The viability of mouse marrow stem cells was dependent on the amount of cryo-preservative at lower cooling rates but independent at cooling rates above the optimum, i.e. 1.5^{0}C/min with 15% concentration of glycerol in the freezing medium. Chinese hamster cells, however, exhibited maximum survival without cryopreservatives at 300^{0}C/min cooling rate, and at 10^{0}C/min with cryopreservatives. Furthermore, a cooling rate of 1.6^{0}C/min was shown to be most deleterious. The optimal cooling rate for unprotected red cells was reported to be approximately 2500 to 3000^{0}C/min; however following the addition of glycerol prior to freezing, the optimum was lowered to 1500^{0}C/min (Rapatz and Luyet, 1965 ; 1968). Mouse embryos do not survive freezing when cooled at rates greater than 7^{0}C/min (Leibo et. al. , 1974).

The rate of cooling interacts with both the cryoprotectants utilized and the rate of thawing. The rate of warming has not been investigated to the extent which freezing rates have. Generally , thawing rate has little effect on survival of cell lines following slow freezing, but becomes a very important parameter following rapid freezing. Mazur and Schmidt (1968) showed that the survival of yeast cells depended greatly on

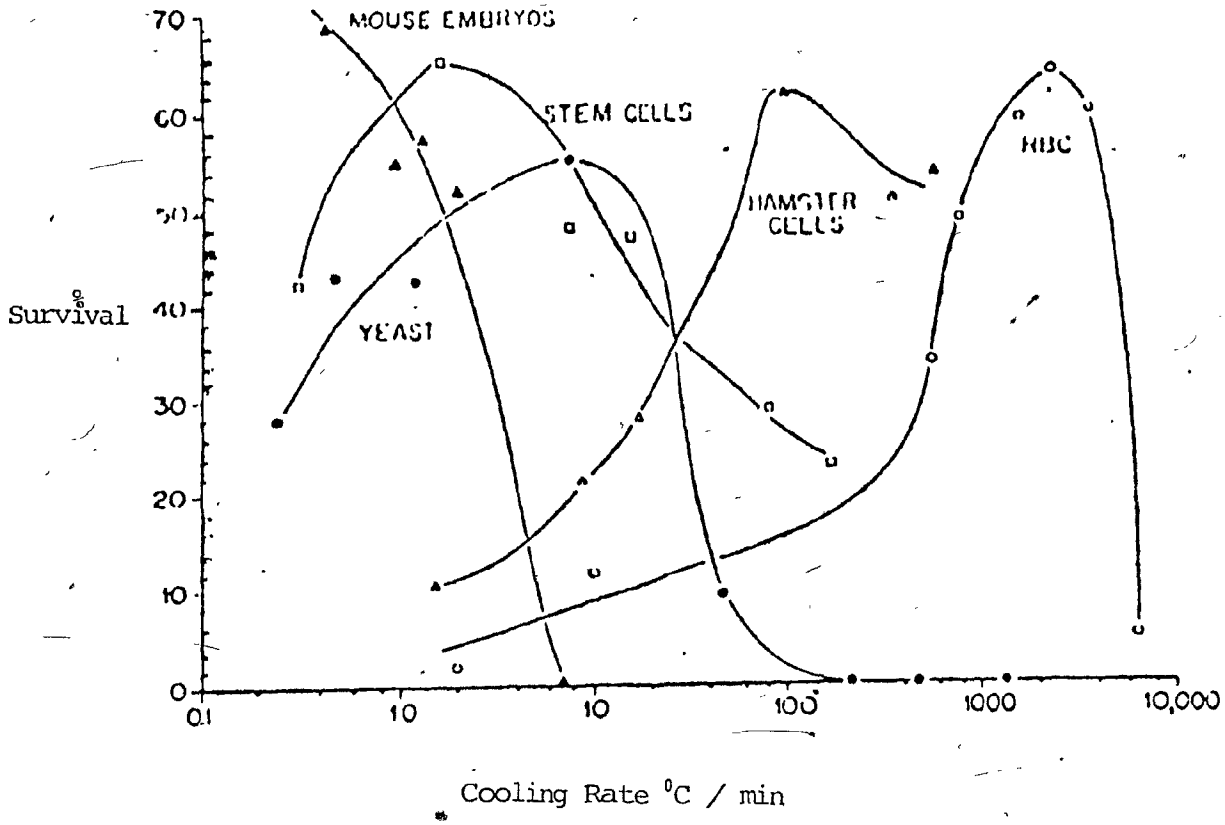

Figure 1. Optimal cooling rates for different cell types. (adapted from Leibo et. al., 1974).

warming rate after rapid cooling of 44.7 to 4730^{0}C/min. If cells cooled at 364^{0}C or 452^{0}C/min were thawed at 1^{0}C min, survival rate was 2 x10^{-6}%. With ultra rapid thawing (48,000^{0}C/min), survival of 4 x 10^{-6}% was obtained, an increase of 100%. Survival after slower cooling (0.24 to 7.2^{0}C/min) was not dependent on the rate of thawing. Mazur et. al. (1970) found that rapidly frozen mouse marrow stem cells were more susceptible to warming rates than slow-cooled cells. Rapidly cooled cells suffered more damage by slow thawing when compared to rapid thawing. Survival after freezing at 1.7^{0}C/min was 64.1% and 62.3% for rapid (910^{0}C/min) and slow (1.8^{0}C/min) warming rates, respectively. Cells cooled at 295^{0}C/min showed survival rates of 23% and 4.6% for rapid and slow warming rates, respectively. Leibo et. al. (1974) tested two and eight-celled mouse embryos for sensitivity to warming rates between 1 and 100^{0}C/min. Two-celled embryos cooled at 0.21^{0}C/min were insensitive to warming rates between 1 and 100^{0}C/min; but proved to be sensitive to low and high warming rates if cooled at 1.8^{0}C/min. Conversely, eight-celled embryos cooled at 0.18^{0}C/min showed high sensitivity to rates of warming, with the optimal warming rate being 2.5^{0}C/min . More rapidly cooled (1.7^{0}C/min) eight-celled embryos were insensitive to warming rates between 1 and 100^{0}C/min.

Reasons for the dependency of some cells cooled above their optimum cooling rate on warming rates and the apparent independence of most cells cooled below the cooling optima are speculative. Smith (1961) suggested that upon thawing of rapid frozen cells

migratory recrystallization, an actual growth of previously small innocuous ice crystals during rewarming, might be the key to all damage. Mazur (1966) stated that when cells are cooled rapidly, crystals of ice within cells are small. Smaller crystals have higher surface energies than large ones, and will grow if warming is slow enough. If warming is rapid, time does not permit this to occur before the melting point is reached. As described above, Farrant et. al. (1977) provided evidence that the critical factor is not the size of intracellular crystals, but the amount per cell which is allowed to achieve equilibrium during rewarming. Rapid thawing does not allow this amount of ice to be achieved.

The fact that each cell type exhibits an optimum treatment upholds the two factor freezing damage hypothesis. There is a point between solution effects and intracellular ice formation where most cell types can survive to a limited extent. This point is dependent on the rate of freezing and rewarming, and on the cryoprotective measures taken. However, there are other aspects of concern which need to be considered as other cell species become the subject of low temperature storage research.

Certain cells were found to be damaged well above freezing temperatures. Spermatoza could not be cooled to $0^{o}C$ without incurring high mortality. Red blood cells cooled rapidly from $37^{0}C$ to 0^{o} showed low survival (Lovelock, 1954). The

term coined for this destructive phenomenon is thermal shock. Lovelock (1954) felt that the effects of thermal shock manifest in the cell membranes at low temperatures as an increase in the ratio of cholesterol, i.e., the cholesterol lipoprotein complex, to lecithin in the cell membrane.

Farrant and Morris (1973) also support the contention that thermal shock is a major contributor to cell damage in the majority of cells. Although cooling to 0^0C is not in itself lethal to many cells, it exerts sublethal damage making the cell susceptible to damage during slow freezing. They added the suggestion that a dilution shock may also cause cellular damage during thawing. Dilution damage occurs by an increase in susceptibility of the cell to solute leaks during the return to the original volume. Thus the dilution shock may be additive to thermal shock, but is probably a less significant component of cell damage. Bank and Maurer (1974) found eight-celled rabbit embryos very sensitive to hypertonic shock requiring a gradual reduction of osmolarity. Dilution was performed at 37^0C on the assumption that the membrane may reseal at higher temperature. Leibo et. al. (1974) feel that an osmotic effect developing from the rapid change in osmotic pressure of the medium during fast thawing might be a problem; especially if a cryoprotective agent is employed. A transient temperature gradient could be imposed by a delay in rewarming rate due to absorption of latent heat of fusion in areas of large amounts of ice (Farrant et. al, 1977). If

this occurred, and all the ice melted on one side of the membrane prior to the other side, an osmotic gradient would be formed. This would damage cells during rapid thawing by fluctuations in the osmotic movement of water.

Because the destructive forces discussed are all exerting their effects concurrently, it is impossible to attribute cell death to any one factor in particular. What seems evident, from the shape of most cooling curves (Figure 1) is that the optimum for cell survival lies between the point where either solution effects due to slow cooling or ice formation due to rapid cooling are both evident, but non-lethal. This delicate balancing point, however , may be manipulated to some extent with the use of cryoprotective additives.

V. Cryoprotection

Luyet suggested that if water could be vitrified in biological systems, the viability of the system would be maintained. To date, it has been impossible to test this hypothesis; in a biological system, no matter how rapid the cooling rate, some form and quantity of ice is always produced. In general, very few cell systems are frozen at rates above 10^0C, and freezing damage is normally attributed to the solution effects - an increase intracellular concentration of solutes and electrolytes which affects protein configuration and solubility, salt precipitation, pH changes and cell dehydration. When one considers the delicate balance that must be preserved when freezing tissue or cells, the structural change in water from liquid to ice forms presents itself as a destructive force both intra-and extracellularly. Acceptance of the present inability to vitrify biological systems dictated that a different approach to freezing was needed whereby more plasticity in the freezing regime would not drastically reduce viability. Cryoprotective agents have proven to expand the range of freezing rates and increase suryival. Rates of freezing and thawing can be manipulated in a effort to establish an optimum post-freeze-thaw survival ; but most cell systems frozen under slow freezing rates do not survive the storage process without the protection of certain additives, or cryoprotective agents (Meryman, 1966). Initially these additives, which consisted of egg yolk or milk derivative pablums, were employed in order to effect a partial dehydration of the cells. This, it was speculated, would

remove some of the cellular water, and prevent formation of intracellular ice. The importance of protective compounds became apparent with the accidental discovery of the beneficial effects of glycerol in protecting rooster spermatozoa from slow freezing injury by Polge et. al.(1949). Since that time many different compounds have been found capable of protecting against freezing damage, glycerol, dimethysulfoxide (Me_2SO), ethylene glycol, polyvinylpyrrolidone (PVP), sucrose and polymers.

The cryoprotective compounds have been traditionally classified into two broad categories (Meryman, 1971). The first group is composed of penetrating agents. These are associated with protection from slow freezing injury and are used in multimolar concentrations. Penetrating agents are assumed to act on a colligative basis, reducing the amount of ice formed and the extent of concentration of nonpenetrating solute. An acceptable agent must penetrate the cell readily, and be nontoxic to the cell. Examples of this group are Me_2SO, glycerol, trimethylamine acetate, ethylene glycol, ethanol, and methanol. The second group is classified as nonpenetrating agents, such as PVP, sugars , sugar alcohols, polymers and proteins. Their mechanism of action is unknown.

Several explanations of the mode of action of the cryoprotective agent have been put forth. Luyet and Keane (1952 , 1953) attributed protection to a partial dehydration of the tissue. They suggested that characteristic chemicals having properties of easy penetration, low toxicity, efficiency of binding water

and a low eutectic temperature could serve as CPA. Mazur et. al. (1970) list the possible ways an additive can protect against freezing injury: 1) by decreasing the magnitude of solution effects, 2) by decreasing the susceptibility of the cell to solution effects, 3) by decreasing the likelihood of intracellular freezing and 4) by decreasing the sensitivity of the cell to freezing damage. To act in one of these capacities, it was thought that the additive must permeate the cell and act on a colligative basis.

Lovelock (1953 , a, b) found that the permeability of the cryoprotectant was most important when using glycerol in a red cell system. If glycerol was able to penetrate the red cell, it had a protective effect. If Ca^{++} - treated red cells were exposed to glycerol, the cells became dehydrated, but were not protected against freezing damage. He proposed that action was of a colligative nature - the non-electrolyte cryoprotective agent lowers the effective concentration of salts in equilibrium with ice at a given temperature and protected the cell. Red blood cells subjected to increasing concentration of salts would mimic freezing conditions and an effective cryoprotectant should have a low molecular weight, low toxicity, be highly soluble, and a good cell permeator. Using these criteria, Lovelock and Bishop (1959) were able to predict the effectiveness of Me_2SO as a cryoprotective agent. In support, Meryman (1968) demonstrated that 4M ammonium acetate, a cryoprotective agent with no demonstrable eutectic point will protect the human red blood

cell; whereas ammonium chloride, with a eutectic of -15.8 ^{0}C, will not protect. The buffering effect was supported by Rasmussen and Mackenzie (1968) who determined that the water - Me_2SO trihydrate has a eutectic point of -63 ± 1 ^{0}C.

Further studies have cast doubt on the necessity of cellular penetration for cryoprotection. Mazur et. al. (1974 a; b) studied the survival of bovine red cells as a function of the permeation of glycerol and sucrose, and of human red cells under similar conditions (Mazur and Miller 1976 , a , b). For both cell types, varying intracellular glycerol concentration did not confer significantly greater protection to cells, indicating that extensive permeation is not required. Freezing cells with the nonpermeant, sucrose, conferred similar protection, but attempts to remove sucrose were deleterious. Furthermore, no information concerning the permeation of glycerol during the freezing process was obtained. These authors concluded that the protection of agents is not due to prevention of excessive cellular dehydration to a minimum cell volume and membrane collapse.

Leibo and Mazur (1974) indicated that glycerol permeation is not a requirement for protection in the eight-celled mouse embryo. A 20 second exposure to glycerol afforded the same protection as a 60 minute exposure. Furthermore, a 20 second exposure prior to freezing gave the same protection at 0, 10, 20, or 37 ^{0}C. Taylor et. al.(1974) studied the permeability of glycerol and Me_2SO in hamster ovarian cells before cooling to -196 ^{0}C. They found that glycerol may be regarded as a non-permeant at 2 ^{0}C

with a 30 minute incubation period; while Me_2SO was considered to be a good permeator under all conditions. Leibo et. al. (1974) measured the cell volume of eight-celled mouse embryos photographically. Even after 90 minutes at 0^0 C, embryos in Me_2SO - PBS solution did not attain original volume unless diluted back to original osmolarity. They concluded that Me_2SO permeates the cell very slowly, if at all, at 0^0 C , and thus protection does not require permeation. Meryman et. al. (1977) froze human red cells in the presence of methanol, ethanol, glycerol, Me_2SO , ammonium acetate, sucrose and PVP, and determined that the action of the cryoprotectant was purely colligative in the case of penetrating cryoprotectants - the percentage of hemolysis was a function of the salt concentration. In addition studies with sucrose indicated no cryoprotective function. Effects of PVP were better than with sucrose, but the results were misleading since PVP was found to retard hemoglobin release . Dilution of the PVP following thawing revealed that it offered no additional cryoprotective properties. Meryman offered the interpretation that cryoprotectants act in two ways : 1) colligatively, by reducing the amount of ice formed, requiring penetration of the cell, and 2) kinetically, by retarding water efflux from the cell through increased solution viscosity.

Using a two - step cooling procedure, McGann (1978) compared survival of Chinese Hamster cells frozen with a penetrating agent, Me_2SO, and a non penetrating agent, hydroxyethyl starch (HES). As the Me_2SO concentration was increased, the holding temperature for maximal survival decreased. Increasing concentration

of HES did not alter the holding temperature for maximal survival. Glycerol added to the cells at 20 ^{0}C to effect penetration exhibited a pattern similar to Me_2SO ; however, if added at 0 ^{0}C , permeation of the cell was poor,and and the pattern was similar to HES. He concluded that both penetrating and non-penetrating agents protect by colligative action- allowing cell survival by decreasing the volume of water and reducing the likelihood of intracellular ice. Penetrating agents reduce cell water content at temperatures low enough to reduce the deleterious effects of increased solute concentration, and protect during slow cooling. Non-penetrating agents act by squeezing water out of the cell at higher temperatures- suggesting little protection from increased solute concentration. In the latter case, slow cooling may be hazardous.

The theory of Lovelock does not, however, explain how nonpenetrating compounds such as sucrose, polyvinylpyrrolidone, or dextran act as cryoprotective agents. Furthermore, it does not explain the specificity of low molecular weight cryoprotective agents, since all will reduce the salt concentration during freezing. The latter objection, however, may in part be due to some intrinsic toxicity of different molecules. The fact that cryoprotective ability varies between different tissues is probably due to the interrelationship between physical factors and biological variability. The solubility of the agent, the eutectic changes it produces in a given system, the viscosity and temperature all affect the diffusion of the agent in a system, and its protection. Keane (1953) suggested that hydrogen binding of the agent can be one factor of its variability.

Karow (1969) believed that the hydrogen binding of cryoprotective agents may relate to the "pseudo toxic" effect of glycerol, Me_2SO and dextran on cardiac muscle. He defined two effects of cryoprotectants-an intrinsic toxicity related to the chemistry of the cryoprotective agent, and a non-specific toxicity related to the concentration, the temperature of administration, and the duration of the exposure to the cryoprotective agent.

Other theories have been suggested to explain the action of cryoprotectants in different freezing systems, especially in view of the protective effect on the cell membrane. The hydrogen bonding theory (Doebbler and Rinfret, 1972) could account for the specificity of the cryoprotective agents. X-ray diffraction studies demonstrated that there is vitreous ice in rapidly frozen solutions of known cryoprotectants, but only hexagonal crystals in frozen solutions of structurally similar compounds which do not protect cells. The growth rate of ice in supercooled liquids with a variety of solutes is correlated with the mole equivalent of potential hydrogen binding sites provided by the solutes (Doebbler, 1966). Multiple binding sites increase the protective action (Doebbler and Rinfret, 1965). The theory is presented that the cryoprotective agents establish hydrogen bonds with water, reducing the amount of water available for ice crystal incorporation, and promote the formation of vitreous ice. Karow and Webb (1965) further theorized that hydrogen binding may act by stabilizing the hydration lattice surrounding proteins and reducing the probability of protein denaturation by dessication during freezing. Slow cooling allows sufficient time for water lattices around proteins to grow and

strengthen, thus protecting. According to Meryman (1971), at -20^{0} C, 1 mole of glycerol binds to 2 moles of H_2O, and 1 mole of Me_2SO binds 3 moles of H_2O. Miller et. al. (1978) tested cryoprotective agents' ability to act as hydroxy radical scavengers, which would prevent oxidation of protein sulfhydryl groups to intermolecular disulfide bonds. They found that both cryoprotectant and scavenger efficiency decreased in the same order within a homologous series.

In a cell-free system, Tsutsayeva et. al. (1978) studied the mechanism of action of low temperature and cryoprotective agents on immune antitoxic antitetanus sera and globulins frozen in the presence and absence of polyethylene oxide. They suggested that the cryoprotective effect was due to the formation of a protective coat preventing protein dehydration precipitated by contact with ice, and glycerol could protect through interaction of its hydroxyl groups with polar amino acid residues on the protein surface. Körber and Scheiwe (1980) investigated the cryoprotective properties of HES by differential thermal analysis. They hypothesized that the protective action of HES differs from the colligative effect attributed to Me_2SO or glycerol - and is due to its water absorptive capacity. Williams and Harris (1977) studied the distribution of cryoprotective agents into lipid interfaces, and proposed that they protect by interaction with the plasma membrane by virtue of surface activity and/or distribution into membrane lipid. Barnett (1978) found that glycerol and Me_2SO inhibit Na^+ K^+ ATPase of pig kidney medulla due to interactions with membrane

lipids. Both agents lower the lipid transition temperature and increase membrane fluidity, rendering the membrane more resilient to osmotic stress.

Alternate theories of cryoprotection do not reduce the importance of a colligative action. They may serve to identify certain characteristics of agents which aid in freeze-thaw protection of cells in conjunction with the ability to prevent intracellular ice and delay solute concentration. Cell water volume, sodium concentration, and potassium concentration were measured in human red cells under hypertonic conditions equivalent to freezing at -37 ^{0}C with NaCl (Farrant and Woolgar, 1972a), sucrose (Farrant and Woolgar, 1972b), and Me_2SO (Farrant and Woolgar, 1972c). They found that the mass of cell water reached minimal values of approximately 0.4 that of total cell water under isotonic conditions with both sucrose and NaCl, while exposure to Me_2SO resulted in only a transient loss of cell liquid which attained near normal values after 5 or 10 minutes. It was shown that sucrose did not permeate the cell above normal isotonic amounts, but labelled Me_2SO did enter the cell and replaced a certain proportion of cell water. Studies found that the onset of cation leakage occurred at approximately 1500,2050, and 5350 milliosmoles for NaCl, sucrose and Me_2SO respectively. It was concluded that a cation leak develops at approximately 2000 milliosmoles even with sucrose as a protective additive; but the nonpenetrating additives such as sucrose and PVP, even though they do not prevent the onset of the membrane leak, moderate the concentration gradient such that the membrane is able to take advantage of the leak, and by remaining impermeable help to prevent swelling of the cation-leaky cell. They

lend osmotic protection to the cell and reduce post hypertonic damage. Penetrating agents prevent cell shrinking and resulting cation leaks, postponing these damaging effects to higher osmolarities.

Alterations to membrane structure of Chinese Hamster Ovary cells under hypertonic conditions were observed by Mironescu and Seed (1977) using scanning electron microscopy. Exposure to 2600 mOsm/kg NaCl produced minimal changes. Above 2600 mOsm/kg, the microvilli and blebbing of the membrane were reduced, and cell locomotion ceased. At 7550 mOsm/kg, the cells were devoid of normal surface detail, and exhibited small perforations. With Me_2SO , at 7550 mOs/kg, microvilli, blebbs and ruffling activity were still present. Substituting sucrose as the hyperosmotic agent produced membrane changes, but were not the same as those produced with NaCl. The authors concluded that increased solute concentration dramatically altered surface microprojections of cells. The extent of alteration was mediated by the osmolality, duration of exposure and type of solute, and was mitigated by Me_2SO, suggesting an ability of Me_2SO to protect cells from hyperosmotic damage in addition to a colligative effect. Further studies failed to correlate survival of cells with cell size or sodium content (Mironescu, 1978), but found a close correlation with potassium loss suggesting that Me_2SO may regulate K^+ loss from the cell, which may be protective at low temperature in hypertonic medium.

VI. Organ Cryopreservation: Problems of Extrapolation from Cell Suspensions to Organs

Karow (1974) lists the numerous agents which have shown cryoprotective properties and the cell types which have been frozen and thawed. It is readily apparent that although the optimal cryoprotection procedures are worked out for many cell types, few organs have as yet been successfully preserved. The graduation from tissue to organ preservation has, except for sporadic successes, been difficult. The assumption has been to apply the same methods of freezing to organs as was successful with cells and tissue. This situation has not changed dramatically over the past 6 years (Ashwood-Smith, 1980). Problems which are associated with organ cryopreservation are different from those associated with cell suspensions or thin slices of tissue. These differences are a result of the unique morphological features of a highly organized structure.

The first differentiating factor between organs and cell suspensions relevent to cryopreservation is the fixed geometry of the organ. This precludes manipulation of the surface area to volume ratio, and poses a problem of non-uniform cooling. It does not allow adequate control of organ core temperature by the method of conduction cooling utilized for cell suspensions frozen in suitably-designed small vessels. To determine the effects of a constant cooling rate imposed on the outer surface of an organ, Rubinsky et. al.(1980) developed an analysis of the thermal stresses exerted on a sphere. When water freezes at some location in an organ, or sphere, compressive stresses develop. For the identical freezing protocol, the stresses which

develop in an organ of 5 mm radius would be ten times greater than a piece of tissue of 1 mm radius.

The effect of cell density is considered to be of particular importance in organ freezing. Nei (1970) suggested that increased concentrations of rabbit red cells lead to tighter packing of cells in inter-ice channels resulting in increased haemolysis. Pegg et. al. (1979) studied the effect of high cell density on cryosurvival of red cells. As the packed cell volume approached 80%, a density similar to that found in organs, cell recovery was drastically reduced. Similar results were reported for HeLa S-3 cells by McGrath et. al. (1975). They found that intracellular ice was present in a larger proportion of cells, at a given cooling velocity, which had a greater packing density. Levin et. al. (1977) used the cell cluster as a first order approximation of organs to predict the amount of intracellular water retained by a group of closely packed cells during cooling and rewarming. At a given cooling rate, the probability of intracellular ice nucleation was increased for cells in the interior of the cluster. The survival curve characteristic of the single cell was shifted to the left - maximal survival was achieved at a lower cooling rate.

Two recent studies of cell packing and cryosurvival suggest that the damaging effects are due to inhibition of cell dehydration. Slow cooling and high concentrations of cryoprotectant would prevent decreased survival (Pegg and Diaper, 1980 ; Meryman et. al., 1980).

The third factor to be considered in organ cryopreservation is the diversity of cell type which compromises the organ. The evidence

for a different optimal rate of cooling and thawing for different cell types was presented in Section IV. of this review. It was also noted that multicellular embryos required different procedures than two-celled embryos (Leibo et. al., 1974). The requirements for optimal cryosurvival of one population of cells in an organ may not fall within the range for adequate survival of another cell type.

In contrast to cell suspensions, the spatial orientation of cells in an organ is of fundamental importance to organ function and viability. The cellular arrangement must be preserved intact. A specific example is the vascular system. Even if the parenchyma is well preserved during cryopreservation, if the vascular system has been rendered incapable of providing oxygen, nutrients or removal of waste products, the organ will not survive. The relatively innocuous effects of extracellular ice in single cell freezing become damaging in organ freezing. Extracellular, intravascular ice expansion could produce damage similar to that found in a pipe which bursts due to expansion of ice.

The volume fluctuations which a cell undergoes during the process of low temperature preservation (Puskar et. al., 1980) could affect the cellular spatial orientation. Uncontrolled fluctuations may promote detachment of cells from the basement membrane or from neighbouring cells.

In spite of the limitations of cell density and diversity, encouraging results have been reported for the cryopreservation of organized tissue fragments and thin organs. Successful cryopreservation has been reported for fetal and neonatal rat hearts (Rajotte et. al.,

1976), parathyroid tissue fragments (Welles et. al., 1977) , and fetal rat pancreas (Kemp et. al., 1977). Some success with spleen (Barner and Schenk, 1966), and intestinal segments (Guttman et. al., 1969) has also been reported.

VII. Cryopreservation of the Kidney

The transition from organized tissue fragments and thin segments to larger, more complex organs has been relatively unsuccessful. The heart and kidney have been the object of intensive studies; as yet, no repeatable procedures for adult mammalian heart or kidney long term freeze-preservation are available (Ashwood Smith, 1980).

At least eight steps are required to achieve successful kidney cryopreservation (Filo et. al, 1976): (1). Harvesting; (2). short term preservation; (3). cryopreparation; (4). controlled freezing; (5). long term storage; (6) controlled thawing; (7). reinstitution of hypothermic ex vivo perfusion; (8). reimplantation. Each step must be accomplished in a manner which causes the least amount of trauma. Kidneys harvested, perfused hypothermically, and reimplanted are capable of supporting life (Small et. al., 1977 ; Belzer, 1977 ; Proctor and Joyce, 1978 ; Belzer and Southard, 1980); however if sublethal damage does occur, the susceptibility to further damage during steps 3 through 7 could be increased and cumulatively prove fatal. Rabbit kidneys perfused hypothermically for relatively short periods exhibit functional impairment during perfusion at 37 ^{0}C (Pegg and Wusteman, 1977).

Steps 3 and 7 involve the introduction of cryoprotectant prior to freezing and the removal following thawing, respectively. To some extent, the permeation of cryoprotectant, and the final concentration achieved dictate the cooling rates needed. It was mentioned in the previous section that the organ size does not permit rapid cooling

rates. Cryobiologists are locked in slow cooling rates given the present technology. The high cell density and structural organization of the kidney render it particularly sensitive to both intra and extracellular ice formation. The most promising route to follow appears to be the treatment with high concentrations of cryoprotectant to minimize excessive buildup of solute concentration and delay the effects of ice formation. The nature of the cryoprotectant, and its concentration dictate the appropriate methods for addition and removal. This process is of considerable interest to kidney cryobiologists, and will be discussed in greater detail in the following section.

Thawing procedures (step 6) are also the subject of research and debate. Advocates of rapid thawing are investigating the use of microwave illumination (Ketterer et. al., 1979). Serious drawbacks to the use of microwaves are non-uniform thawing, lack of directional control of the radiant energy and the possibility of lethal effects of microwaves themselves and in conjunction with cryoprotectant. Temperature sensing during microwave thawing poses serious problems; application of electrodes to the organ surface produces burning due to contact resistance. Some of these problems are surmountable. Burdette et. al. (1978) obtained uniformity of thaw of dog kidneys by implanting electroseeds (steel spheres inserted in the renal pelvis) and illumination with both 2450 MHz and 7 MHz. Guttman et. al. (1980) have developed a method of non-contact temperature sensing which permits monitoring of organ temperature to 0^{0} C.

Pegg and coworkers argue that rapid thawing is unnecessary in organ preservation (Pegg et. al., 1978 ; Pegg et. al., 1979 ;

Jacobsen and Pegg, 1979). It was postulated that the increased concentration of intracellular solutes during slow cooling would not be maintained in equilibrium with extracellular fluid during rapid thawing. During the phase change, water would rush into cells and produce osmotic cell lysis.

The final step is the reimplantation of the kidney. The majority of researchers have relied on this stringent test of kidney viability, due to a lack of appropriate in vitro tests which reflect the functional integrity of the kidney (Sell, 1967). Reimplantation has serious drawbacks. The procedure is expensive and requires highly skilled surgical help. Technical failures due to the long and repeated procedures are often cited. The time interval between experimentation and conclusion of results is prolonged. The procedure does not lend itself readily to identification of the specific areas of damage to the kidney precipitated by the various procedures. The need to employ a suitable in vitro test of kidney function prior to transplantation is discussed more fully in section VIII of this review.

Very limited and sporadic successes in kidney freeze-preservation have been reported. After freezing to -50 ^{0}C for 15 minutes in 2.2 M glycerol, Halasz et. al. (1967) found 25% of dog kidneys had life- sustaining function. Mundth et. al. (1965) reported 2 of 14 kidneys survived freezing to -20 ^{0}C with 1.6 M Me_2SO. Dietzman et. al. (1973) reported function in 2 of over 150 kidneys frozen to -20 ^{0}C for 15 minutes with 1.6 M Me_2SO. Using 1.4 M Me_2SO

and cooling to -80 ^{0}C for 15 minutes, Guttman et. al. (1977) found 9 of 18 kidneys could support life. The kidneys were cooled with intra-arterial helium and thawed by microwave illumination. These findings could not be repeated by Pegg et. al. (1978), or by Guttman himself (Guttman et. al., 1979); however Toledo-Pereyra (1980) did obtain survival of 3 out of 10 kidneys frozen to -80 or -120 ^{0}C using similar techniques. In the latter case the kidneys were cooled very rapidly and held at the minimum temperature for only 3 to 5 minutes.

The common factor in these reports was the use of low concentrations of cryoprotectants and very short holding times at the lowest temperature reached, not allowing equilibrium freezing to occur. The experiments were empirical studies to obtain results using techniques extrapolated from freezing cells in suspension without providing information of the mechanisms operating in this largely different system.

VIII. Cryoprotection of the Kidney

Pegg (1973) suggested that due to organ size and heterogeneity, freezing of organs will remain locked in a slow cooling system. Therefore, we will need to find the appropriate cryoprotective agent in the highest tolerable concentrations in order to achieve success. A review of the literature reveals that there is no agreement as to the agent of choice, its concentration, the method of addition and removal, and the temperature at which these manipulations are carried out (Pegg , 1972 ; Jacobsen, 1978 ; Karow, 1974).

Early work with the kidney focused on the use of the cryoprotective agent , glycerol (Huggins, 1959). He was unsuccessful when trying to perfuse dog kidneys with progressively increasing and decreasing concentrations of glycerol in Tyrode solution. Failure was due to massive renal swelling and cortical necrosis. In vitro immersion of rabbit kidney slices in 5 or 10% glycerol in Tyrode solution showed a decrease in tissue weight after 50-80 minutes which gradually increased and equilibrated at a weight greater than the original. The higher glycerol concentration showed greater increases. Transfer from glycerol to heparinized blood increased weights to over 100% of the control after 3.5 hours. This could be checked if the slices were placed in 20% sucrose in Tyrode solution after placing in blood for 10 minutes. The temperature of these manipulations was not mentioned.

Rivers et. al. (1961) recognized that the effective osmotic pressure of glycerol resulted in acute changes in weight from rapid gain or loss of water , depending on whether the higher concentration of glycerol is within or outside the vascular space. The rate and degree of

weight change reflects this concentration gradient. He suggested that edema and swelling from glycerol removal would contribute to resistance to blood flow and may represent a potential for cell destruction by lysis. Thus, deglycerolization probably represents a greater threat to organ viability than glycerolization. Dog kidneys perfused with 15 and 30% glycerol decreased in weight over 5 minutes and then steadily increased in weight achieving a 50% increase after 6 hours, without stabilization, reflecting cellular damage. Control kidneys perfused with blood or Balanced Salt Solution with albumin reached a constant weight level. Kidneys were perfused in vivo with 15% glycerol in saline at 6 ^{0}C for 1 hour, followed by decreasing concentrations to 10% and 5% for 20 minutes respectively. Of 27 dogs, 11 survived longer than 1 month; 6 of which had normal kidney physiology. The remaining 5 showed plasma cell nephritis and pyelonephritis. Short term survivals (2-3 days) showed vascular changes similar to reversible osmotic nephrosis. All survivors showed elevated BUN and albuminuria for 3-7 days. This indicated that although kidneys were affected, toxicity of glycerol was minimal if osmotic changes were controlled. One kidney, frozen at -14 ^{0}C for 5 hours following perfusion with 15% glycerol, failed to function.

Brada and Shloerb (1965) perfused dog kidneys at 18.3 ml/min with 5, 10 and 15% glycerol in a salt solution, containing labelled glycerol and water for 5, 10, 15, 30 and 60 minutes. They found that equilibration was complete at 30 minutes. Perfusion with 10% glycerol for 10 minutes resulted in survival after immediate contralateral nephrectomy.

Makin and Howard (1965) attempted to prolong the storage of canine kidneys by supercooling with 7.5% Me_2SO in salt solution perfusate. Ten kidneys were perfused at -5^0 C for 20 minutes and stored at that temperature for 4 hours. Nephrectomy followed reimplantation 1 to 6 weeks post implant. An additional 5 kidneys were stored for 12 hours at -5^0 C, and 2 were stored at 5^0 C for 4 hours. No kidneys were observed to freeze; all exhibited a uniform shrinkage predominently in the cortex, and all secreted urine following contralateral nephrectomy. All kidneys appeared swollen and discoloured following reestablishment of blood flow. Two of 10 dog kidneys stored 4 hours at -5^0 C might have survived since BUN levels started to fall, but died of extrarenal causes. All other kidneys were necrotic. Histologically, 10 minutes after reimplantation the kidneys showed proximal convoluted tubule and glomerular damage, with tubular casts appearing after 3 days. After 11 days, there was some evidence of regeneration of tubules; after 6 weeks, fibrosis was marked. Nevertheless, the authors claimed to be the first to report the conservation of urine production after subzero storage, where other authors had not.

Halasz et. al. (1966) perfused dog kidneys in situ at 20^0 C with glycerol, propylene glycol and Me_2SO alone and in combination. The perfusate contained 6% low molecular weight dextran in a balanced salt solution. Each kidney was perfused for 10 minutes at 70 mmHg at a rate of 60-75 ml/min. All control kidneys survived. Kidneys perfused with 15% glycerol exhibited swelling and cyanosis. Outflow blockage was diminished if the kidney was washed with 50 ml of basic

perfusate, and by releasing the vascular clamp slowly. Perfusion with glycerol or propylene glycol in concentrations of up to 20% resulted in reversible damage. Three out of five kidneys functioned after perfusion with up to 15% Me_2SO . In combination, 10% glycerol and 10% propylene glycol were tolerated, but 15% of each were not. Solutions of all three cryoprotective agents at 5% concentrations each gave 4 out of 4 dogs surviving after contralateral nephrectomy, with survival decreasing until 11% concentrations of each in combination resulted in 0 out of 4 survivals. They concluded that damage was reversible and due to the hyperosmolarity and not the toxicity of the agents. Damage was mainly confined to the tubules, and swelling and outflow block was probably due to the hypertonic medium.

There is conflicting evidence of an actual cytotoxic effect of cryoprotective agents, specifically Me_2SO . Malinin (1973) incubated primary kidney cells from Rhesus monkey embryos in TC-199 and 10% FCS with Me_2SO . Lots were incubated in 7.5 or 15% Me_2SO in Earles Salts at 4^0C or 25^0C for 10,20,40 and 60 minutes, then fixed and examined under the electron microscope. Structural differences were observed after 10 minutes at 4^0C with 7.5% Me_2SO -evidence of lipid accumulation, mitochondrial swelling and damage to the mitochondrial membrane and cristae. At 25^0C and 7.5% Me_2SO after 10 minutes , mitochondrial swelling, RER dilatation and degranulation, autophagic activity, and changes in the nuclear membrane occurred. As incubation time with Me_2SO increased there were increased numbers of liposomes, and cell ruptures, regardless of temperature.

Although viability was not tested, the Me_2SO did not appear innocuous. In contrast, Small and Ide (1976) could not detect any deleterious effects of Me_2SO on kidney function in rats chronically injected with Me_2SO over a 28 day period.

To differentiate between toxic and osmotic damage of Me_2SO, Hak et. al. (1973 a) examined the effects of Me_2SO on cultured beating rat heart cells at 4, 24 and 37 ^{0}C. The method of addition and removal of Me_2SO was varied. A single addition and removal of 1.5 M Me_2SO was tolerated well at most temperatures, determined by myocardial contractility studies and trypan blue staining. Me_2SO in 2.0 M concentrations had more drastic deleterious effects, especially at lower temperatures. With a slow elimination of Me_2SO , higher temperatures increased toleration; 24 ^{0}C was optimal. If the exposure time to Me_2SO was lengthened (60 minutes vs 10 minutes), lower temperatures (4 ^{0}C, 24 ^{0}C) were more favorable. Further studies (Hak et. al., 1973b) indicated that sucrose and sorbital in concentrations up to 1.5 M was not tolerated as well as NaCl at similar concentrations. Me_2SO was best tolerated. The authors felt this was due to a dehydration of cells to a minimum volume, causing death.

Pegg (1972) perfused rabbit kidneys with 2 molar concentrations of glycerol, Me_2SO or ethylene glycol. The perfusate contained 4.5% dextran 70 and 1.5% BSA, and the organs were perfused at 37^o C or 5 ^{0}C at 40 mmHg pressure. After an initial washout for 5 minutes, the cryoprotectant was added and the kidney perfused for 2 hours. Following perfusion, the kidney was homogenized and the extent of equilibration with cryoprotective agent determined. Control

perfusions exhibited a 50% increase in vascular resistance at 37°C during the first 40 minutes, which decreased to original values by 90 minutes. At 5°C, only a small initial increase was observed. At both temperatures, weight gain was similar, at 0.1% per minute. Glycerol perfusion caused a decreased resistance at 37°C and 5°C, greater at 5°C; weight gain transiently decreased at 37°C initially, then increased in both cases to 40% of initial weight after 2 hours. Ethylene glycol perfusion was similar, but at 5°C, weight gain was minimal. Me_2SO perfusion resulted in a decreased resistance at the beginning, followed by an increased resistance throughout perfusion especially at 5°C. Weight followed a similar pattern. Histological examination showed edema, tubule dilatation, and capillary dilatation which corresponded to weight gain. Edema was most marked with glycerol at 5°C, least with ethylene glycol. Glycerol showed the least change. Me_2SO, and to some extent ethylene glycol perfusion resulted in striking changes in the capillary vascular epithelium manifested as cell swelling and detachment from the basement membrane. In certain instances this damage was so widespread that only nuclei remained. The extent of equilibration of cryoprotectant agent after two hours varied with temperature. Glycerol equilibration at 5°C was 72%, while at 37°C it was 102%. Me_2SO equilibration at 5°C was superior to 37°C, 67% and 58% respectively.

Other reports of Me_2SO perfusion do not demonstrate the same toxicity. Jeske et. al. (1974) perfused rabbit kidneys at 37°C with 10, 15, and 20% (1.4, 2.1 and 2.8M) Me_2SO for 50 minutes

followed by electron microscopic evaluation. They found that the kidney tolerated Me_2SO perfusion ultrastructurally, except for clarification of proximal tubule cytoplasm. The proximal convoluted tubule seemed most sensitive to damage; glomeruli appeared to be extremely durable. It was suggested that the early resistance changes observed in Pegg's report (1972) were due to changes in smooth muscle tone, not ultrastructural changes; whereas after prolonged perfusion (2 hours), these changes may have been due to capillary degeneration.

Karow and Jeske (1976) presented a more complete evaluation of renal function during Me_2SO perfusion. A colloid-free K -Mg rich perfusate at 37 ^{0}C was used containing 10,15, or 20% (1.4,2.1, or 2.8 M) Me_2SO. Kidneys were perfused for 10 minutes with Me_2SO-free perfusate followed by 50 minutes with Me_2SO . No attempt was made to introduce Me_2SO in a stepwise manner. Kidney function at 15 minute intervals was compared to control function at similar intervals. Perfusion with 1.4M Me_2SO increased flow at 50 minutes, but decreased at 60 minutes. Glomerular filtration rate increased significantly at 35 minutes; sodium clearance increased at 30 minutes. Weight gain was 56.8%. The kidneys perfused with 2.1M Me_2SO did not differ significantly in function from controls except paraaminohippurate (PAH) clearance, which decreased at 30, 50 and 60 minutes. With 2.8M Me_2SO, renal flow was higher than controls at all times. Resistance did not show significant changes, but did increase. PAH clearance was severely depressed. The authors concluded that changes induced by Me_2SO were due to osmotic fluid shifts, edema, and vasoaction of Me_2SO on smooth muscle cells.

Further studies were done with rabbit and dog kidneys with

1.4M Me_2SO (Karow et. al., 1979). In rabbit kidneys perfused at 37°C, maximum tissue Me_2SO distribution was achieved in 30 minutes and washout was 90% complete in 30 minutes. Prolonged washout did not reduce residual Me_2SO. In dog kidneys perfused at 37, 25 or 10°C, similar values were obtained; lower temperatures reduced the saturation rate, not the final concentration after 30 minutes. Dog kidneys were reimplanted after Me_2SO administration. After perfusion at 25°C, 5/12 dogs survived compared to 3/7 controls. Results at 37°C were better - 2/3 survived after perfusion with 1.4M Me_2SO, compared to 4/5 controls. Gradual addition and removal of Me_2SO at 25°C increased survival (8/9) compared to one-step addition and removal (2/6).

Small et. al. (1977)perfused canine kidneys with Me_2SO to test (1) the agent's effects on renal tubule function, and (2) the equilibration of Me_2SO with renal tissue. Kidneys were perfused with 10% Me_2SO in cryoprecipitated canine plasma at 60 mmHg, 0.7 ml/min/g kidney weight, at 8-10°C. The Me_2SO was added and removed gradually . Exposure to 10% Me_2SO was 20 minutes. The kidneys were reimplanted and clearance studies were done after reimplantation, and repeated one week later just prior to contralateral nephrectomy. In the kidneys perfused without Me_2SO, only renal concentrating ability was impaired. In the Me_2SO-perfused group, urine flow, glucose reabsorption, inulin clearance, PAH extraction, and free water clearance were all impaired for 2 days following reimplantation. At 7 days, only the renal free water clearance defect remained. Me_2SO tissue content expressed as a percentage of perfusate content

revealed that at highest perfusate Me_2SO concentration (10%), Me_2SO content of outer, inner cortex, and medulla was 96.9 ± 1.5, 88.3 ± 2.5, and 60.8 ± 4.0% respectively. At low perfusate concentrations of Me_2SO (2.5%) corresponding values were 115.4 ±2.5, 113.7 ± 1.6, and 113.2 ± 4.6%.

Recognizing the possible contribution of osmotic effects to injury during the introduction and removal of cryoprotectant, Pegg and Wusteman (1977) perfused rabbit kidneys at 5 ^{0}C with increasing glycerol concentrations to 2M at rapid rates of addition, at 80 mM min^{-1} and at 30 mM min^{-1}. Deglycerolization was accomplished at the same rate as addition. Rapid and 80 mM min^{-1} additions produced large increases in renal vascular resistance during 5 ^{0}C perfusion and deleterious effects were observed during assessment of function by <u>in vitro</u> perfusion at 37 ^{0}C. Including mannitol ameliorated the damaging effects to some extent. After addition and removal at 30 mM min^{-1}, kidneys retained a level of function at 37 ^{0}C similar to cold-perfused controls, but reduced in comparison to freshly isolated kidneys.

Using the slow addition and removal technique, Jacobsen (1978) showed that rabbit kidneys perfused with up to 4M glycerol could function after autotransplantation. Characteristic of all these experiments was an increased renal vascular resistance during the deglycerolization process. Further reduction in rates of addition or removal, and increased perfusate colloid did not improve the perfusion characteristics, or post-transplant function (Jacobsen, 1978). Attempts to freeze rabbit kidneys containing 3M glycerol to -80 ^{0}C

(Jacobsen, 1979) were unsuccessful; Fahy (1980) was able to elicit an alpha adrenergic response after storing 3M glycerolized kidneys at -30 ^{0}C, but subsequent studies suggested the kidneys were not viable.

Conclusions which might be drawn from the literature are confounded by different experimental procedures which each investigator has chosen. There does not appear to be ample justification for a shift to glycerol in lieu of Me_2SO as Pegg has suggested is necessary. Karow has shown that Me_2SO does not severely alter kidney ultrastructure or function. Unfortunately, time and temperature conditions in both experiments were very different and a direct comparison of the work of both authors is not possible. The partial successes achieved using Me_2SO supports its use as cryoprotectant; and especially the success reported by Guttman and Toleydo-Pereyra should reinforce belief in Me_2SO as the cryoprotectant of choice. It remains to properly identify where the harmful effects lie and try to circumvent these. To quote Jeske et. al.(1974): "There exists an obvious need to reduce the severity of alterations produced by cryoprotectant perfusion, especially if such alterations predispose the renal parenchyma to an inability to withstand the stresses of cooling to very low temperature and rewarming".

Pegg and co-workers have contributed admirably in the development of methods of glycerol addition and removal with attention to

osmotically induced fluid shifts. This method is not without osmotic trauma; an alternate method is still needed. Similar work using Me_2SO has not been reported; yet it does have advantages. Glycerol is a poor permeator at low temperatures and slowly deglycerolizing may not be adequate since efflux of cellular glycerol is very slow (Dooley, 1980). To date, there is little known about the extent of intracellular permeation of Me_2SO or glycerol; information which may serve to identify optimal procedures for introduction and removal of cryoprotectant.

IX. Assessment of Kidney Viability and Function

Filo (1976) proposed that the hypothermic perfusion step following thawing (Section VII) could serve a dual purpose: (1) The removal of the cryoprotectant ; (2) an opportunity to assess organ viability prior to reimplantation. Assessment of organ function prior to reimplantation would be advantageous in both clinical and experimental applications (Calman, 1974). As the number of potential kidney donors increases, selection of kidneys which will function immediately following transplantation would improve success and reduce economic factors. Experimentally, an *in vitro* test would save both time and animals.

Malinin and Perry (1967) reviewed the different viability tests useful for cells and tissues. These included vital staining, nucleotide incorporation, cell culture, and analysis of the enzyme spectrum. Use of these methods has not resulted in routine application in renal studies for prediction of organ status prior to transplantation. Sell (1967) indicated the unreliability of the tetrazolium dye test. Turner *et. al.* (1967) found oxygen consumption of rat kidneys, following ischemia or freeze-thaw treatment, inappropriate as an index of the functional integrity of the kidney. Total nucleotide levels correlated with survival of rat kidneys following *in vivo* clamping of blood vessels (Calman, 1974); however, it was impossible to distinguish between vascular or metabolic function as the site of lethal damage.

The possibility of utilizing a number of *in vitro* parameters to obtain an "*in vitro* index" of viability was explored by Abbott

(1969). Ten parameters were examined, and the resultant index was useful for delineating tissue slice damage as the result of cryomanipulation. Correlation of this index with whole organ function was not reported. Fahy (1980) found the ability of kidney cortical slices to transport PAH and re-establish cation ratios was a useful indicator of slice viability.

Abouna (1974) compared measurements of twenty parameters during hypothermic perfusion and nine parameters immediately following transplantation of canine kidneys. These potential viability tests were compared between kidneys which functioned following transplantation and those which failed to support life. Of all parameters measured during in vitro hypothermic perfusion, only perfusate flow rate and vascular resistance were significantly different in the two groups. During post-transplant studies, only urine volume and creatinine clearance differed between survivors and non-survivors. It did appear that renal tubular function in kidneys eventually shown capable of supporting life increased during the second clearance period, in contrast to non-viable kidneys.

The problem of employing a simple viability test is the inability to assess the extent of damage to the vascular system as well as the transport functions of the kidney. Sells (1967) described the problem as the need to determine organ transplantability, not viability. In vitro normothermic perfusion is an alternative to transplantation which permits assessment of the functional capacity of the kidney. It is not subject to the problem of diminished metabolic activity during hypothermic perfusion, and retains the

ability to measure vascular integrity. Perfusion of the whole organ at normothermia permits determination of integrated organ function and separation of vascular and tubular impairment.

Utilization of the normothermic, in vitro perfused kidney has contributed to the understanding of renal physiology and pharmacology. It is subject to limitations (Nizet, 1975), and can become a very complicated procedure in certain species. For larger species whole blood is primarily used as the perfusate (Nizet et. al., 1967 ; Waugh and Kubo, 1969 ; Berkovitz et. al., 1968; Conrad et. al., 1973 ; Nizet, 1975 ; Vanherweghem et. al., 1976).

The rat kidney has been used extensively as a model to study in vitro organ function by several groups (Ross et. al., 1973 ; 1975 ; Shurek et. al., 1975 ; Merkens et. al., 1978 , Trimble, 1979 ; Cohen et. al., 1980 ; Maack, 1980). Although function was impaired in comparison to in vivo values (deMello and Maack, 1976 ; Maack, 1980), a considerable and acceptable level of function using cell-free balanced salt solutions with bovine serum albumin (BSA) as colloid was obtained. Ross (1972) suggested the usefulness of this preparation to study methods of renal preservation. The method is rapid; in contrast to survival experiments, where two or three workers may require several days to accomplish an experiment, a single investigator could perform a similar experiment in one day with an isolated preparation. One could follow a number of parameters using the isolated kidney; a survival experiment offers no extra information and could render reasons for

success or failure difficult to determine. The use of smaller animals would lessen genetic variability at less cost. Finally, similar studies would be relatively inexpensive in comparison to larger animals which utilized reimplantation for the assessment of different treatments. This method has been applied to the problem of development and analysis of different flushing solutions used in cold storage of kidneys (Bishop and Ross, 1978 ; Marshall et. al., 1978 ; Ross et. al, 1979 ; Jablonski et. al., 1980).

The rat perfusion model is subject to limitations. Techniques of cannulation and organ retrieval are difficult due to the small size. The potential for future transplantation as the ultimate test of function is limited. The small size may not be applicable to cooling and thawing problems associated with larger organs. The rabbit kidney offers advantages similar to the rat with the added benefits of ease of handling and greater size. Physiological studies with this model are rare, and, as in the rat, few reports of its use for preservation studies are available.

Rabbit kidney in vitro perfusion with whole blood was described by Rosenfeld and Sellers (1960). In vitro . kidney function was within the range reported for in vivo studies. Levin et. al.(1965) compared whole blood, washed red cell, and acellular electrolyte perfusates for in vitro rabbit kidney perfusion. Washed red cells and electrolyte perfusates resulted in low rates of glomerular filtration and high perfusate flow rates. Renal function with whole blood as perfusate was approximately 1/4 to 1/2 that observed in vivo ; the other perfusates were

inferior. Hollerman and Malinin (1970) perfused rabbit kidneys with a balanced salt solution. They found a rapid deterioration of renal function which they believed was the result of changes in vascular patency and permeability. They noted red blood cells trapped in capillaries and blockage of glomerular tufts by material deposits.

Fonteles et. al. (1973) perfused rabbit kidneys at 37 °C with solutions rich in potassium and magnesium and with Tyrode solution. Initial vasospasm was blocked by including isoxsuprine in the perfusate. Flow rates were low, with high renal vascular resistance. Including BSA and dextran resulted in higher flow rates, but no urine production. Glomerular filtration rates (GFR) were low in all groups. Sodium reabsorption averaged 50% of the filtered load ; actual sodium transport was low.

Ultrastructural evaluation of the kidney cortex after perfusion correlated with the physiological function reported above (Jeske et. al., 1973). After two hours of perfusion with potassium-magnesium rich solution, approximately half the proximal tubules were necrotic, the remainder exhibited vacuolization. Following perfusion with Tyrode solution, nearly all the proximal tubules were severely altered. The vascular system remained normal. This system was used to study the effects of Me_2SO on renal function at normothermia (Karow and Jeske, 1976) as described in Section VIII .

Pegg and co-workers have contributed significantly to the development of the in vitro rabbit kidney perfusion assay. Earlier work was focused on the maintenance of stable perfusion characteristics .

Pegg and Farrant (1969) perfused rabbit kidneys at 37°C with solutions similar to rabbit plasma electrolyte concentrations. Dextran or PVP (6% w/v) was included as the source of colloid. PVP was not as effective as dextran in the control of edema. Increasing concentrations of dextran resulted in proportional decreases in renal vascular resistance, but dextran was incompletely retained by the vascular system and evidence suggested a damaging effect of dextran on the vascular endothelium. Further evidence of dextran-induced damage was reported by Pegg (1970). Dextran 70 was not more effective than dextran 40; BSA (6% w/v), or mixtures of BSA and dextran (2% and 4% respectively) prevented edema to a greater degree. It was concluded that the use of dextran as the sole colloid source should be avoided. The use of BSA as a colloid for rabbit kidney perfusion was a problem as well. When attempting to perfuse kidneys hypothermically, Pegg and Green (1972) noted that several kidneys performed poorly. The reason was traced to variation between batches of BSA.

Pegg (1971) examined other parameters of importance for the maintenance of acceptable perfusion characteristics at 37°C. These results indicated that preliminary blood washout should be as complete as possible to avoid the effects of residual red blood cells and platelets; a filter of 1μ pore diameter should be included in the circuit, and bubble oxygenators avoided. Kidneys perfused for one or two hours at 37°C with a similar perfusate containing BSA and dextran were reimplanted (Pegg and Green, 1973). Survival of kidneys perfused for one hour was good; poor survival was obtained

after 2-hour perfusion. A characteristic of these reports was low perfusion pressure and high colloid concentration. Physiological function of tubules was not examined in vitro.

Fuller et. al. (1977) studied the physiology of rabbit kidneys perfused with different colloids at 37 ^{0}C and 100 mmHg pressure. Kidneys perfused with 5% BSA, 5% Dextran 70, or a mixture of 5% Dextran 70 and 0.7% BSA (w/v) exhibited low GFR and large amounts of protein leakage at the glomerulus. Electron microscopic evaluation revealed extensive damage to the capillary endothelial lining. Perfusion with 3% HES or 3% Pluronic F108 yielded higher GFR and lower albumin leakage. HES produced extensive lesions similar to the dextran-perfused kidneys, therefore Pluronic F108 was chosen for further study. Perfusate flow rates were high (13.3 ± 0.5 ml $min^{-1}g^{-1}$) after 1 hour of perfusion, erasing the corticomedullary concentration gradient. Oxygen delivery was adequate to maintain the ATP/ADP ratio close to values obtained for control kidneys. Glucose reabsorption increased to 80% of the filtered load during the first hour of perfusion. Sodium reabsorption increased to 67%, but decreased significantly during the second hour. The sodium load transported remained constant during the second hour.

Wusteman (1978) examined the effects of other colloids, HES, Dextran 150, and Haemaccel on kidney function using the same system to compare function with Pluronic F108. Dextran 150 produced

higher GFR than Haemaccel or Pluronic F108. Protein leakage decreased during the first hour of perfusion, but remained high with HES. Sodium and glucose reabsorption rates were depressed in comparison to *in vivo* studies, and decreased with time. Overall, Dextran 150 was considered to be the most satisfactory source of colloid.

The usefulness of this assay system was demonstrated by Fuller and Pegg (1976). They compared the different methods of rabbit kidney hypothermic storage procedures by functional assessment during normothermic perfusion. The results of this experiment were in close agreement with conclusions drawn from transplantation studies.

Wusteman (1977) established the reliability of the system by studying the sensitivity of the preparation to ischemically-induced damage. Comparing function of freshly isolated control kidneys to groups which received varied ischemic periods showed that damage was easily detectable. The extent of impairment of renal handling of sodium and glucose by 60 or 90 minutes of ischemia was distinguishable.

The rabbit kidney *in vitro* normothermic perfusion assay described above has proven fruitful in studies of preservation. Pegg and Wusteman (1977) were able to delineate a procedure which allowed the introduction and removal of 2M glycerol at 5 ^{0}C. A subsequent study (Jacobsen *et. al*, 1978) proved that the method of glycerol treatment predicted to be optimal by *in vitro* perfusion allowed kidneys to support life after transplantation - providing further support of the predictive ability of this assay system.

MATERIALS AND METHODS

I. Kidney Retrieval

a). Animals: New Zealand White Albino rabbits weighing between 2.5 and 3.5 kg were obtained from a local breeder (Berci's Farm Reg'd, Athelstan, Quebec). The animals were housed in stainless steel metal cages (63.5 x 45.7 x 39.3 cm) in an isolated room maintained at 18 ^{0}C, 12 hours light and 12 hours dark. Food (Supersweet Rabbit Chow, D.N. Racine Inc., Athelstan, Quebec) and water were supplied ad libitum. The animals were housed under these conditions for a minimum of three days prior to use as donors.

b). Preparation for Surgery: The rabbits were placed in a restraining box and brought to the surgical preparation area. A preanaesthetic (0.8 ml/kg body weight) containing 75 mg/ml ketamine (Ketaset, Rogar STD, London, Ontario) 6.25 mg/ml acepromazine maleate (Atravet, Ayerst Laboratories, Montreal, Quebec) and 0.004 mg/ml atropine (Sterlab, Downsview, Ontario) was administered intramuscularly.

The rabbit remained in the restraining box for 10 minutes following the injection and before handling. The abdomen was shaved and the animal brought to the operating room.

c). Anaesthesia: The rabbit was placed on the operating table in the supine position. General anaesthesia was induced with a halothane (Fluothane, Ayerst Laboratories, Montreal, Quebec) and oxygen mixture administered through a transparent self-seal small animal mask. The anaesthetic circuit was a semi-closed system assembled in our laboratory using a Fluotec Mark 2 vaporizer (Cyprane Ltd., Keighly, England) and small bore tubing. Initially the mixture was adjusted to administer 2% halothane in 0_2 which was subsequently decreased to 1% after the incision was made.

d). Intravenous (I.V.) Administration: A 21 guage (0.8 mm) butterfly infusion set (Abbott Laboratories, Montreal, Quebec) was placed in a prominent ear vein and secured with adhesive tape. A solution of 0.85% NaCl (Abbott Laboratories, Montreal, Quebec) was attached and administered at a slow rate to maintain patency. Approximately 15 minutes prior to removal of the kidney the animal received 2 g (10 ml of a 20% w/v solution) of mannitol. Five minutes prior to kidney ligation, heparin (1000 IU/kg) was injected followed by a saline bolus (10 ml/kg) to promote diuresis.

e). Kidney Isolation: A midline skin incision approximately 20 cm long extending from sternum to pubis was made with a number 15 scalpel blade. The wall of the peritoneum was nicked in the

midline and the incision extended with a scissors. The intestine was deflected to the side and placed in a plastic bag filled with warm saline. The perivascular tissue of the renal pedicle was infiltrated with 0.5 ml of a solution containing 15 mg/ml papaverine HCl (Charles E. Frosst and Co., Kirkland, Quebec) and 5 mg/ml lidocaine hydrochloride (Xylocaine 1%, Astra Pharmaceuticals, Mississauga, Ontario), administered with a 26 gauge needle attached to a 1 ml tuberculin syringe.

The ureter was dissected free of connective tissue approximately 5 cm from the point of entry into the bladder. A length of 2-0 silk ligature was placed under the ureter and tied loosely. A small nick in the ureter exposed the lumen.

The ureter cannula was a modified teflon catheter of a 16ga (1.7 mm) or 18ga (16 mm) x 3.81 cm intravenous catheter placement unit (Angiocath, Deseret Inc., Sandy,Utah). The plastic hub was removed and the catheter was gently shaped into an elongated "S" without kinking. The shape was maintained with tape. The taped catheter was placed in hot (80-90 ^{0}C) water for 2 minutes, and immediately transferred to the freezer (-20 ^{0}C) for 2 minutes. The tape was removed after rewarming and the cannula maintained its shape. The tapered end of the cannula was introduced into the lumen and the ligature was tightened securely around the ureter and cannula.

Isolation of the renal vasculature was accomplished with minimal disturbance to the kidney. The fat and connective tissue surrounding the artery and vein were teased away with a gauze pad and fine forceps. A loose ligature (2-0 silk) was placed around

the artery 1.5 cm from the point of entry into the hilus. The ligature around the vein was positioned distal to the adrenal vein. The heparin and saline were administered and urine flow observed for five minutes to allow recovery from handling during the isolation.

f). Kidney Extirpation: The kidney was removed as quickly as possible to minimize ischemia. The arterial ligature was tied and the artery severed distally. The procedure was repeated for the vein. The kidney was freed from the surrounding tissue and lifted from the body cavity. The arterial lumen was exposed with forceps and the cannula placed in the artery. The cannula used was an etched teflon vessel tip (model T-418,419 or 420, Extracorporeal Specialties, Pa.) of varied interior diameter (1.6, 1.5 or 1.4 mm) depending on the size of the arterial lumen. The non-tapered end of the vessel tip was fitted into the syringe attachment adaptor of a 20" extension set (#4429, Abbott Laboratories, Montreal, Quebec) and secured with silastic adhesive (Dow Corning, Mississauga, Ontario). The tubing from the bottle containing the oxygenated washout solution was attached and the kidney flushed by gravity. During the flush the arterial cannula was secured with 2-0 silk. The kidney was suspended over a collection dish until the washout was complete.

If the contralateral kidney was to be used, the body cavity was inspected for bleeding. The intestine was placed in the body cavity, the skin flaps closed and the halothane reduced to 0.5% concentration. Removal of the contralateral kidney was achieved in exactly the same manner as described. The animal was sacrificed with a saturated KCl solution.

II. The Normothermic (37°C) Perfusion Circuit

A. Circuit Design and Construction

a). Introduction: The circuit for 37°C perfusion of the rabbit kidneys is outlined in Figure 2. This system was designed and constructed in our laboratory with the exception of the organ chamber, return funnel, and heat exchanger. With this system perfusions could be run under either constant pressure or constant flow. The temperature of the perfusate was maintained at 37°C by a circulating water bath (Forma Scientific Model 2095 FCR) with the capacity for heating and cooling and a variable flow rate. A tank of 95% O_2: 5% CO_2 (Union Carbide, Montreal) served as the source of oxygen and to maintain the pH of the perfusate at pH 7.4. Temperature of the perfusate was monitored using a small animal esophageal probe placed in the circuit and connected to a telethermometer (Yellow Springs Inst., Yellow Springs, Ohio). Pressure was monitered using a Statham P 23Db strain guage transducer and recorded on a Beckman Dynograph S Recorder (Beckman Instruments, Montreal, Quebec).

The perfusate was pumped from the reservoir by a Watson Marlowe MHRE-200 roller pump (Fred Dungey, Toronto, Ontario). It flowed through a series of filters arranged in parallel, and into the oxygenator. From the oxygenator the perfusate flowed through the heat exchanger and into the bubble trap before flowing into the renal artery. Pressure and temperature were monitored at a point after the perfusate left the bubble trap just before entering the renal artery. Perfusate which exited the vein was funnelled into the

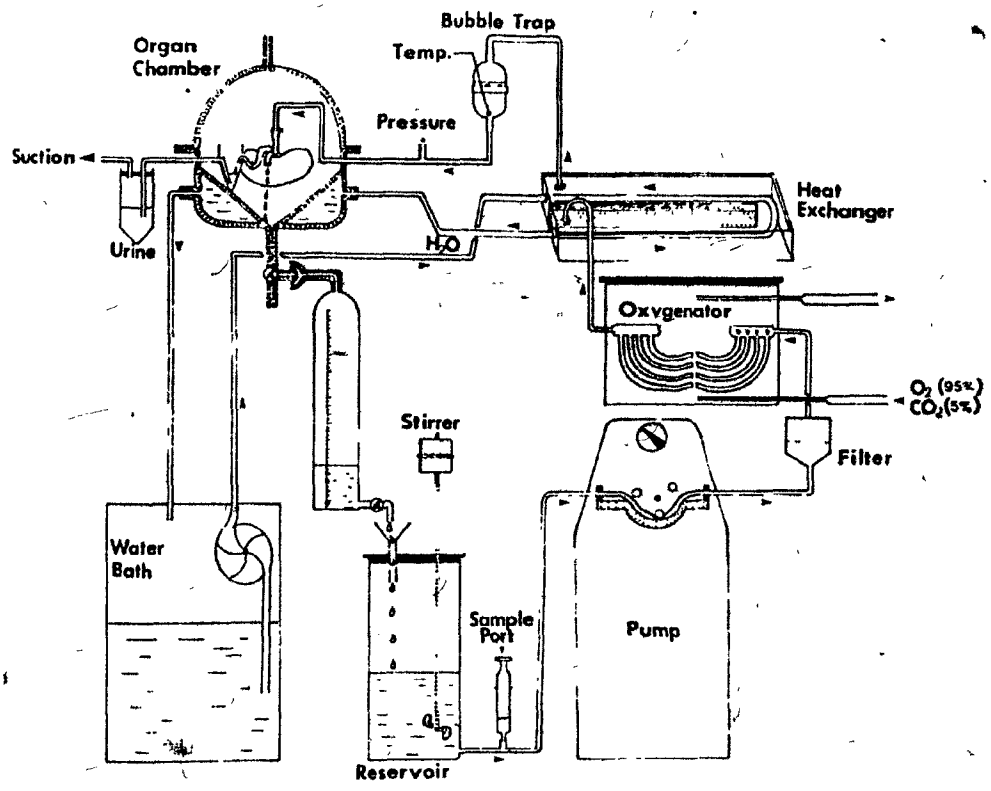

Figure 2. Diagram of the 37°C perfusion circuit.

return column and returned to the reservoir. Perfusate samples were drawn from the sampling port between the reservoir and the pump such that pressure was not altered during perfusion. Urine produced was aspirated from a small collecting vessel inside the organ chamber and collected in a larger vessel. A detailed description of each element of the system follows.

b). Perfusate Reservoir: The reservoir (Figure 3) was constructed using a plexiglass base 17.5 cm x 7.5 cm x 0.4 cm thick. A 10 cm length of plexiglass tubing (O.D.= 14.7 cm, wall thickness = 7 mm) formed the outer reservoir. A 23 cm length of tubing (O.D.= 8.4 cm, wall thickness = 7 mm) formed the inner reservoir. The two lengths of tubing were glued to the plate with methylene chloride ethylene dichloride ethyl acetate (K- lux, KSH Inc, St. Louis, Missouri) forming an inner and outer reservoir. Two right angled connectors from a Mox-100 organ perfusion cassette (Waters Instruments, Minneapolis, Minnesota) were fitted to the bottom of the base plate to serve as reservoir outlets.

The connectors were joined to the syringe attachment end of a 50.8 cm extension set by short lengths of 6.35 mm O.D. polyethylene tubing. The extension set tubings were joined into a common line with a 3.17 mm Y-connector (Nalgene, Canlab, Montreal, Quebec).

The slide clamps which were furnished with the extension sets alternately closed and opened the reservoirs. The common line was connected to the sampling port assembly.

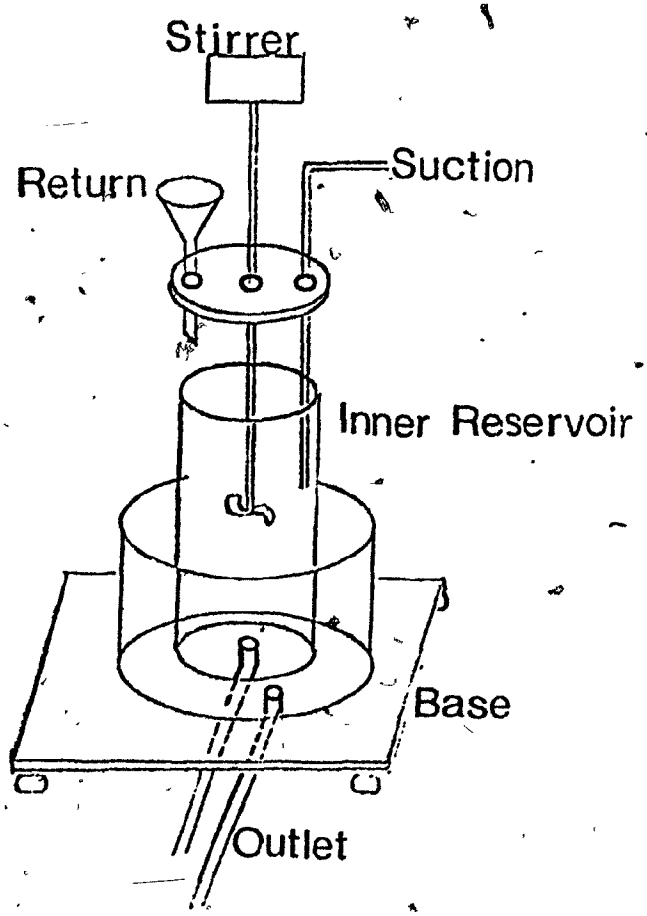

Figure 3. Diagram of the perfusate reservoir assembly for the 37^0 perfusion circuit.

c). Sampling Port: Perfusate was pulled through a 6.35 x 6.35 mm straight connector with a side luer lock. Two 3-way stop cocks were fitted together such that both syringe attachment sites were facing upwards(Figure 4). One stopcock was fitted into the side luer lock of the straight connector. Two 10 ml plastic disposable syringes were attached to the stopcock. One served to draw the perfusate sample from the main circuit; the other had the piston removed and the barrel served as a small reservoir to pipette the sample. By opening the entry to the circuit, a sample was withdrawn. Closing the stopcock such that the luer side was shut opened the connection between the two syringes and allowed the sample to be pushed into the small reservoir where it was pipetted. Any perfusate remaining could then be returned to the main circuit in the opposite manner.

d). Filters: From the straight connector the perfusate was pulled to the pump. At this point a length of silastic tubing 6.35 mm O.D. and 20 cm in length was interposed and used in contact with the pump rollers. The flow was then channelled into 4 lines and pushed through four 47 mm diameter filter holders (Swinnex 47 mm, Millipore, Mississauga, Ontario) which contained Gelman type AE glass fiber prefilters (Gelman Instruments, Montreal, Quebec). The entrance and exit ports of the filters were connected into the circuit with the luer attachment of the 50.8 cm extension set which formed a tight connection, but was easy to remove.

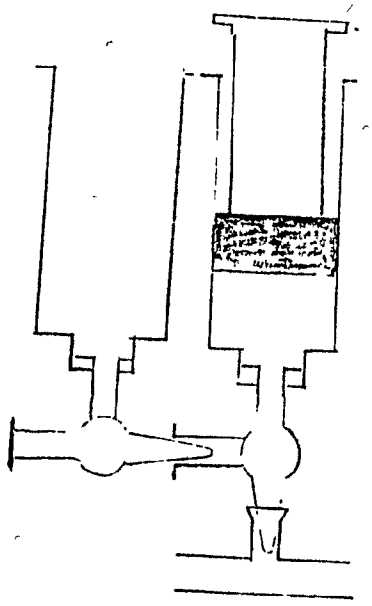

Figure 4. The sampling port assembly for the 37°C perfusion circuit.

e). Oxygenator: After flowing through the filters, the four perfusate channels were collected into one channel which entered the oxygenator. The container used for the oxygenator was a 0.66 ℓ clear plastic food saver with hermetic pressure seal. Two holes (5 mm diameter) were drilled in the wall approximately 2 cm from the top and opposite each other. Two stainless steel tubing manifolds with inlet diameter of 5.16 mm and 4 outlets of 2.41 mm (TCM-6-13/4, Small Parts Inc., Miami, Florida) fit snugly into the holes such that the outlets (2.4 mm) were inside the vessel suspended by the inlet tube (5.16 mm). The four small tubes were connected to their counterparts by four 3.6 m lengths of silastic medical grade tubing, I.D. 1.473 mm, O.D. 1.956 mm (No.602-235, Dow Corning Corp.,Michigan). The lengths of silastic tubing were coiled and placed in the container. In this way flow from the filters entered the first manifold, was split into four channels of silastic tubing and collected into one channel by the second manifold as it exited the oxygenator. At the top and bottom of the oxygenator vessel two small holes were drilled into which Radiopaque 14 ga I.V. teflon catheters 6.4 cm in length (Abbocath T, Cat. No 4534-14, Abbott, Chicago, Illinois) were inserted and the hubs glued to the vessel with epoxy glue. The gas mixture of 95% O_2: 5% CO_2 flowed into the oxygenator through the lower catheter and exited through the top catheter.

f). Heat Exchanger: Perfusate flowed through a short length of 6.35 mm tubing into the heat exchanger. This was a countercurrent exchange system taken from a Mox-100 organ perfusion cassette.

g). Bubble Trap: The warmed, oxygenated perfusate from the heat exchanger entered the bubble trap. This was constructed using a blood pump from a hemoset (No. RX 171054 (3), Abbott Laboratories, Montreal, Quebec). The blood pump was fitted with an outlet to facilitate filling, and a Yellow Springs small animal esophageal probe (model 402 A-41915-A, Yellow Spring Instrument Co., Yellow Springs, Ohio) with the junction situated at the exit. The bubble trap was connected to the arterial cannula assembly of the organ chamber. A T-connector 3.17 mm O.D. lead to a Statham P 23Db strain guage transducer to moniter the perfusion pressure prior to entering the organ chamber and renal artery.

h). Organ Chamber: The organ chamber and return column was designed in our laboratory. A plastic model was used initially for testing and the final product was made of glass by a local glass blower (Kado Glass Co., Montreal, Quebec). The chamber consisted of a funnel surrounded by an outer water jacket. The diameter at the top was 15 cm. The total height was 10 cm. The kidney was suspended by the renal artery over the centre of the chamber. Venous effluent dripped to the bottom of the funnel, which was fitted with a 2-way teflon stopcock. If the perfusate was to be recirculated it flowed into a graduated column which could be closed to determine the perfusate flow rate. The return column capacity was 100 ml. With the stopcock in the open position the perfusate returned to the reservoir and was recirculated.

i). Urine Collection Assembly: A small polypropylene centrifuge tube was attached to the inlet on the opposite side of the arterial inlet of the organ chamber. The ureter cannula was placed in the small hole in the tube at the 1.5 ml level. Urine dripped into the tube and was aspirated by vacuum from the organ chamber into a larger collection tube outside the chamber.

B. Operation of the 37 ^{0}C Circuit

a). Initial Manipulation: Prior to preparation of the donor, the apparatus, which had been previously cleaned and dried , was assembled. Filters were placed in the filter holders. The pressure transducer and line linking it with the perfusion circuit were filled with saline. The circulating water bath was turned on with the circulating pump valve closed. The recorder was turned on and the calibration verified. Syringes were fitted into the sampling port assembly. The slide clamp from the outer reservoir chamber was closed and the inner chamber opened. The pump segment of silastic tubing was placed on the roller stage.

b). Priming the Circuit: The top of the inside reservoir was removed and the perfusate poured in. The top was replaced and the stirrer motor turned on . The perfusate was stirred for 2-3 minutes. The pump was activated and set at low speed to draw perfusate from the reservoir and to fill the circuit. The course of the perfusate was observed to ensure that all filter holders were filled and devoid

of air bubbles and that all four channels of the oxygenator system were operating. If one oxygenator channel was not operating, the most probable cause was a kink in the silastic tubing. An increase in the flow of the pump overcame the resistance to flow. The heat exchanger was filled by alternately tilting from horizontal to force air out and into the bubble trap. The bubble trap was filled to half-volume by opening the air intake and clamping the outlet. At this point the valve between the tank and oxygenator was opened and the oxygenator was continuously flushed with 95% O_2: 5% CO_2 at 2 ℓ /min. The circulating water bath temperature was set to 43 ^{0}C. The circulating pump valve was opened to half-maximal flow rate. The water flowed through the heat exchanger in the direction opposite to the perfusate flow rate. It then flowed into the water jacket surrounding the organ chamber and returned to the bath. Any additions to the perfusate (specifically the H^3- inulin) were made after inspection for leaking connections. The perfusate was circulated at 40 ml/min to allow equilibration of O_2, CO_2, pH and temperature for a minimum of 15 minutes before attachment of the kidney.

c). Kidney Attachment: While the kidney was being flushed, the pump speed was decreased. The renal arterial cannula was plugged into the arterial outlet. The artery was examined closely to ensure it was not twisted and that the kidney was hanging freely from the artery. The ureteral cannula was hooked into the collection vessel, and the ureter was positioned to avoid twisting or occlusion of the lumen by the cannula.

The pressure was continuously monitored as the flow rate was slowly increased to the desired perfusion pressure. The temperature was maintained at 37 ^{0}C by (1) varying the flow rate of the water through the heat exchanger; (2) increasing or decreasing the temperature of the bath or (3) turning on the compressor to cool the bath. The organ chamber cover was replaced. The perfusion continued for 10 minutes to allow kidney stabilization. The pressure was maintained constant by varying the speed of the roller pump. The urine was aspirated into a collecting vessel.

d). Kidney Perfusion: After the initial stabilization period, the initial flow rate was determined by closing the one way teflon stopcock at the bottom of the return cylinder and measuring perfusate venous effluent flow for a one minute interval. The flow rate and pressure at that point were recorded. The urine collection vessel (outside the chamber) was changed and the 15 minute collection period commenced. The pressure and temperature were constantly monitered and controlled. At the midpoint of the collection period a 5 ml sample was drawn from the sampling port, pipetted into a prelabelled test tube and sealed with parafilm. Two minutes prior to the completion of the collection period, the venous effluent flow rate and corresponding pressure were recorded. At the end of the collection period the collecting vessel was replaced and the next period began without interruption. The urine vessel was capped, mixed and the volume recorded. A 10 ml aliquot was set aside for biochemical determinations.

C. Cleaning Procedure

After completion of the perfusion, the kidney was removed. The outer reservoir chamber was filled with normal saline. The inner chamber medium was aspirated into a liquid radioactive disposal can. The slide clamp to the inner chamber was closed and the outer chamber slide clamp opened. The saline was pumped through the system without recirculation, collected in the inner reservoir and aspirated into the radioactive can. If the perfusion circuit was to be used immediately for the next experiment, one litre of saline was pumped through the system in this manner. The outer reservoir was emptied by the pump and air was pumped through to flush the saline from the circuit. The circuit was then primed as described.

If the circuit was not used immediately afterwards, the saline wash was followed with a wash of 2 litres of distilled H_2O and flushed with air. The filter holders were opened, the filters discarded into a radioactive waste can, and the holders reassembled. The outer chamber was filled with a strong alkali dishwashing detergent in distilled H_2O. The solution was circulated without aspiration until the entire system including the organ chamber was filled. The inner reservoir was then opened and the solution was recirculated at high speed for 30 minutes. The pump was stopped and the detergent solution was left in the circuit. If the circuit had previously been used for five experiments, the apparatus was emptied and dismantled for scrubbing and rinsing of the individual components with distilled H_2O under pressure. The apparatus was air dried and reassembled for use. If not, the

detergent was emptied and the circuit flushed with 10 litres of distilled H_2O at high speed without recirculation. A vacuum source was attached to the arterial line and the system was left to dry.

III. The Hypothermic (10 °C) Perfusion Circuit

A. Circuit Design and Construction

a). Introduction: This system was designed for hypothermic perfusion at 10 °C with multiple reservoir access and provision for the addition of cryoprotective agent. The circuit for hypothermic perfusion is outlined in Figure 5. The pressure was maintained constant at 60 mmHg by establishing a column of perfusate over the kidney at a fixed height. Perfusate was either recirculated or flowed once through the kidney. Cooling was achieved with a circulating bath identical to the one used for the 37 °C perfusion circuit. A mixture of water and ethylene glycol was continuously circulated through the outer jacket of the condenser and through the outer chamber of the organ chamber. Perfusate was pumped through filters up to the condenser where it was cooled. After the condenser the perfusate flowed into a bubble trap and into the renal artery. The venous and ureteral effluent flowed through a graduated column and back to the reservoirs.

b). Perfusate Reservoirs: The reservoir system was designed to accommodate up to 3 reservoirs, but could be readily adapted to accommodate more. The reservoirs were arranged to facilitate switching from one to the other without interruption of flow to the kidney; and to enable the aspiration of perfusate from one reservoir while collecting with a second if the perfusate was not recirculated, in order to remove cryoprotectant progressively. Saline glass bottles (500 ml) were fitted with two-hole No 6 rubber stoppers. A 1 ml plastic pipette was placed in one hole and pushed to the bottom of the bottle. The

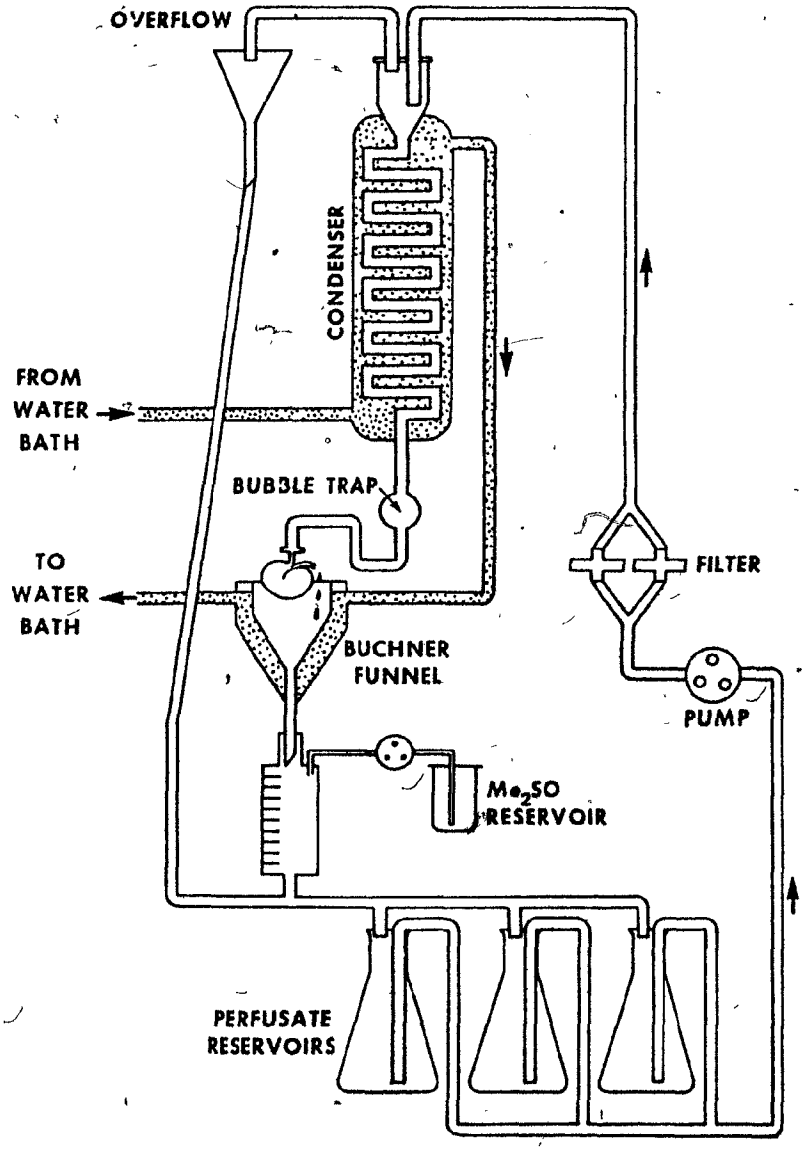

Figure 5. The 10°C perfusion circuit.

male adapter of a venotube extension set was forced halfway down the second hole from the top of the bottle. The protruding end was connected to a 6.35 mm T-connector with a length of 6.35 mm O.D. tubing. The female adapter of the extension set was trimmed to 6 cm of tubing length. This was forced into the second hole of the stopper from the bottom and locked into the male adapter. I.V. tubing from the extension set with a slide clamp was forced over the top end of the pipette. Three reservoirs were joined together by T connectors. The aspirator tubes were joined with 3.17 mm Y connectors into a single line. Day Pinchcocks were used to close the return tubing at each T connector; slide clamps regulated the aspiration tubes. A separate reservoir placed outside the circuit was used for the addition of dimethylsulfoxide (Me_2SO). The reservoir was identical to that used for perfusate, but the return system was absent.

c). Pumps: The pump circulating the perfusate to the top of the condenser was similar to that used in the 37 ^{0}C circuit (Watson Marlow MHRE 200). The speed did not control the pressure or flow as before; since the amount of perfusate delivered to the condenser was maintained at a rate which allowed sufficient overflow of perfusate to ensure proper mixing and maintained a constant pressure head over the kidney. When the reservoir was changed, or in a non-recirculating mode, the pump speed was adjusted to equal the flow through the kidney, thus maintaining the level in the condenser without loss of perfusate through the overflow.

The Me_2SO was delivered into the graduated burette with a Gilson

Minipulse II precision volume roller pump (Gilson, France).

d). Filters: The perfusate was pushed through two parallel 47 mm prefilters. The filter holders, filters and connectors were similar to those included in the 37°C circuit.

e). Condenser Assembly: The top of a Graham Coil-Type pyrex condenser (jacket length = 500 mm, Canlab, Montreal, Quebec) was fitted with the rubber stopper from an intravenous drip bottle. Both a 1 ml and 5 ml plastic pipette were shaped into a U by gentle heating and forced into the stopper. The I.V. tubing from the filters was connected to the 1 ml pipette. The larger pipette served as an overflow tube which emptied into a polypropylene funnel. The funnel was suspended beneath the opening of the overflow tube. A length of tubing 9.52 mm O.D. connected the funnel to the return line at the point where the graduated burette emptied into the reservoirs.

f). Bubble Trap: The bubble trap was identical to that described for the 37°C perfusion circuit. It was connected to the condensor by a 12.7 mm x 6.35 mm connector. A glass laboratory thermometer was placed in the bubble trap chamber and sealed to the wall with millipore M.F. cement (Millipore, Mississauga, Ontario).

g). Organ Chamber: The kidney was suspended by the renal artery in a double-walled Buchner funnel (81 mm O.D.). A wide stem (17 mm) glass filling funnel (75 mm O.D.) with the stem shortened

served as a cover. The stem of the Buchner funnel was attached to the graduated burette by a 12.7 mm x 6.35 mm connector.

h). Graduated Column: Venous and ureteral effluent from the Buchner funnel flowed into a 100 ml calibrated burette. The burette was adapted from a graduated precision volume I.V. set (soluset 100 x 60, Abbott Montreal, Quebec). The diaphragm was removed through a small opening made in the wall of the burette which was resealed with millipore MF cement. The drip chamber and microdrip outlet were removed and a 6.35 mm O.D. tubing fitted in place. This tubing was then joined with the overflow to the reservoirs. A 14 ga teflon catheter with hub was inserted into the injection site of the burette. Me_2SO was pumped into the circuit at this point.

B. Operation of the 10 ^{0}C Circuit

a). Priming the Circuit: Prior to attachment of the kidney to the system, the circulating water bath was cooled to the desired temperature. In this system maintaining the bath temperature at 8 ^{0}C was sufficient to cool the perfusate to 10 ^{0}C with the circulating valve opened to maximum. The bottle containing perfusate was attached to the stopper of the return system and the pipette pushed to the bottom. This reservoir was isolated by the appropriate location of pinchcocks and slide clamps. The pump was turned on and perfusate was pushed through the filters and into the condenser. A screw clamp at the point of arterial cannula attachment was closed to decrease

the flow rate through the condenser and allow it to fill completely. The excess perfusate then exited through the overflow and returned to the reservoir. The bubble trap was inverted and filled to half-volume to cover the thermometer bulb. The perfusate was recirculated for 10 minutes to equilibrate at 10 ^{0}C.

b). Kidney Attachment: The kidney was isolated, cannulated, and flushed as described. After the washout period, the arterial cannula was connected to the circuit and the kidney was suspended by the artery in the Buchner funnel. The glass funnel was lowered to close the chamber. The ureter was left to drip inside the funnel. The screw clamp was opened completely to allow maximum flow. The pump speed was adjusted to ensure sufficient flow through the overflow to maintain the pressure constant and mix the perfusate.

c). Kidney Perfusion: The accessory reservoir containing the cryoprotectant solution was attached and the Gilson Minipulse II turned on to fill the tubing up to the point of entry into the return column. When the additions were to begin, the pump was restarted.

If the perfusate from the main reservoir was changed, the new media contained in a 500 ml bottle was placed in position and the aspiratory pipette lowered to the bottom. The outlet tube from the previous reservoir was clamped and the tube of the new reservoir opened. The speed of the pump was adjusted to maintain the column of perfusate with minimal loss through the overflow. When a sufficient volume of perfusate from the second reservoir had been circulated and collected

in the 1st reservoir, the return clamps were rearranged to allow recirculation. The pump speed was increased to allow mixing by increasing circulation through the overflow.

Timed flow collections were made by clamping the return line at a point above the junction with the overflow line. The venous and ureteral effluent flow rate was measured in the burette, while the overflow returned to the reservoir.

C. Cleaning Procedure

The cleaning procedure was similar to that described for the 37°C circuit, however the circuit was not disassembled for cleaning. If the system was not used for extended periods of time it was left filled with detergent solution. The entire circuit was rinsed with distilled water and a vacuum source was attached to the arterial line to dry the system. The reservoirs were removed and washed with normal laboratory glassware.

IV. Media Preparation

A. Perfusion Media for Assessment of Kidney Function at 37°C

All media for perfusion were prepared with double glass distilled deionized H_2O ($dd\ H_2O$). The salts used in preparation were of analytical quality whenever they could be obtained. The formulations were weighed using an analytical balance accurate to 0.5 mg.

The media formulations used in the experiments are shown in Tables 1 and 2. These formulations were used for the 37°C perfusion circuit to test kidney function. Individual variations in the formula are described, but did not differ in salt concentrations.

The perfusion medium was prepared in batches of 10 or 20 litres. In most cases 4 stock solutions of 10X concentration were prepared as follows: Solution 1: $CaCl_2$, $MgCl_2$, Glucose, Phenol Red: Solution 2: NaCl, $NaHCO_3$, Inulin; Solution 3: K_2HPO_4, KH_2PO_4, KCl, Lactate. The fourth solution depended on the final formulation. If Bovine Serum Albumin (BSA) was included the BSA (Sigma Chemical, St. Louis, Missouri) was layered over ddH_2O in a large beaker and stirred slowly to prevent foaming. If dextran was included, it was treated in a similar manner to BSA. Lactate was weighed out into a 100 ml beaker and diluted with ddH_2O. The Na salt was prepared by titration with 5N NaOH to pH 7.4 before adding to solution 3. Butyrate was treated in the identical manner as lactate. Inulin was dissolved in 250 ml ddH_2O by warming to 40°C with constant stirring. Once the inulin was completely dissolved, it was added to solution 2.

The solutions were blended together in a 12 ℓ boiling flask in the following exemplary sequence: 2000 ml ddH_2O, 1000 ml Solution 1, 1000 ml ddH_2O, 1000 ml Solution 2, 1000 ml ddH_2O, 1000 ml

Table 1: Medium composition for normothermic perfusion (grams per litre of perfusate).

Component g/l	Experiment 1	Experiment 2	Experiment 3	Experiment 4,5,7
$CaCl_2 \cdot 2H_2O$	0.1470	*	*	*
$MgCl_2 \cdot 6H_2O$	0.0441	*	*	*
$NaHCO_3$	2.1003	*	*	*
KH_2PO_4	0.0381	*	*	*
K_2HPO_4	0.1463	*	*	*
NaCl	7.3050	*	*	*
KCl	0.2416	*	*	*
Inulin	2.0000	*	*	*
Inulin (methoxy-^3H) µCi	20µCi	*	*	*
Glucose or	1.0	1.00	1.0	1.0
Mannitol	--	1.01	--	--
Lactate (88.6%)	--	0.0508	0.0508	0.0508
or Butyrate	0.4405	0.4405	--	--
Dextran	30.0	--	--	--
BSA 0%	--	--	0	--
0.5%	--	--	5.0	5.0
1.0%	--	--	10.0	--
Insulin	100 I.U.	100 I.U.	100 I.U.	100 I.U.

*Concentrations did not vary between experiments.

Table 2: Medium composition for normothermic perfusion (milliequivalents or millimoles)

Component	mEq/l	mM
Na^+	150	
K^+	5.2	
Cl^-	130.8	
HCO_3^-	25.0	
HPO_4^{2-}	1.68	
$H_2PO_4^-$	0.28	
Ca^{2+}	2.0	
Mg^{2+}	0.6	
Glucose or Mannitol		5.56
Lactate or Butyrate		5.0

Solution 3, 1000 ml ddH_2O, 1000 ml Solution 4 and 1000 ml ddH_2O to a final volume of 10 ℓ. An aliquot of perfusion medium was taken for determination of osmolality , sodium and potassium. The medium was sterilized by pressure filtration through a glass fibre prefilter, 0.45 μ and 0.22 μ membrane filters using a small batch filtration system (Gelman Instruments, Montreal, Quebec). The filtered medium was stored in sterile bottles at 5^0 C until use.

Prior to addition of the perfusate to the circuit, the desired volume of medium was measured in a volumetric flask. Insulin (100 I.U./ℓ , Insulin Toronto, Connaught Laboratories, Willowdale, Ontario) was added. The medium was added to the reservoir and the circuit was primed as described in Section II B. The H^3-Inulin (20 μCi, Inulin-{methoxy-^{3}H}, New England Nuclear, Lachine, Quebec) was pipetted into the reservoir.

The medium for flushing the kidney after nephrectomy was placed in an intravenous drip bottle and fitted with a stopper and intravenous administration set. Insulin was added at the same concentration as in the perfusion medium. The medium was equilibrated to room temperature and was bubbled with 95% O_2: 5% CO_2. Isoxsuprine HCl, 0.05 mg/ml, (Vasodilan, Mead Johnson, Canada) was added in some experiments discussed at the relevent point in the text. The bottle was capped and suspended at a height of 40 cm above the operating table. The arterial cannula was attached to the male luer end of the administration set and the line filled with medium. Immediately after the renal artery was cannulated, the cair clamp was opened to allow maximal flow and the kidney was perfused.

B. Perfusion Media for Hypothermic (10 C) Perfusion

The media for hypothermic perfusion was a modification of the formulation, Renal Preservation Solution II, described by Fahy et. al. (1979). The composition of the medium is shown in Table 3. The initial perfusate was prepared at twice normal concentration (2X). The pH was adjusted to pH 7.2 with 5N NaOH. The solution was filtered as described for the 37 ^{0}C perfusate, and stored at 5 ^{0}C. The stock solution (2X) was diluted with the appropriate amounts of ddH_2O and/or a 20% (w/v) solution of mannitol to a final concentration of 1X and an osmolality of 400, 600 or 800 mOs/kg. Table 4 illustrates the preparation of one litre of hypothermic perfusion medium of 400, 600 or 800 mOs/kg by dilution of 2X stock solution. To obtain 500 and 700 mOs/kg media, a given volume (500ml) of the 600 mOs/kg medium was blended with an equal volume of 400 or 800 mOs/kg medium respectfully.

The exact volume of each of the media required for the experiment was measured into pre-sterilized 500 ml glass bottles which served as the reservoirs for the cold perfusion apparatus. The bottles were stored at 5 ^{0}C until use. The media was discarded if not used within one week.

The medium which contained Me_2SO was formulated using the 2X concentration stock solution described above. Me_2SO (Fisher Scientific, Montreal, Quebec) was diluted to 12 M with ddH_2O at room temperature. The 2X concentration stock solution was placed in a beaker and stirred. The glass electrode was placed in the beaker and the pH was adjusted to pH 7.0 with 2N HCl. A volume of 12 M Me_2SO

Table 3: Media composition for hypothermic (10°C) perfusion

Osmolality mOs/kg	Component	g/l	mM
	Glucose	32.43	180.0
	KCl	2.10	28.2
	K_2HPO_4	1.26	7.2
	$NaHCO_3$	0.84	10.0
	*Glutathione (reduced)	1.53	5.0
	$MgCl_2 \cdot 6H_2O$	0.40	2.0
	$CaCl_2$	0.111	1.0
	*Adenine	0.135	1.0
	*HEPES	5.957	25.0
	**Chlorpromazine	0.025	8.0
400	Mannitol	20.039	110.0
600	Mannitol	56.473	310.0
800	Mannitol	92.908	510.0

*Sigma Chemicals, St. Louis, Missouri

**Largactil, Poulenc Ltd., Montreal, Quebec

Table 4: Dilution of 2X stock solution* to yield one litre each of 1X perfusate of 400, 500, 600, 700 or 800 mOs/kg

Osmolality (mOs/kg)	Volume of 2X stock solution (ml)	Volume of ddH_2O (ml)	Volume of 20% mannitol** (ml)
400	750	750	--
600	1000	635.66	364.34
800	750	203.49	546.51
500	500 ml of 600 mOs/kg and 500 ml of 400 mOs/kg		
700	500 ml of 600 mOs/kg and 500 ml of 800 mOs/kg		

*Stock solution (2X) contained twice the amount of solute of 400 mOs/kg medium described in Table 3 per litre.

**Abbott Laboratories, Montreal, Quebec

solution equal to the 2X concentration solution was slowly added to the beaker while constantly stirring. The pH was continuously monitered and maintained at pH 7.2 at 25°C. A slight flocculent precipitate, $CaPO_4$, was produced. This was removed by filtration of the resulting 6 M Me_2SO in 1X perfusion medium through a glass fibre prefilter. The solution was stored in 500 ml glass bottles, 200 ml of solution per bottle, at -20°C.

V. Chemical Determinations

a). Inulin; Tritiated inulin content of perfusate and urine samples was estimated by Liquid Scintillation spectroscopy. Duplicate aliquots (0.05 ml) of samples were pipetted into 15 ml of liquid scintillation cocktail (triton-toluene and Ominflour, New England Nuclear, Lachine Quebec) in glass vials (Wheaton, New England Nuclear, Lachine, Quebec). Samples were counted for 10 minutes on an Inter-technique SL 30 liquid scintillation spectrometer (Canatech, Montreal, Quebec), and the results coded onto a punch tape. The tape was read into a Wang 700 series advanced programmable calculator (Wang, Montreal, Quebec) programmed with the appropriate quench curve to calculate the disintegrations per minute.

b). Glucose: The glucose concentration in the samples was determined colorimetrically using the glucose-oxidase-per oxidase method obtained in the form of a kit (Glucinet, Sclavo Diagnostics, New Jersey). Samples were assayed in duplicate and the optical density read from a Gilson 300 spectrophotometer at 510 nm. Concentrations were calculated using a calibration curve prepared for each assay.

c). Sodium and Potassium: Sodium and potassium concentrations were determined using emission flame photometry. A flame photometer (Radiometer FLM I, Radiometer, Copenhagen) was calibrated with the appropriate standards (A & C Chemicals, Montreal, Quebec).

d). Osmolality: The osmolality of the solutions was determined as a function of the vapour pressure of the solution using a Wescor Model 5100 A vapour pressure osmometer (Wescor Inc., Utah).

e). Protein: Protein determinations were done using the method described by Lowry et. al. (1951). A standard curve of known concentrations of BSA were prepared with each assay.

f). Phenol Red: Samples were rendered alkalotic with 5 N NaOH (1 ml of sample, 1 ml of 5 N NaOH) and the optical density read at 560 nm . The concentration was calculated from a standard curve processed with each assay.

VI. Determination of Kidney Weight

Once the perfusion was completed, the kidney was removed from the apparatus. The capsule was slit along the dorsal axis and peeled back to the pelvis. The capsule, artery, vein and ureter were cut at the point of entry into the pelvis. The kidney was cut longitudinally, blotted for 3 seconds with a gauze pad and placed in preweighed liquid scintillation vials. The vials were capped and weighed to determine wet kidney tissue weight.

VII. Histology

a). Electron Microscopy: Small sections of tissue approximately 1 mm^3 were fixed for 2 hours in 3% glutaraldehydre in Na-cacodylate buffer, rinsed and stored in cacodylate buffer at 4 ^{0}C until processing. The tissue was post-fixed in 1% OSO_4 in cacodylate buffer, dehydrated in graded alcohols, infiltrated with a 1:1 mixture of Epon and propylene oxide, and embedded in Epon 812. Ultrathin sections were cut on an LKB Ultratome III using a diamond knife and collected on 300 mesh copper grids. The sections were stained with uranyl acetate and Reynolds lead citrate, examined and photographed in a Hitachi HU-12 electron microscope.

b). Light Microscopy: Sections for light microscopy were fixed in 10% buffered formalin and embedded in paraffin. Thin sections were mounted on slides and stained with haematoxylin and eosin. Photomicrographs were obtained using a Leitz Wetzlar microscope equipped with a Leitz Orthomat camera. The magnification of the photographs was calculated as the product of the objective magnification, camera magnification (3.2) and the enlargement factor after development of the negative.

VIII. Calculations

a). Introduction: This section describes the calculations to quantitate kidney function during normothermic perfusion. The determinations were performed on pooled samples of urine collected over each 15 minute clearance period. The perfusate sample taken at the midpoint of the clearance period represented the mean values for the perfusate during that period. The perfusate flow rate and corresponding perfusion pressure were recorded at the beginning of each clearance period and following the final clearance period.

A computer programme was prepared to perform the calculations on a Commodore PET 2001 microprocessor. A printout of the programme, using BASIC language is included in Appendix A. The computer repeated the calculations for each clearance period employing the following formulae. The values were expressed per gram kidney wet weight. The symbol U_x represents the value obtained for substance X in the pooled urine sample. The symbol P_x represents the value obtained for substance X in the perfusate sample drawn at the midpoint of the collection period. The symbol V represents the urine volume in $ml min^{-1}$ produced during the 15 minute collection period.

b). Glomerular filtration rate (GFR): The GFR was assumed to be equal to the inulin clearance (C_{in})

$GFR = C_{in} = \{ U_{in} / P_{in} \} \times V$

P_{in} & U_{in} expressed as dpm

c). Sodium Reabsorption: As a % of filtered load (% R_{Na})

$$\%R_{Na} = \{ 1 - \{ \frac{U_{Na} \times V}{GFR \times P_{Na}} \} \} \times 100$$

As total sodium transported ($\mu Eq\ min^{-1}$)

$$\{P_{Na} \times GFR\} - \{ U_{Na} \times V\}$$

d). Glucose Reabsorption: As a % of filtered load

$$\% R_{Glu} = \{1 - \{U_{Glu} \times V / GFR \times P_{Glu} \} \} \times 100$$

e). Protein Leakage: As the ratio of albumin clearance (C_{alb}) to C_{in}

$$C_{alb} / C_{in} = \{ \{ U_{alb} / P_{alb} \} \times V \} / C_{in}$$

f). Phenol Red Secretion: As the ratio of phenol red clearance (C_{PR}) to C_{in}

$$C_{PR} / C_{in} = \{ \{ U_{PR} / P_{PR} \} \times V \} / C_{in}$$

g). Renal Vascular Resistance (RVR)

RVR = P/ Flow

where P is the pressure in mmHg

Flow is the perfusate flow rate in $ml min^{-1} g^{-1}$

Perfusate viscosity was not determined. The RVR is not a true indication of resistance, but of vascular conductance. In these experiments the two were assumed to be similar; but the RVR was not compared between groups in which media viscosity varied.

IX. Statistical Analysis

Statistical analyses were performed on each set of parameters of kidney function measured using methods of analysis of variance (Snedecor and Cochran , 1967). For experiment 1, the effects of treatment and perfusion time were examined for differences using a two-way classification with proportional subclass numbers. For experiments 2,3,4,5 and 7 the effects of treatment and perfusion time were examined for differences using a programme provided with the Wang Programmable Calculator for two-way analysis of variance in observations per cell. For experiment 6, the results were analyzed using the Wang programme for three-way analysis of variance. The Scheffé-test was employed for comparison between treatment means (Scheffé , 1953).

Experiment I: Rabbit Kidney Function In Vitro: Normothermic Perfusion Study of the Effect of Colloid.

Introduction

Simple tests used to assess damage to cells or tissues such as dye exclusion, adenine nucleotide levels, enzyme release, have not proved completely satisfactory for the prediction of kidney status following preservation treatments (Sell, 1967). Auto-transplantation, although the ultimate criterion, is both costly and demands highly skilled help. The *in vitro* model of normothermic kidney perfusion with a defined media has provided a useful alternative in preservation studies in rat and rabbit kidneys. We have constructed a simple system for the *in vitro* assessment of rabbit kidney function by normothermic perfusion.

The following experiment examined rabbit kidney function *in vitro* with and without the inclusion of dextran as a source of colloid osmotic pressure (COP). *In vivo* , colloid osmotic pressure is exerted by serum proteins, predominantly serum albumin. *In vitro*, the inclusion of rabbit albumin for perfusion is not economically feasible. Although highly purified preparations of human serum albumin have proven to be a reliable nonspecies specific colloid source for dogs,(Claes and Blohmé, 1973) the cost is prohibitive. Bovine Serum Albumin (BSA), as an alternative, is not entirely reliable due to batch variation (Pegg *et. al.*, 1977).

A number of other possible synthetic colloids have been studied. Polyvinylpyrrolidone (PVP) utilized for rat kidney

perfusion, (Bullivant , 1978b), was not retained by rabbit glomeruli (Pegg and Farrant , 1969). Experiments using a combination, dextran and BSA, while being more effective in controlling edema, resulted in low rates of glomerular filtration in subsequent studies (Fonteles et. al. , 1973 ; Fuller et. al. , 1977).

In a study of the effects of four different colloids on glomerular structure and function, perfusates containing BSA, BSA and Dextran 70, Dextran 70, Hydroxyethyl starch (HES) or Pluronic F108 produced structural alterations in the capillary endothelium (Fuller et. al. , 1977). In a similar study, which compared pluronic F108, HES, haemaccel and dextran 150, dextran 150 was the most satisfactory (Wusteman , 1978).

The following experiments were performed to evaluate the effects of high or low molecular weight dextrans in our system and to investigate the possibility of omitting colloid if the hydrostatic pressure was adjusted to compensate for the absence of colloid.

Methods

1). Perfusate

The composition of the perfusion media (perfusate) for experiment I is shown in Table 1. The media were identical for all groups; only the type of dextran utilized was varied.

2). Perfusion

The 37 ^{0}C perfusion circuit was assembled and the circuit was primed with one litre of perfusate. The kidney was removed from the rabbit as previously described; however the washout by gravity was not performed. The kidney was immediately attached to the circuit and the stopcock at the base of the organ chamber was opened to drain off the first 150 ml of perfusate. When dextran was included in the perfusate, the pressure was adjusted to 110 mmHg by controlling the flow rate at the pump. If dextran was not included the pressure was adjusted to 50 mmHg. After the first 150 ml of perfusate had flowed through the kidney, the stopcock was closed and the medium recirculated for 10 minutes to allow stabilization prior to commencement of the first collection period. The kidneys were perfused for 135 minutes.

3). Treatment Groups

Kidneys were randomly assigned to one of the following four

treatment groups:

A. Colloid-free: Six kidneys were perfused without dextran for 135 minutes. The pressure was maintained at 50 mmHg throughout the perfusion period.

B. Dextran 180: Seven kidneys were perfused with 3% (W/V) dextran MW = 180,000 (Sigma Chemicals, St. Louis). The pressure was maintained at 110 mmHg.

C. Dextran 80: Six kidneys were perfused with 3% (W/V) dextran MW = 80,000 (Sigma Chemicals, St. Louis), at 110 mmHg.

D. Dextran 70: Three kidneys were perfused with 3% (W/V) dextran MW = 70,000, (Pharmacia) at 110 mmHg.

Perfusate flow rate and pressure were recorded at the beginning of each collection period, and following the last collection period. Urine was collected over 15 minute intervals. A perfusate sample was drawn at the sampling port at the midpoint of each collection period. Urine and perfusate samples were assayed for inulin, sodium and glucose as previously described.

Results

1). Perfusion Characteristics

The perfusate flow rates at 30 minute intervals are presented in Table 5. During the initial half hour of perfusion, kidneys perfused with dextran-containing perfusate at 110 mmHg pressure exhibited significantly higher flow rates than kidneys perfused without dextran at 50 mmHg (Dextran 70 $p < .005$, Dextran 80 $p < .025$, Dextran 180 $p < .05$). The flow rates in the dextran groups were not significantly different. The renal vascular resistance (RVR) increased in all three groups with dextran over the initial value (Figure 6); in contrast, the RVR in the group without dextran, at reduced pressure, remained stable over the 135 minute perfusion period and did not exhibit the large variation observed in the other groups.

As the perfusion period progressed, a marked change in the morphological appearance of the kidneys occurred. In all cases where dextran was included in the perfusate, a pronounced edema was noted, concurrent with marked subcapsular accumulation of fluid. This was not observed in those kidneys perfused at 50 mmHg without dextran.

Urine production in dextran perfused kidneys was very low (Table 6). Kidneys perfused with Dextran 70 produced negligible amounts during the first 60 minutes of perfusion; therefore, we could not perform biochemical tests. All three groups were lower than the kidneys perfused without dextrans in which observed urine

Table 5: Perfusate flow rates of kidneys perfused at 37°C with and without dextran. (ml $min^{-1}g^{-1}$)

	Perfusion Time (min)			
Treatment	30	60	90	120
No colloid	1.73 ± 0.49*	1.87 ± 0.21	2.32 ± 0.30	2.64 ± 0.33
Dextran 180	4.92 ± 0.94	4.28 ± 0.90	3.89 ± 1.10	2.94 ± 1.16
Dextran 80	5.45 ± 0.95	4.65 ± 0.76	3.65 ± 0.78	2.58 ± 0.97
Dextran 70	7.42 ± 1.33	6.31 ± 0.72	5.06 ± 0.92	3.42 ± 1.12

*Mean ± SEM (between 4 to 6 observations per group)

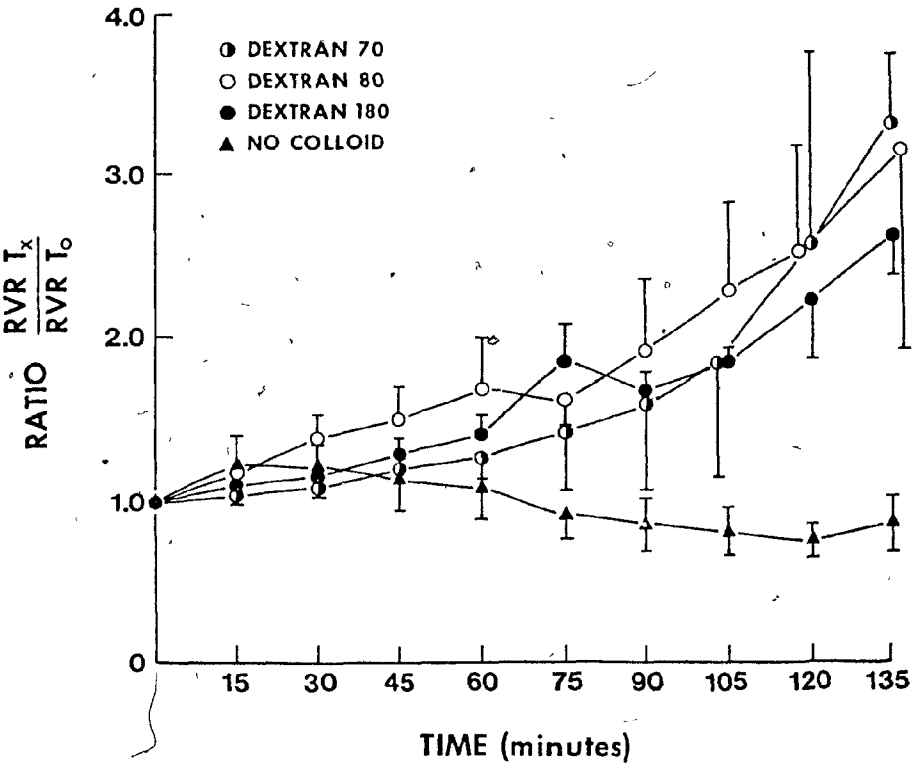

Figure 6. The ratio of the renal vascular resistance (RVR) at different times of perfusion to the initial renal vascular resistance at time 0. Each point represents the mean ± SEM of between 3 and 7 observations.

Table 6: Urine flow rates of kidneys perfused at 37°C with and without dextran. (ml $min^{-1}g^{-1}$)*

	Perfusion Time (min)			
Treatment	30	60	90	120
No colloid	0.113 ± .020**	0.132 ± .014	.145 ± .015	.144 ± .016
Dextran 180	0.040 ± .014	0.025 ± .008	.026 ± .004	.022 ± .004
Dextran 80	0.020 ± .009	0.026 ± .011	.020 ± .008	.022 ± .008
Dextran 70	0.005 ± .004	0.017 ± .016	.026 ± .022	.025 ± .006

*Averaged over 15 min collection period
** Mean ± SEM (between 4 to 6 observations per group)

flows were stable. The presence of dextran depressed the GFR (Figure 7). After 15 minutes of perfusion, the GFR in the dextran-free group was significantly greater than Dextran 80 or Dextran 70 groups ($p < .005$). The dextran 180 group GFR at 15 minutes was significantly greater than the other dextran-containing groups ($p < .025$); during the following 45 minutes, the GFR in the dextran group declined rapidly and for the remaining collection periods all dextran-perfused groups were equally depressed in comparison to the dextran-free group ($p < .005$), which maintained a stable GFR throughout the perfusion period.

2). Tubular Function

Tubular reabsorption of sodium and glucose as a percentage of the filtered load is presented in Figures 8 and 9. Without dextran, sodium reabsorption was stable over the 135 minute perfusion period. In both the dextran 80 and dextran 180 groups, the sodium reabsorption declined with time and exhibited larger variation. Glucose reabsorption was essentially the same in all three groups. The actual sodium load transported (Figure 10) was stable in the group perfused without dextran, and greater than the dextran perfused groups.

3). Histology

Histologic examination of kidneys perfused with dextrans revealed

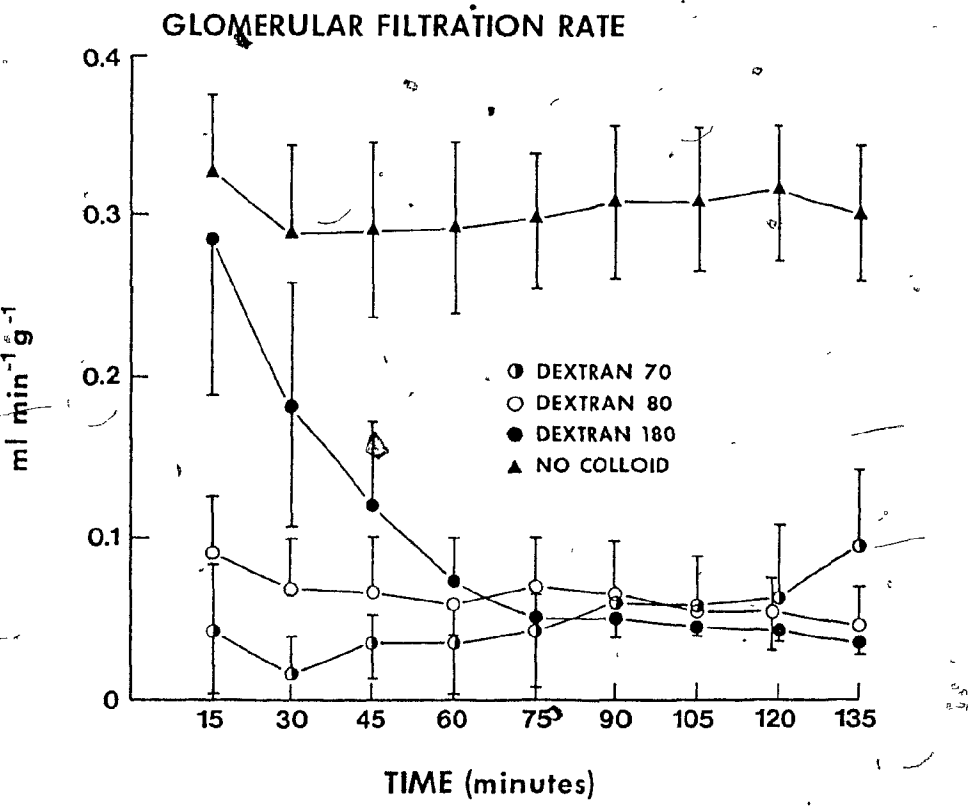

Figure 7. The glomerular filtration rate of kidneys perfused with and without dextran as a function of perfusion time. Each point represents the mean ± SEM of between 3 and 7 observations.

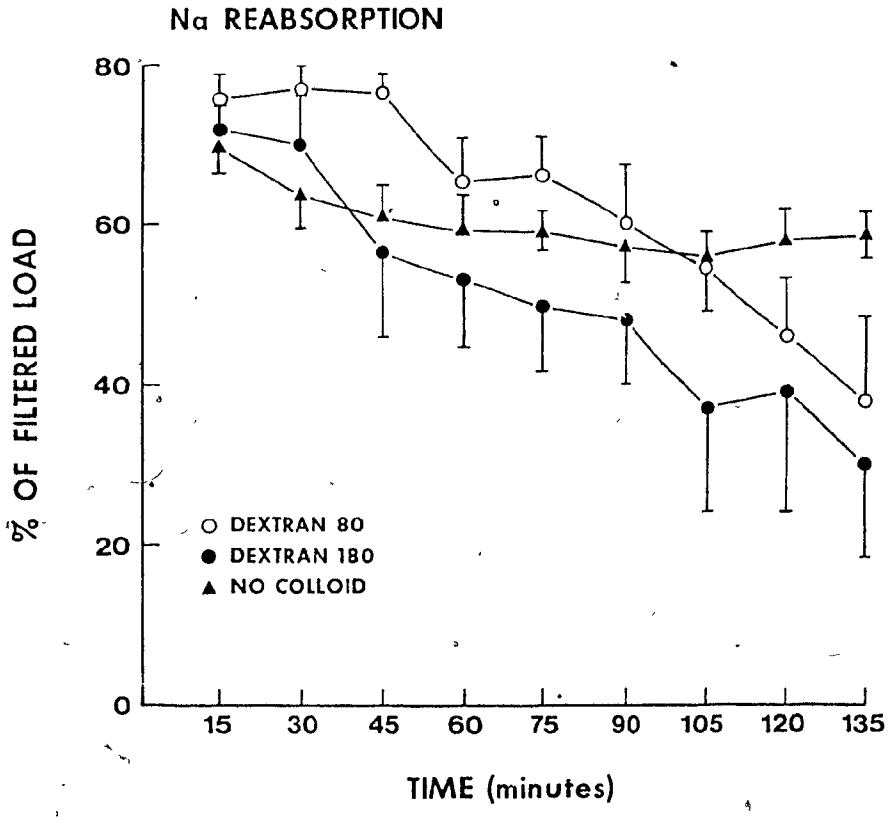

Figure 8. The percentage of filtered sodium reabsorbed by kidneys perfused with and without dextran as a function of perfusion time. Each point represents the mean ± SEM of 6 or 7 observations.

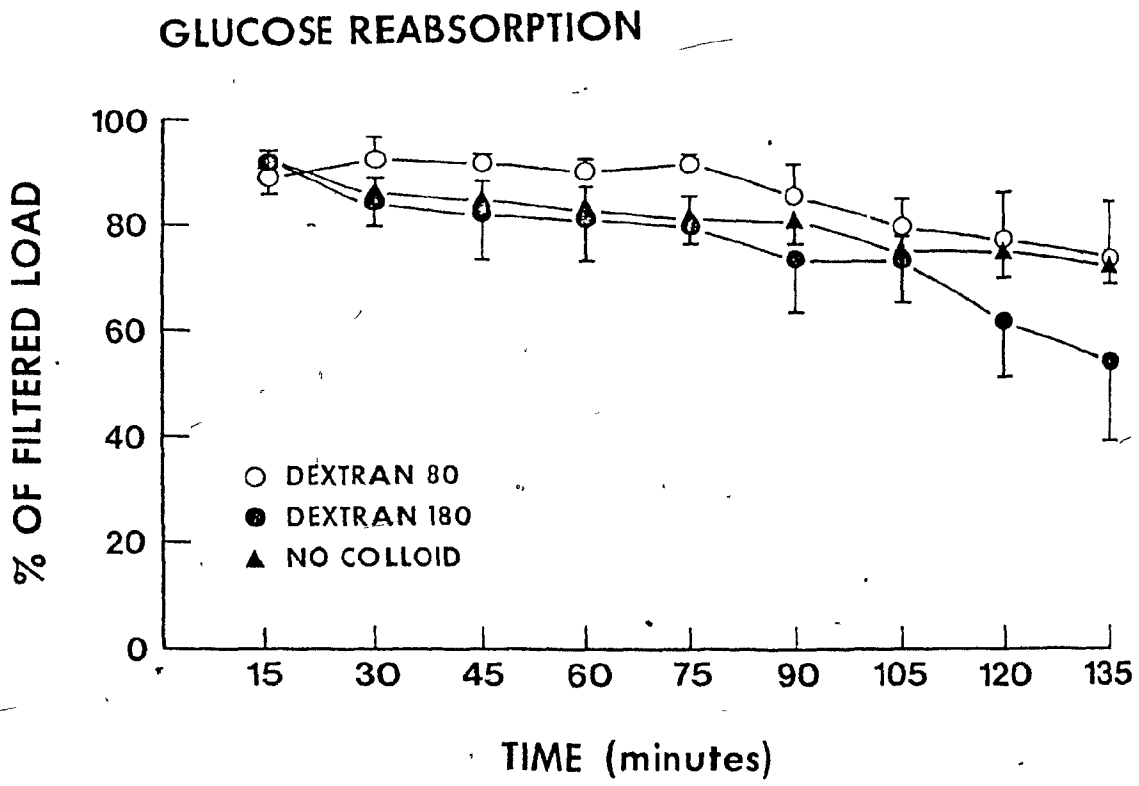

Figure 9. The percentage of filtered glucose reabsorbed by kidneys perfused with and without dextran as a function of perfusion time. Each point represents the mean ± SEM of 6 or 7 observations.

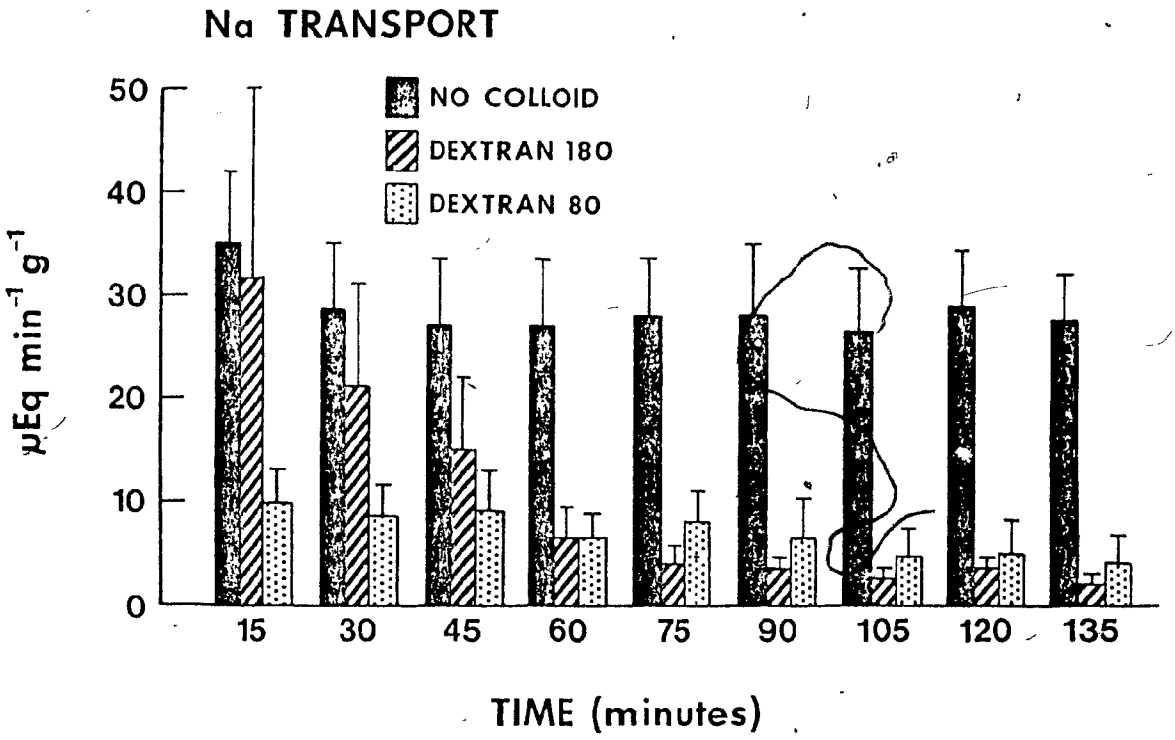

Figure 10. Sodium transport by kidneys perfused with and without dextran as a function of perfusion time. Each point represent the mean ± SEM of 6 or 7 observations.

glomerular and tubular alterations. In certain regions of dextran-perfused kidneys, glomeruli appeared shrunken, with marked distension of Bowman's space (Figure 11A). The tubular lumen contained debris. In kidneys perfused without dextran, glomeruli remained intact with minimal distension of Bowman's space and relatively clear tubular lumen (Figure 11B). Electron microscopic examination of dextran-perfused kidneys indicated that glomerular structure was maintained (Figure 12B); capillary lumen size was reduced in comparison to controls (Figure 12A). Proximal tubules had undergone marked changes (Figures 12C and 12D). The brush border lining the lumen appeared disrupted. Mitochondria were swollen and dense. The cytoplasm and lumen contained large numbers of vacuoles, with evidence of loss of cytoplasm. While portions of peritubular capillaries appeared intact (Figure 13A), portions contained debris and the basement membrane lining tubular cells was devoid of endothelium (Figure 13B). Mitochondria were enlarged with disorganized cristae.

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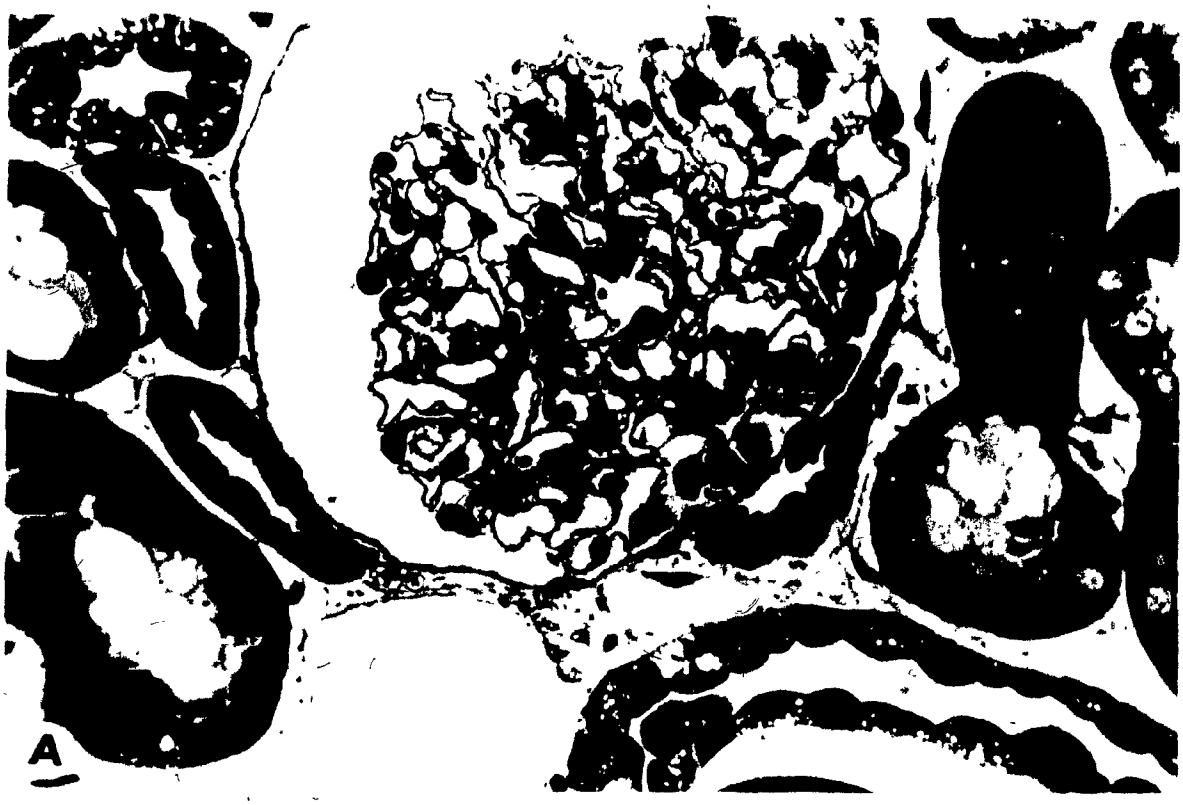
Figure 11

A. Photomicrograph of a section of kidney perfused with dextran (MW 80,000). Distension of Bowman's space and tubule debris are evident (608 X).

B. Kidney perfused without colloid at reduced pressure (H & E, 608 X).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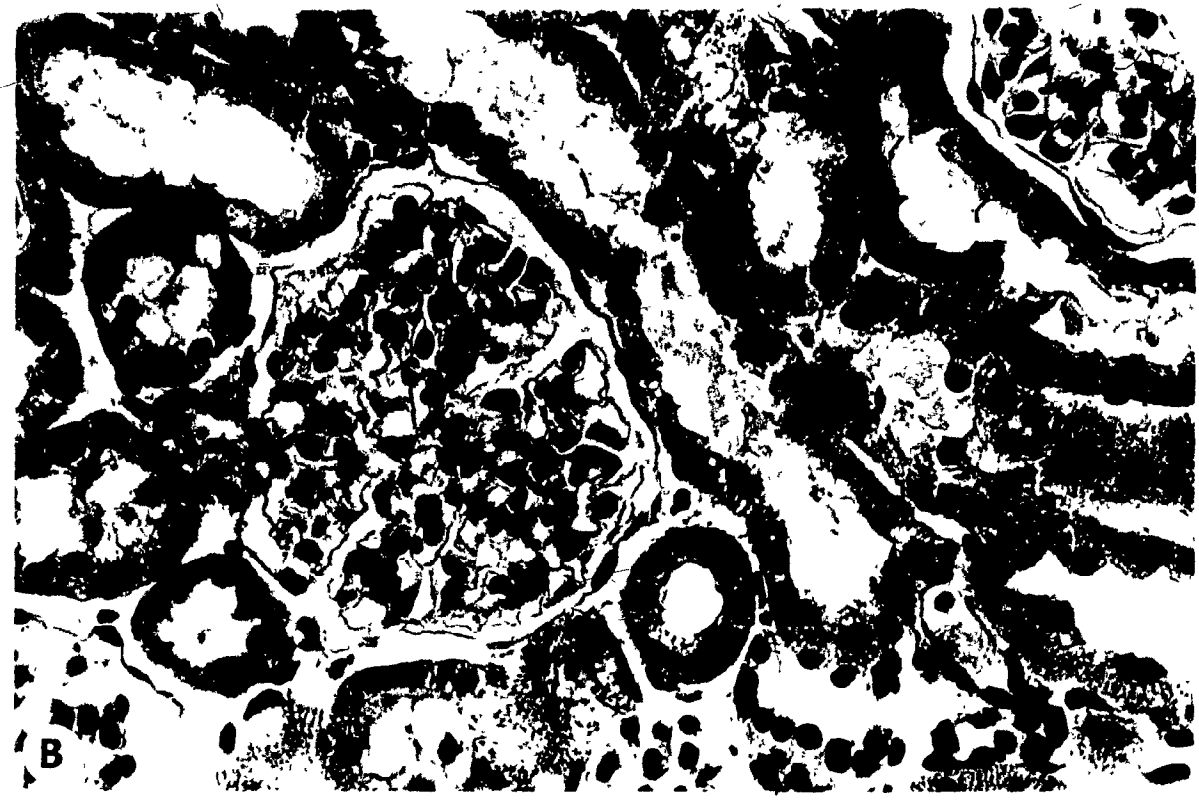
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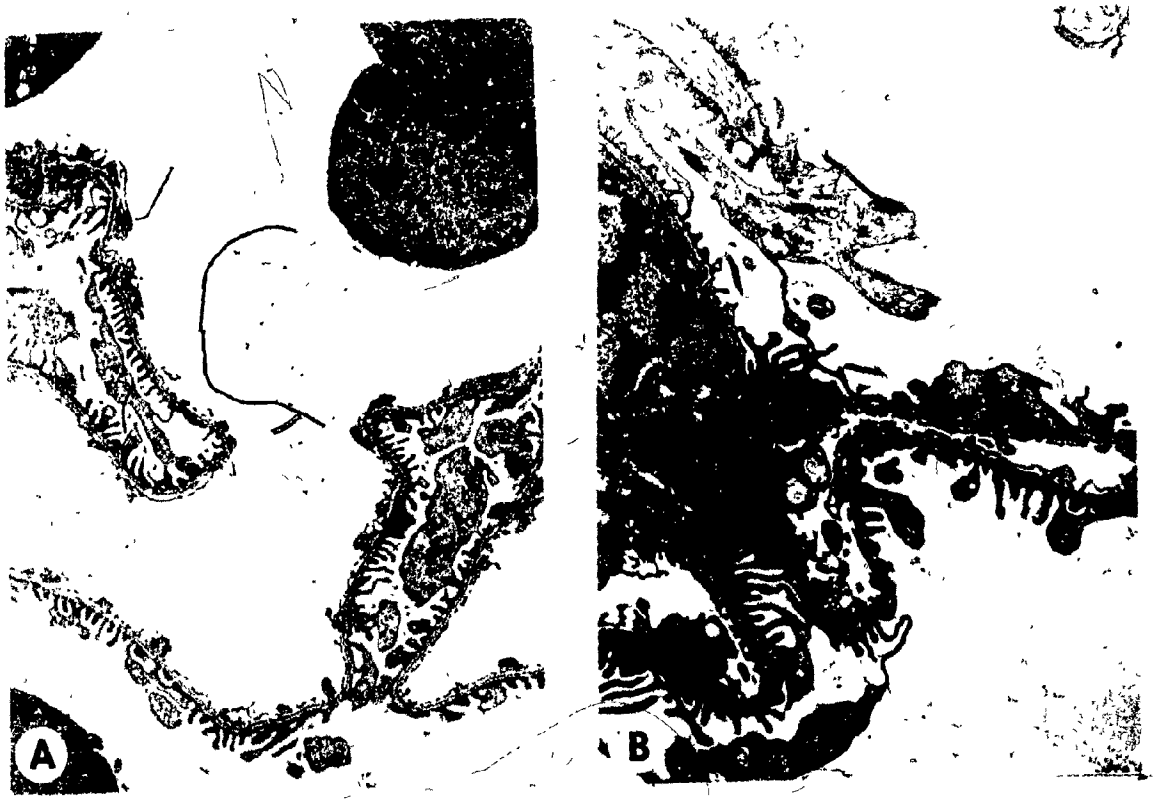
Figure 12

A. Electron micrograph showing glomerular ultrastructure of a kidney sectioned immediately following removal. (400 X).

B. Electron micrograph of a kidney section following perfusion with dextran (MW 180,000). Bowan's space is distended with partial occlusion of glomerular capillaries (8000 X).

C. Electron micrograph of a kidney section following perfusion with dextran (MW 80,000), exhibiting disruption of brush border and swollen mitochondria (4100 X).

D. Electron micrograph of a section of kidney proximal tubule following perfusion with dextran (MW 180,000). Tubule cells exhibited loss of cell cytoplasm, disruption of the brush border, cytoplasmic vacuolization and dense mitochondria (5500 X).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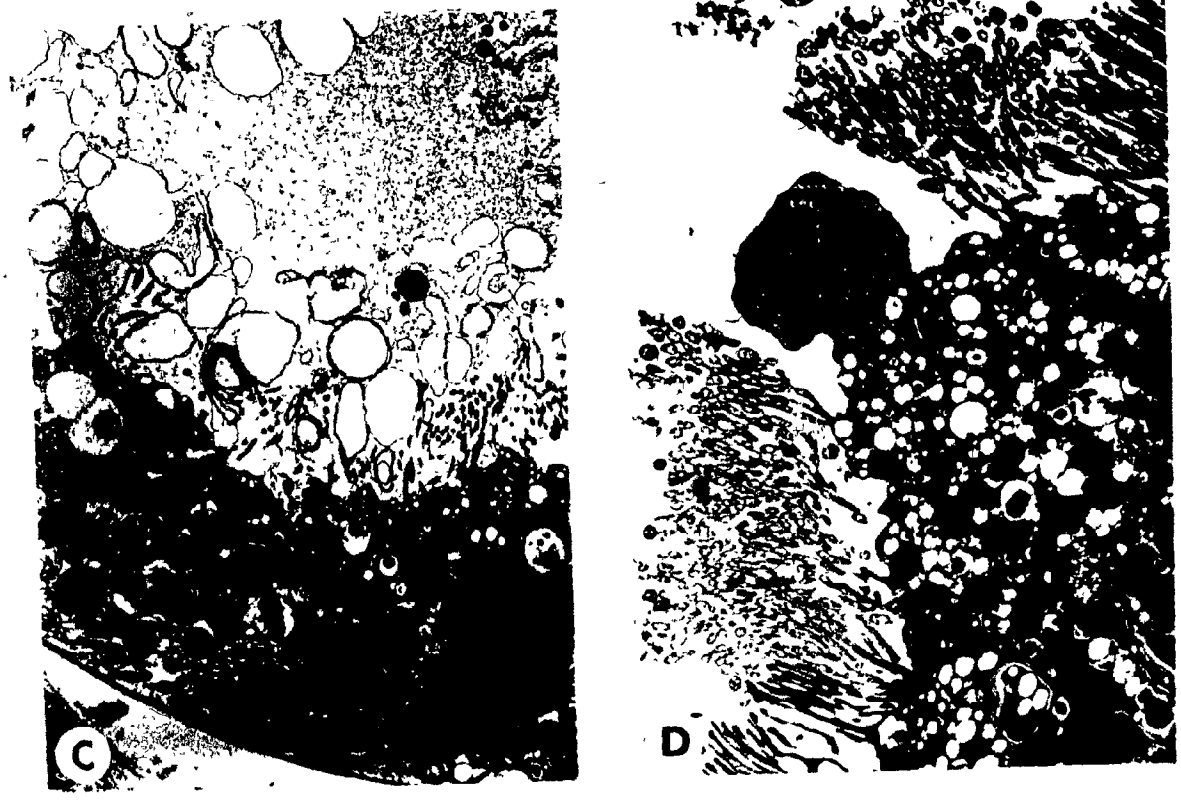
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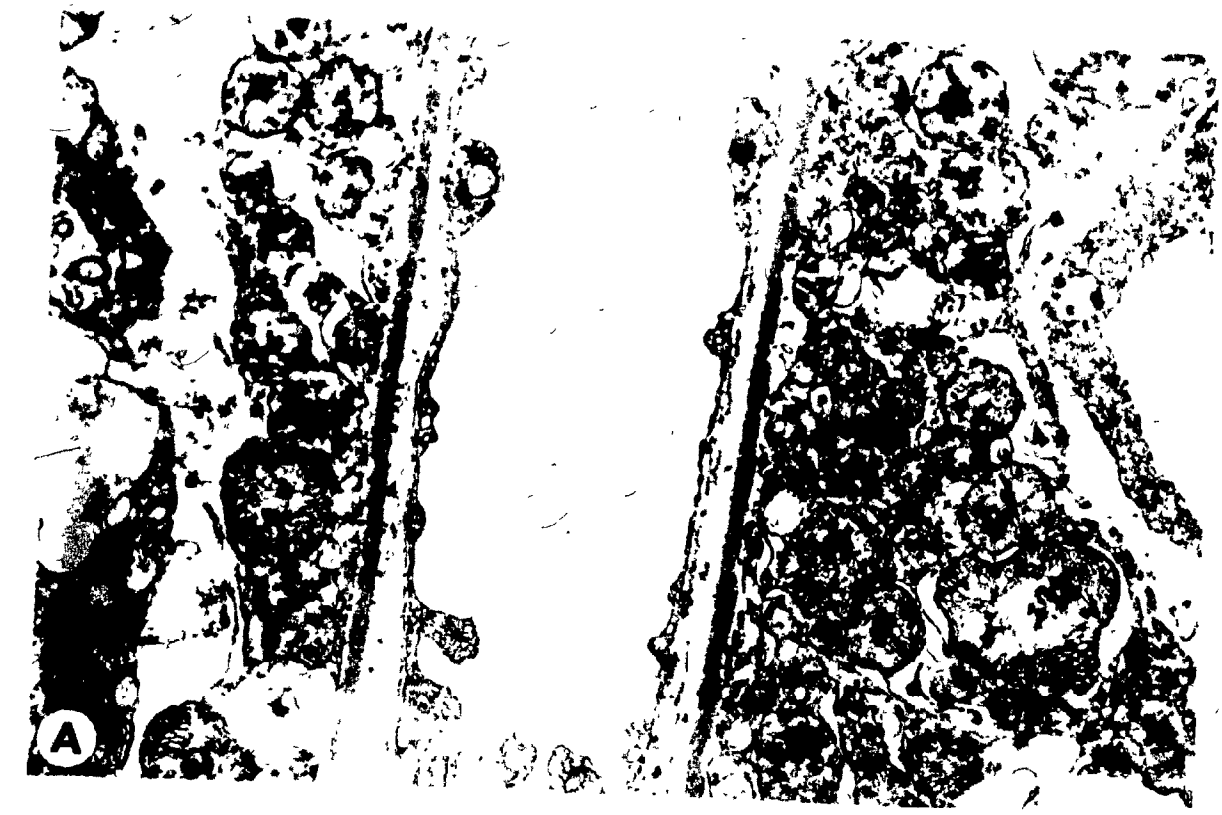
Figure 13

A,B. Electron micrograph series of a kidney section following perfusion with dextran (Mw 180,000) showing (A) intact peritubular endothelium and (B) loss of endothelial integrity along the same length of capillary (9720 X).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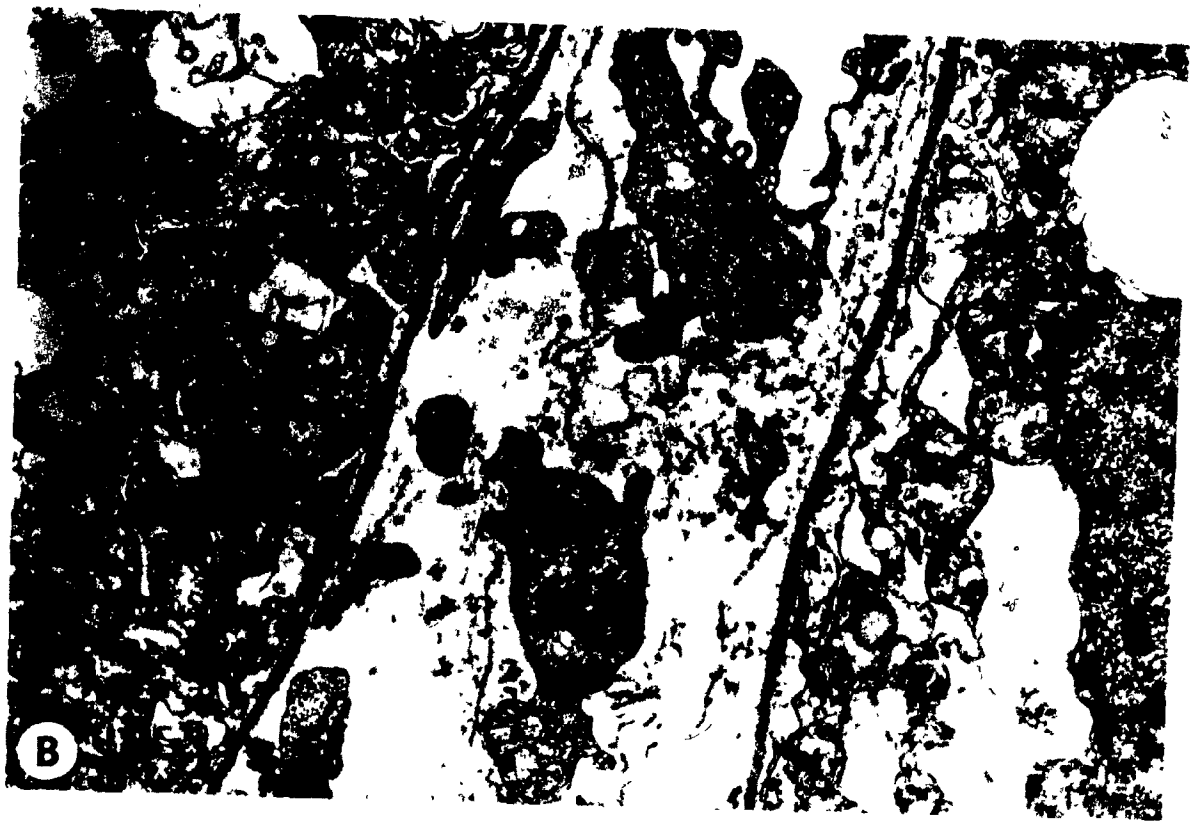
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Discussion

The perfusion of rabbit kidneys *in vitro* at 37 ^{0}C with dextran included as colloid resulted in markedly depressed rates of glomerular filtration and increasing renal vascular resistance throughout the perfusion period. In contrast, kidneys perfused without colloid at reduced pressure exhibited a higher GFR and a stable resistance. If the intraglomerular pressure was approximately two thirds of the arterial pressure (Renkin and Gilmore, 1973) and the colloid osmotic pressure exerted by the dextrans was estimated at 25 mmHg (Fuller *et. al.* , 1977), the resulting net filtration pressure at 110 mmHg was 45-50 mmHg. In the colloid-free perfusion, the net perfusion pressure was approximately 33 mmHg. It appears that the low values for GFR in dextran-perfused kidneys were not the result of lower pressure at the glomerulus, but an effect due to the presence of dextran in the perfusate. The effect was observed in both high and low molecular weight dextrans, as well as in dextrans obtained from different sources. Histological examination of dextran-perfused kidneys showed areas of glomerular structural alteration. Electron microscopic studies revealed extensive damage to proximal tubules-disruption of the brush border, intracellular and intra-luminal vacuole formation and mitochondrial swelling.

The possible effect of dextran on the vascular endothelium was noted by Pegg and Farrant (1969)and by Pegg (1970). They found an increased renal vascular resistance and suggested that dextran may precipitate vascular endothelial degeneration. Supportive evidence was provided by Fuller *et. al.* (1977) who noted extensive

lesions in the glomerular endothelium, not only with dextran but with other colloids tested. In the present study, the effect of dextran was apparent in glomeruli and in the tubule as well. It is possible that the tubular damage may have been a secondary effect of uneven perfusion of areas where the vascular system was affected by dextran; however dextran has been reported to have a direct damaging effect on proximal tubule cells (Imai and Kokko , 1974).

The function of tubules as measured by sodium uptake indicated a progressive decline in function as compared to the colloid-free perfused kidneys. The edema which was observed suggested that the dextran was not completely retained within the vascular bed. These observations indicated that during the course of perfusion with dextran, kidney function progressively declined as a result of a damaging effect of dextran on the vascular system and a toxic effect of dextran, filtered at the glomerulus, on tubule epithelial cells . Other reports have noted that the severity of damage varied with different dextran preparations, but the occurrence is frequent (Pegg et. al. , 1977 ; Pegg and Wusteman , 1977).

Although a perfusion system including a source of COP represents a more physiological approach the colloid-free reduced pressure system described offers an attractive alternative to the problems of variation of synthetic colloids, and the prohibitive cost of suitable albumin preparations. The kidney perfused under these conditions achieved an acceptable level of function during the initial 15 minutes of perfusion which was maintained for the entire perfusion period. The values for GFR were similar to those reported, for kidneys

perfused with Pluronic F-108 (Fuller et. al., 1977), but lower than dextran 150 (Wusteman 1977 ; 1978). The fractional reabsorption of sodium and glucose were also comparable.

In our study, in the absence of colloid, sodium reabsorption occurred without the facilitating effect of COP in the peritubular capillaries. For the perfusion of rat kidneys, the fractional reabsorption of sodium at 0-2% BSA concentration was 55%, and increased to 95% with increasing BSA concentration to 8% (Little and Cohen , 1974). In isolated rabbit proximal tubules, water reabsorption was increased if 3.5% PVP was added to the bathing medium in the presence of ouabain (Imai and Kokko ,1974). Sodium uptake in our system was still comparable to rabbit kidneys perfused with colloid (Fuller and Pegg , 1976 ; Fuller et. al. , 1977). The results reported here indicate that the colloid-free, reduced pressure system of rabbit kidney perfusion should prove useful as an in vitro model for the evaluation of kidney function.

Experiment 2: The Effects of Energy Substrate on In Vitro Rabbit Kidney Function During Normothermic Perfusion.

Introduction

The principal fuels of renal metabolism include glucose fatty acids, and lactate (Yoshida, 1976). Experiments with the rat kidney *in vitro* perfusion model have shown that tubular reabsorption requires the exogenous supply of glucose (Gregg *et. al.* , 1978) and supplementation with lactate enhances function (Cohen and Little , 1976 ; Merkins *et. al.* , 1978 ; Cohen *et. al.* , 1980). The contribution of shortchain fatty acids during *in vitro* rat kidney perfusion is more speculative (Trimble and Bowman , 1973 ; Ross *et. al.* , 1973). In the perfused rabbit kidney, supplementation of glucose-containing perfusate did not enhance Na reabsorption, but maintained stability of the preparation (Fuller *et. al.* , 1977). The previous experiment indicated that the isolated rabbit kidney was able to reabsorb significant amounts of sodium and glucose during *in vitro* perfusion with a simple salt solution. The following experiments were conducted to determine if the rabbit kidney perfused in our system was capable of responding to exogenous substrate; and to ascertain if supplementation of the perfusate with lactate or fatty acid could increase kidney function.

Methods

1). Perfusate

Two solutions were prepared for this study (Table I). One included glucose, 5.56 mM as sole energy substrate. The other contained no energy substrate; mannitol, 5.56 mM was substituted for glucose. The added substrates, lactate, or butyrate were prepared as the Na salt in 2.5 mM quantities. The 2.5 mM quantities were adjusted to pH7.4 with 5N NaOH, diluted to 5 ml with distilled water, and added to the perfusate which contained glucose. Similarly, 2.5 mM quantities of NaCl were prepared and added to the perfusion circuit when glucose-free medium was used.

2). Perfusion

The 37^{0} perfusion circuit was assembled as described in the Methods section. The circuit was primed with exactly 500 ml of the media containing either glucose or mannitol. Insulin (50 IU) was added to the reservoir. If the Na-lactate or Na-butyrate was included, the 5 ml aliquot was added to the glucose containing perfusate in the reservoir. For perfusions with glucose or with substrate-free perfusate, a 5 ml aliquot of NaCl was added. The perfusate was recirculated with constant stirring of the reservoir for 15 minutes prior to kidney attachment to allow mixing and equilibration at 37^{0}C. The kidneys were removed from the rabbit as previously described. The pre-perfusion flush by gravity

was initiated as soon as the cannula was introduced into the renal artery. If the kidney was destined to perfusion with medium containing substrate, the flushing solution consisted of 150 ml of oxygenated, glucose-containing perfusion medium. Kidneys to be perfused with substrate-free medium were flushed with 150 ml of oxygenated medium containing mannitol. The kidneys were attached to the perfusion circuit and the perfusion pressure was adjusted to 50 mmHg. The perfusate was recirculated through the kidney for 10 minutes to allow stabilization prior to commencement of the first 15 minute collection period.

3). Treatment Groups

Seven kidneys in each treatment were perfused at 37 ^{0}C and 50 mmHg pressure for 6 consecutive 15 minute clearance periods with the following perfusates:

A. Substrate-free perfusate (5.56 mM mannitol);

B. Perfusate containing 5.56 mM glucose;

C. Perfusate containing 5.56 mM glucose and 5 mM Na-butyrate;

D. Perfusate containing 5.56 mM glucose and 5 mM Na-lactate.

The perfusate flow rate was recorded at the beginning of each collection period and after the final period. Perfusate samples (5 ml) were drawn from the sampling port at 7.5 minute intervals beginning at the start of the first collection period. Urine produced was collected over each 15 minute period.

The glucose concentration of all samples was determined. The

inulin and sodium determinations were done on the samples collected at the midpoint of each collection period for calculation of GFR, sodium and glucose reabsorption. The glucose utilization for each kidney was calculated according to the method of Gregg et. al. (1978).

Results

1). Perfusion Characteristics

The perfusate flow rates are shown in Table 7. Similar flow rates were observed in groups perfused with either lactate, butyrate or no substrate. The group perfused with glucose alone exhibited higher initial vascular resistance. Problems which occurred during the removal and cannulation of two kidneys in this group resulted in a prolonged ischemic period prior to initial flushing by gravity. This may have contributed to the higher vascular resistance observed. The flow rates in this group were lower than the other three groups ($p < .005$), and although the resistance decreased during the perfusion, the flow rates after 60 minutes of perfusion were still significantly depressed. The pattern of resistance in all four groups was similar; a slight increase during the first collection period followed by a decrease for the remaining perfusion time (Figure 14). The perfusate flow rates after 90 minutes were similar to the initial flows.

The glomerular filtration rate was similar in all four groups (Figure 15). The pattern was stable between 0.2 and 0.3 ml $min^{-1}g^{-1}$. The GFR did not increase as did the flow rates during the final 45 minutes, but tended to rise during the second 30 minute interval, falling slightly during the final 30 minutes.

Urine flow rates (Table 8) exhibited a pattern similar to that for GFR with the exception of the substrate-free perfused kidneys. Whereas urine production decreased slightly during the final 30

Table 7: Perfusate flow rates of kidneys perfused at 37°C with different sources of energy substrate (ml $min^{-1}g^{-1}$)

Treatment	Perfusion Time (min) 0	15	30	45	60	75	90
Glucose & Lactate	2.31* ± 0.33	2.10 ± 0.25	2.10 ± 0.10	2.11 ± 0.33	2.17 ± 0.39	2.30 ± 0.37	2.50 ± 0.46
Glucose & Butyrate	2.35 ± 0.47	1.80 ± 0.34	2.14 ± 0.41	2.44 ± 0.50	2.76 ± 0.49	2.79 ± 0.50	2.70 ± 0.51
Glucose	1.20 ± 0.21	1.12 ± 0.19	1.14 ± 0.19	1.30 ± 0.22	1.51 ± 0.32	1.73 ± 0.33	1.87 ± 0.33
No substrate	2.21 ± 0.36	2.10 ± 0.33	2.16 ± 0.34	2.26 ± 0.30	2.31 ± 0.29	2.43 ± 0.32	2.49 ± 0.42

* Mean ± SEM (7 observations at each time interval)

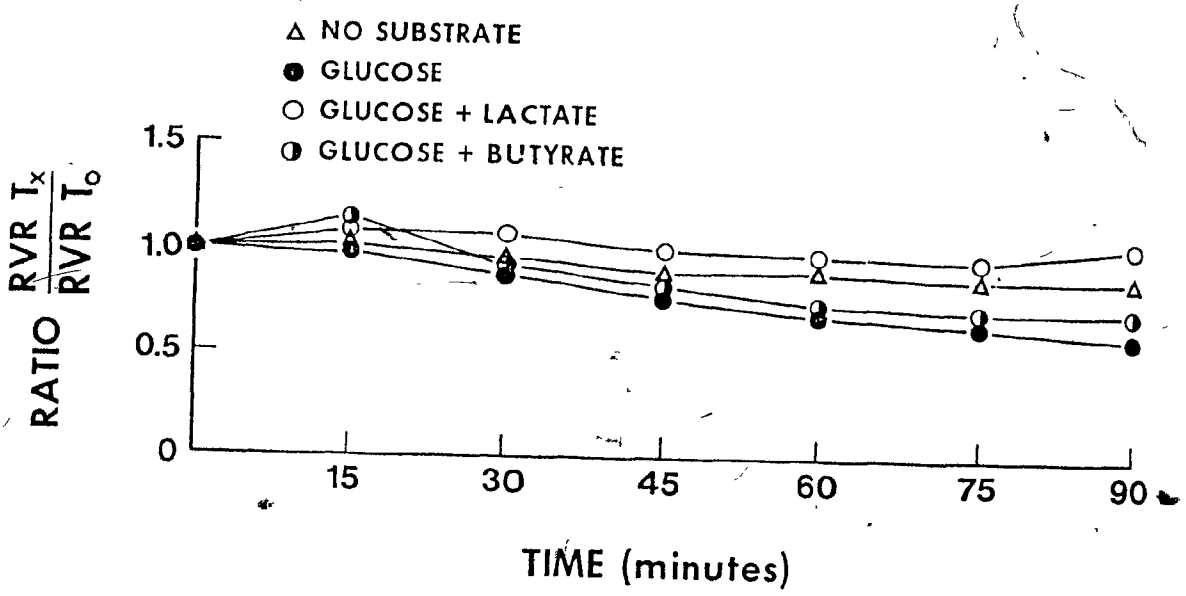

Figure 14. The ratio of renal vascular resistance (RVR) at different times of perfusion to the initial RVR at time 0. Each point represents the mean ± SEM of 7 observations.

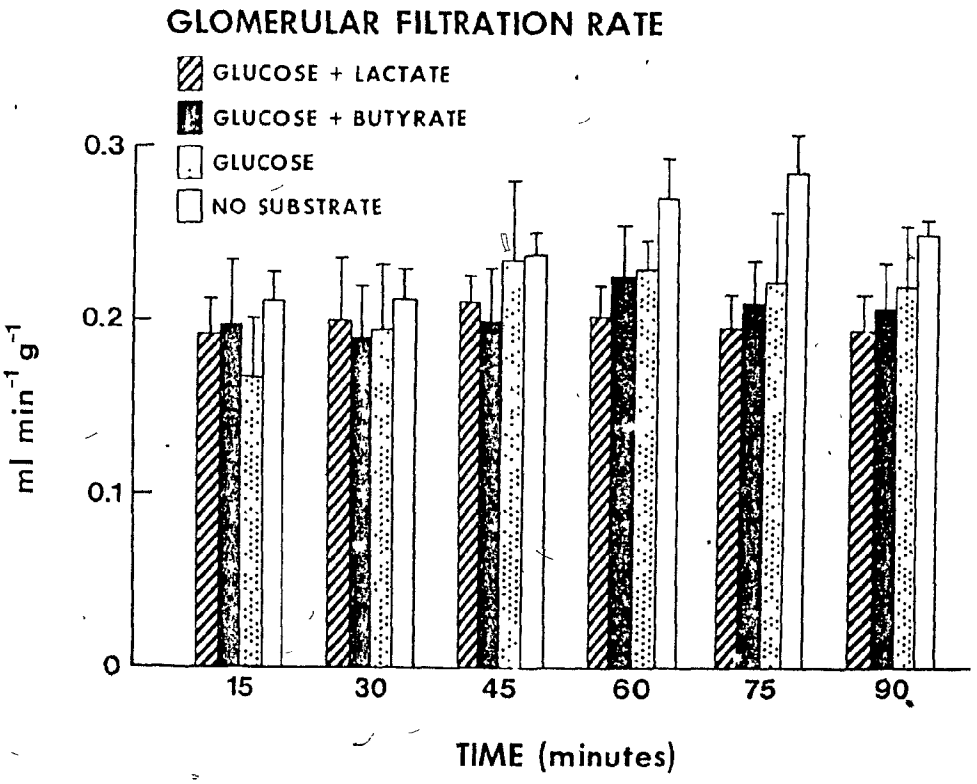

Figure 15. The glomerular filtration rate of kidneys perfused with different energy substrate as a function of perfusion time. (Mean ± SEM, 7 observations at each point).

Table 8: Urine flow rates of kidneys perfused at 37°C with different sources of energy substrate (ml $min^{-1} g^{-1}$)*

	Perfusion Time (min)					
Treatment	15	30	45	60	75	90
Glucose & Lactate	0.071** ± 0.013	0.083 ± 0.016	0.106 ± 0.011	0.103 ± 0.017	0.105 ± 0.010	0.097 ± 0.015
Glucose & Butyrate	0.078 ± 0.020	0.094 ± 0.016	0.117 ± 0.013	0.131 ± 0.013	0.128 ± 0.012	0.119 ± 0.015
Glucose	0.101 ± 0.029	0.132 ± 0.034	0.149 ± 0.038	0.151 ± 0.034	0.140 ± 0.031	0.129 ± 0.027
No substrate	0.146 ± 0.030	0.168 ± 0.034	0.193 ± 0.031	0.222 ± 0.030	0.237 ± 0.026	0.232 ± 0.024

*Averaged over each 15 min collection period
**Mean ± SEM (7 observations at each time interval)

minutes in kidneys perfused with a source of energy, it remained high in the substrate-free perfused kidneys. Urine production, in groups containing an energy source, was lower than in the group without substrate ($p < .005$). Kidneys perfused with glucose alone had higher urine flow rates than kidneys where lactate was added (0.132 ± 0.032 vs 0.085 ± 0.014 ml $min^{-1}g^{-1}$, $p < .01$).

2). Tubular Function

The percent of filtered sodium reabsorbed in all groups perfused with an energy source was greater than in kidneys perfused without substrate, (Figure 16). Kidneys perfused with 5 mM lactate ($54.37 \pm 4.63\%$) exhibited higher rates of reabsorption than butyrate ($46.59 \pm 3.61\%$, $p < .25$), glucose alone ($37.62 \pm 3.66\%$, $p < .005$) or substrate-free ($30.65 \pm 5.76\%$, $p < .005$) perfused kidneys. For kidneys perfused with butyrate, the added substrate was less effective in enhancement of sodium reabsorption over glucose alone ($p < .01$); the differences were not apparent during the initial clearance periods. Similarly, perfusion with glucose as sole energy source was slightly better than without ($p < .05$), and only became evident in the last 30 minutes of perfusion. Overall, all groups exhibited a decrease in sodium reabsorption during the first 45 minutes, followed by a stable period and a rapid fall off in the group perfused without substrate, which decreased 50% by the end of the perfusion, ($41.94 \pm 5.56\%$ during the first clearance period to $21.14 \pm 5.06\%$ during the final clearance period, $p < .05$).

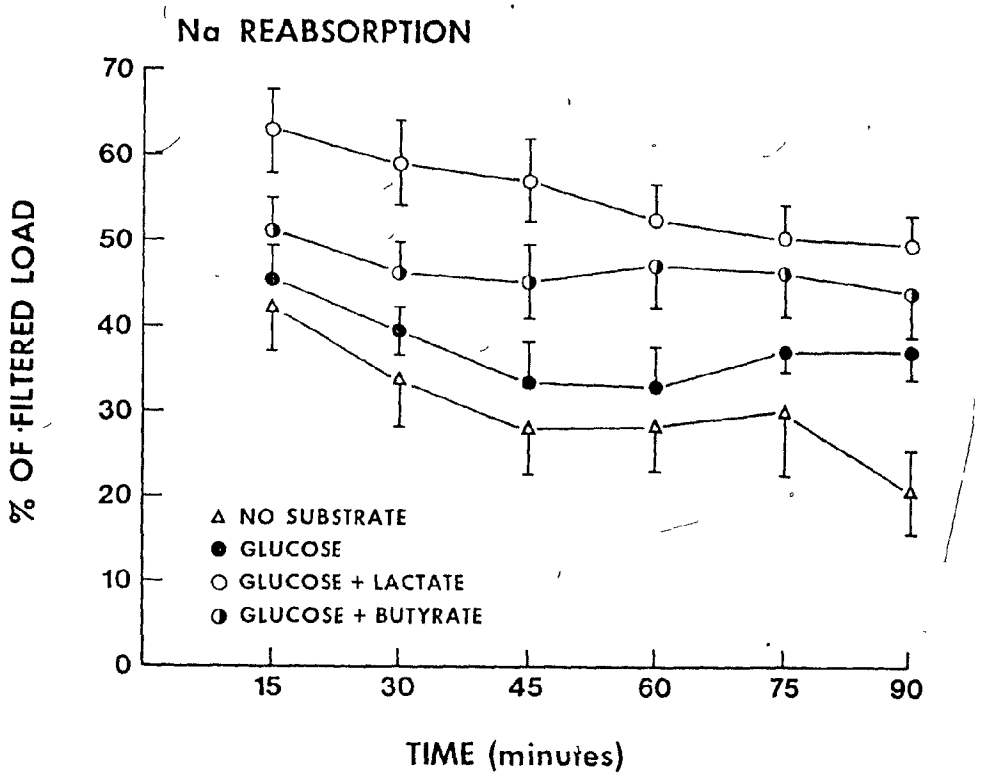

Figure 16. The percentage of filtered sodium reabsorbed by kidneys perfused with different substrate (Mean ± SEM, 7 observations at each point).

The effects of substrate on glucose reabsorption were not as apparent (Figure 17). In the lactate-perfused kidneys, glucose reabsorption (85.13 ±3.33%) was slightly increased over the butyrate (79.07 ± 4.91%) and glucose (78.98 ± 3.95%), $p < 0.1$. Generally, the glucose reabsorption declined with time ($p \leq .05$) in all three groups which contained glucose; however, the decline was greatest in groups perfused with butyrate and glucose and glucose alone ($p \leq .025$ between the first and final clearance periods).

Total glucose utilization during the 90 minutes of perfusion is expressed as the mean of the glucose utilization per μEq of sodium transported during the six clearance periods of perfusion when glucose was present in the perfusate (Table 9). Kidneys perfused with glucose as the sole energy source utilized the most glucose; whereas addition of lactate or butyrate significantly reduced net glucose utilization ($p < .01$ and $p < .05$ respectively). During the perfusion, the phenol red content of the perfusate diminished; by the end of the experiments, the recirculating perfusate was almost colourless. The phenol red was concentrated in the urine produced by the kidney. Although the extent of the concentration of phenol red in the urine was not quantitated in this experiment, it provided an indication of active secretion by the kidney.

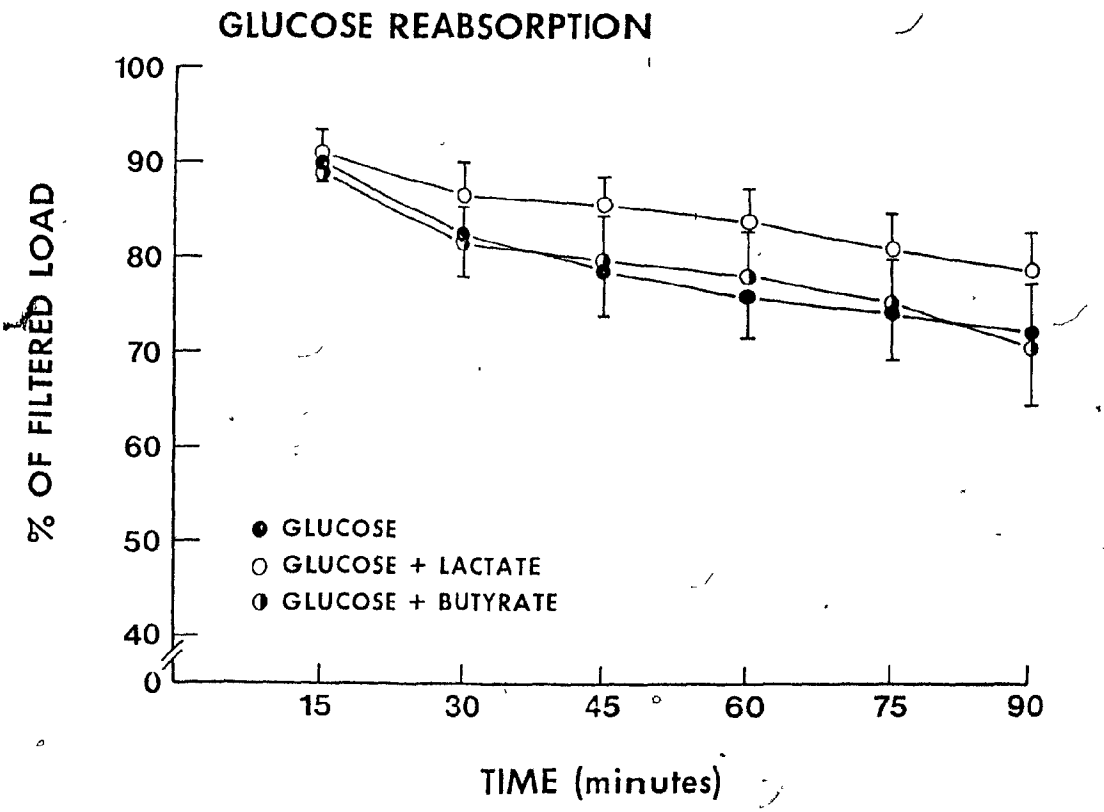

Figure 17. The percentage of filtered glucose reabsorbed by kidneys perfused with different energy substrate as a function of perfusion time. (Mean ± SEM, 7 observations at each point).

Table 9: Glucose utilization of kidneys perfused at 37°C with different sources of energy substrate

Treatment	Glucose Utilization mmol μEq^{-1} Na g^{-1} min^{-1}
Glucose & Lactate	0.0652 ± 0.042*
Glucose & Butyrate	0.0933 ± 0.0321
Glucose	0.1355 ± 0.0378

*Mean ± SEM (42 observations in each group)

Discussion

Rabbit kidneys perfused with a colloid free solution at reduced pressure are capable of sustaining a significant amount of active sodium reabsorption and glucose transport. The lower perfusate flow rates observed in the glucose-perfused kidneys in this series was a result of extended ischemia during cannulation which may have precipitated an initial vasospasm preventing complete removal of blood from the vascular system during the initial flush-out. All four groups of kidneys did in fact exhibit a slight increase in RVR during the early stages of perfusion. This phenomenon has been observed in both rat (Shurek et. al. , 1975) and rabbit (Fonteles et. al. , 1973) kidney perfusions. The most probable cause is an increased sympathetic tone during surgical isolation since α-receptor blocking agents can prevent this (Shurek et. al. , 1975; Bullivant , 1978 a). It could be advantageous to include a vasodilator in the flushout medium to prevent this and promote a more rapid and total clearing of blood from the vascular system prior to attachment to the perfusion circuit. The decrease in RVR during the latter periods of perfusion indicates a stable preparation; in hypothermic perfusion, a stable or decreasing RVR is considered to be an important criterion when deciding the fate of a transplantable human kidney (Belzer et. al. , 1968).

In contrast to the performance of rat or rabbit kidneys during perfusion with colloid, the kidneys perfused in this study establish

a stable GFR and perfusate flow rate at the onset of perfusion. The difference may be due to the initial effects of a foreign colloid substance on the kidney, perhaps by changes in glomerular permeability (Wusteman , 1978) or to a transient swelling of outer medullary cells (Bullivant , 1978b). An important distinction to be considered when attempting a comparison is the net pressure favoring filtration across the capillary membrane which is lower in our system than in most colloid-containing systems. Inclusion of colloids results in supra-physiological flow rates at physiological pressure , due in part to the physical characteristics of the perfusate (Maack , 1980). The net result could be the progressive opening of vascular channels-either previously closed, or by distension of open vascular pathways. This could ultimately lead to more efficient regional perfusion of, and oxygen delivery to, the tissues.

The results obtained indicate that the kidney perfused under the conditions of our system is sensitive to the availability of substrate and utilizes substrate obtained from the perfusion medium for sodium reabsorption. Both lactate and butyrate increased sodium reabsorption with a decreased glucose utilization, lactate being the most beneficial. Results published for the rat kidney are in agreement with these findings. Glucose as the sole source of fuel did not provide adequate energy for a sufficient, steady state, sodium reabsorption in rat perfusion studies, while supplementation of lactate increased sodium reabsorption and oxygen uptake (Shurek et. al. , 1975 ; Cohen and Little , 1976 ; Merkins et. al., 1978 ; Cohen et. al. , 1980). The effect of butyrate was not

as apparent over glucose (Ross et. al. , 1973). The beneficial effects of lactate have been attributed to increased hydrogen delivery to the electron chain (Shurek et. al. , 1975), to increased glucose conservation and/or production (Cohen and Little, 1976), and to stimulation of synthesis of necessary structural components of the cell (Merkins et. al. , 1978). The lactate effect was immediately recognized during the first clearance period, whereas the effects of glucose and butyrate, or glucose alone became evident after 45 minutes of perfusion. Endogenous substrate was steadily depleted in kidneys perfused with glucose alone or without substrate at a more rapid rate than observed with glucose and lactate or butyrate together. During the final minutes of perfusion all three substrate-containing groups had plateaued, while the substrate free group continued to decline. This pattern was similar to that described in the rat kidney (Trimble and Bowman , 1973 ; Ross et. al. , 1973). This would indicate that the kidney relies on endogenous substrate at the onset of perfusion with glucose as the only energy source and is capable of switching to a possible anaerobic metabolism after depletion, whereas lactate and butyrate may prevent this switch-over.

These results indicate the potential usefulness of this system for evaluating the effects of preservation treatments on the structural and functional integrity of the kidney. The preparation is sensitive to changes which are modest in comparison to the anticipated effects of long term storage procedures.

Experiment 3: The Effects of a Vasodilator, Perfusion Pressure and Bovine Serum Albumin (BSA) on In Vitro Rabbit Kidney Function During Normothermic Perfusion.

Introduction

Several questions have been introduced based on the results of the previous experiments. The first is the need for addition of a vasodilatory agent to the flushing medium to reverse vasospasm precipitated by surgical manipulation of the kidney, and facilitate removal of blood and blood components during the initial washout prior to perfusion. The second is the choice of perfusion pressure. In the previous two experiments, kidneys were perfused without colloid at pressures which achieved a lower net filtration pressure than kidneys perfused with a colloid source. An increase in the perfusion pressure would increase perfusate flow rate and oxygen delivery to the kidney, and increase the filtered load. The response of the kidney would indicate whether or not it was capable of increased function under these perfusion conditions.

The third question is the effect of addition of small amounts of bovine serum albumin (BSA); not in concentrations to mimic the oncotic pressure of blood, but to provide protein necessary for stabilization of normal permeability characteristics of blood vessels (Landis and Pappenheimer , 1963), as a source of amino acids (Galaske et.al. , 1975), and to permit the measurement of albumin leakage at the glomerulus (Wusteman , 1977).

Methods

1). Perfusate

The perfusate was made up as previously described. All perfusion media contained 5 mM lactate added as the Na salt. The amount of bovine serum albumin included in the medium varied according to the treatment groups (Table 1).

2). Perfusion

Twenty-four kidneys were obtained in the manner previously described. The kidneys were flushed by gravity with 150 ml of solution used for the perfusion, with isoxsuprine-HCl (0.05 mg/ml), an α -adrenergic blocking agent added after the solution had been oxygenated. Following the flush , the kidneys were attached to the 37 ^{0}C perfusion circuit. The perfusion pressure was adjusted to 50 mmHg. A 10 minute stabilization period preceded the beginning of the first collection period. Following this, the kidneys were perfused at 50 mmHg for 3 consecutive 15 minute collection periods. After 45 minutes of perfusion, the perfusion pressure was increased to 75 mmHg by increasing the pump speed. The kidneys were perfused for 3 consecutive 15 minute collection periods at 75 mmHg pressure.

3). Treatment Groups

A. No BSA: Eight kidneys were perfused for 90 minutes as described above at 50 and 75 mmHg perfusion pressure.

B. 0.5% BSA: Eight kidneys were perfused for 90 minutes at 37 ^{0}C according to the protocol described above of altering the perfusion pressure after 45 minutes. In this group, the perfusate included BSA (Sigma Chemicals, St. Louis, Missouri) 0.5% (W/V).

C. 1.0% BSA: Eight kidneys were perfused as for groups I and II. The perfusate included BSA, 1.0% (W/V).

Urine and perfusate samples were collected, counted for inulin, and assayed for sodium, glucose, protein and phenol red as previously described. Perfusate flow rates were determined at the beginning of each clearance period and following the last period.

Results

1). Perfusion Characteristics

Increasing the perfusion pressure from 50 to 75 mmHg resulted in a corresponding increase in flow rate from 3.79 ± 0.28 at 50 mmHg to a mean of 6.49 ± 0.27 ml $min^{-1}g^{-1}$ at 75 mmHg pressure (Table 10). This increase was independent of the effects of including BSA in the medium, and was significant ($p < .005$). The mean perfusate flow rates for the 0.5% BSA (5.73 ± 0.37) and the 1.0% BSA groups (5.65 ± 0.42 ml $min^{-1}g^{-1}$) did not differ from each other; however, both were greater than the group perfused without BSA (4.36 ± 0.26 ml $min^{-1}g^{-1}$ $p < .01$). With the increased perfusion pressure, the GFR increased in all three treatment groups perfused at 75 mmHg as compared to 50 mmHg ($p < .005$), as seen in Figure 18. The group without BSA increased 77.9% with increased pressure from a mean of 0.282 ± 0.018 to 0.502 ± 0.027 ml $min^{-1}g^{-1}$. The group with 0.5% BSA increased 124% from 0.218 ± 0.019 to 0.487 ± 0.036, and the 1.0% group increased 64.3% from 0.161 ± 0.025 to 0.368 ± 0.038 ml $min^{-1}g^{-1}$. The mean GFR for the group without BSA (0.392 ± 0.022) was similar to the 0.5% group (0.352 ± 0.028) but significantly greater than the 1.0% group (0.264 ± 0.020 ml $min^{-1}g^{-1}$, $p < .005$). The 0.5% group was also greater than the 1.0% group ($p < .025$), the difference becoming more apparent during perfusion at 75 mmHg pressure.

The ratio of the clearance of albumin to the clearance of inulin

Table 10: Perfusate flow rates of kidneys perfused at 37°C with different concentrations of BSA and pressure (ml min^{-1} g^{-1})

	Perfusion Time (min)						
Treatment	0	15	30	45	60	75	90
No BSA	2.23* ± 0.35	2.41 ± 0.35	2.94 ± 0.38	4.56 ± 0.36	5.50 ± 0.39	6.18 ± 0.28	6.85 ± 0.35
0.5% BSA	4.60 ± 0.93	3.79 ± 0.71	3.66 ± 0.57	5.29 ± 0.60	6.39 ± 0.61	7.71 ± 0.83	8.65 ± 1.05
1.0% BSA	4.80 ± 0.67	4.13 ± 0.74	3.68 ± 0.66	5.40 ± 1.00	6.24 ± 1.12	7.37 ± 1.41	7.95 ± 1.39

* Mean ± SEM, (8 observations at each time interval)

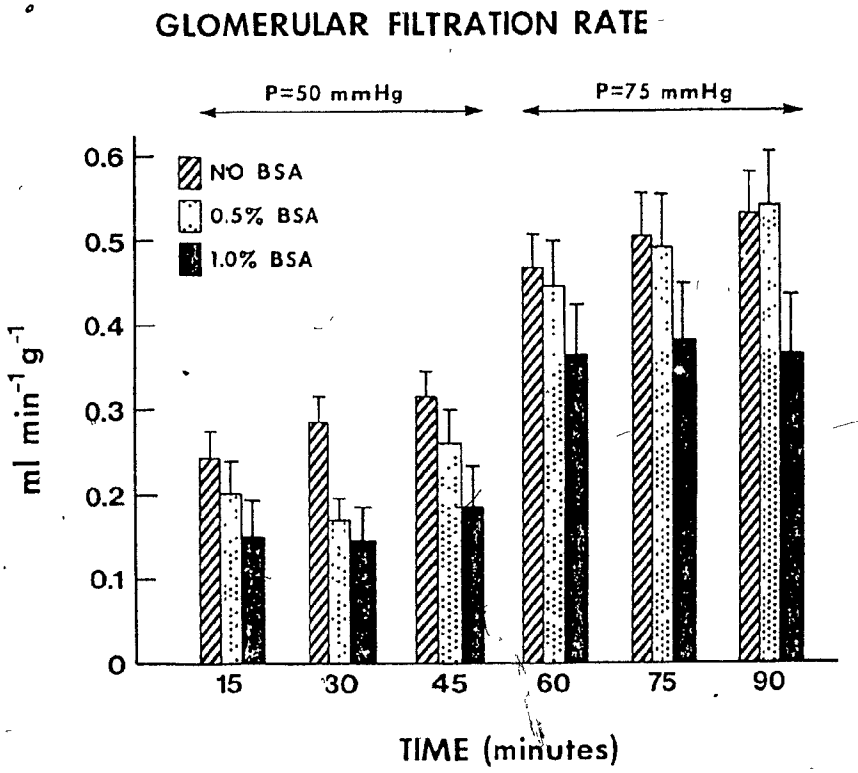

Figure 18. The glomerular filtration rate of kidneys perfused with 0, 0.5% or 1.0% BSA at 50 or 75 mmHg pressure. (Mean ± SEM, 8 observations per point).

(C_{alb}/C_{in}) is an indication of the amount of protein lost at the glomerulus. The higher the ratio, the greater the amount of protein leakage at the glomerulus. In both groups perfused with albumin the amount of leakage was very low (Figure 19).

2). Tubular Function

The percentage of filtered sodium reabsorbed (Figure 20) was higher in the 1.0% (62.34 ± 2.3%) and 0.5% (62.40 ± 2.13%) BSA perfused kidneys than kidneys perfused without BSA (38.65 ± 1.19%) ($p < .005$). In all three groups the proportion of the filtered load reabsorbed with increased perfusion pressure decreased from 44.68 ± 1.09% to 32.63 ± 1.72% in kidneys perfused without BSA ($p < .05$); from 68.17 ± 2.71% to 56.63 ± 2.89% in kidneys perfused with 0.5% BSA ($p < 0.1$), and from 68.62 ± 2.7% to 56.05 ± 3.3% in the 1.0% BSA group ($p < .05$). The sodium load transported by the tubules (Figure 21) was similar in all three groups during the period of perfusion at 50 mmHg pressure; however, with an increased pressure of 75 mmHg, the addition of 0.5% BSA (41.26 ± 2.8 μEq $min^{-1}g^{-1}$) resulted in higher rates of Na transport compared to the group perfused without BSA (24.30 ± 1.39 μEq $min^{-1}g^{-1}$, $p < .005$). A similar, but not as dramatic effect was observed with addition of 1.0% BSA (34.25 ± 3.55 μEq $min^{-1}g^{-1}$, $p < .025$). Sodium transport in the group perfused without BSA was only slightly increased with increased pressure. With BSA, at either concentration, the amount of Na^+ handled by the tubules was significantly increased ($p < .005$) at 75 mmHg over 50 mmHg

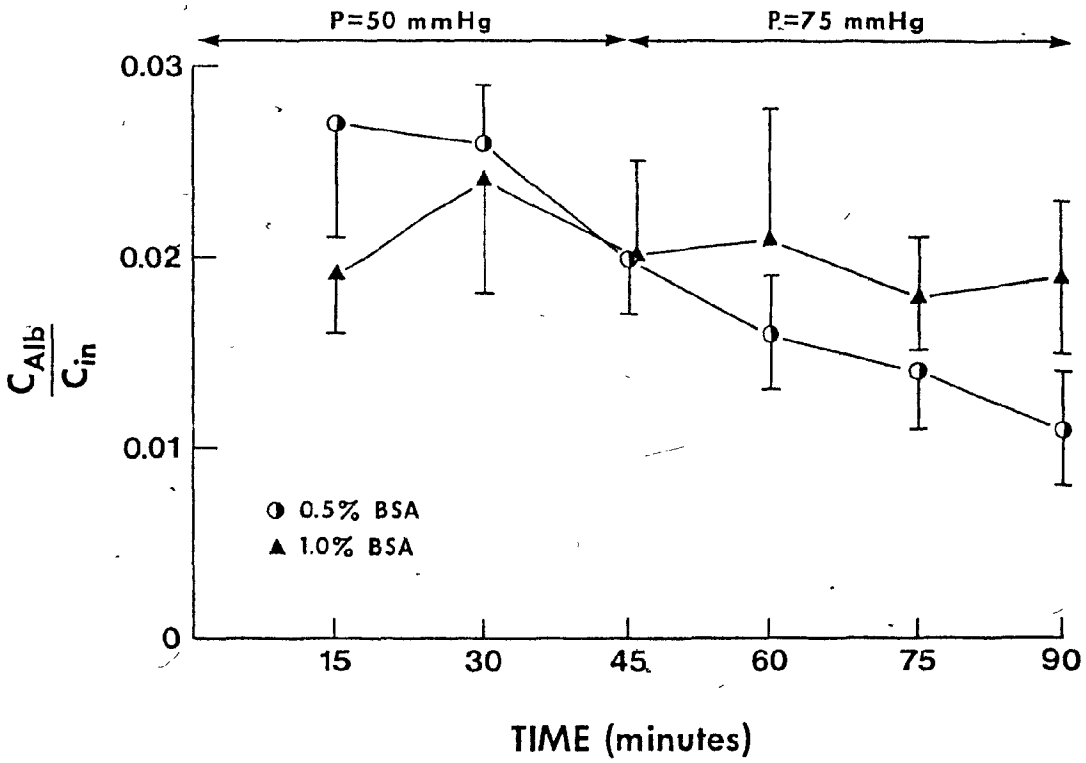

Figure 19. The ratio of the albumin clearance (C_{alb}) to inulin clearance (C_{in}) in kidneys perfused with 0.5% or 1.0% BSA as a function of perfusion time. (Mean ± SEM, 8 observations per point).

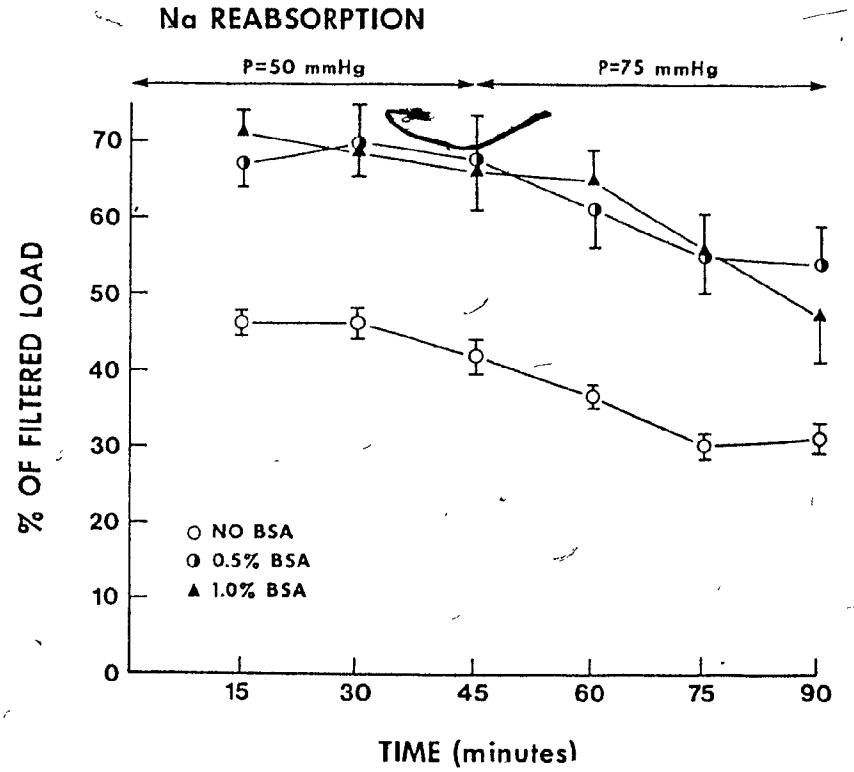

Figure 20. The percentage of filtered sodium reabsorbed by kidneys perfused with 0, 0.5 or 1.0% BSA, at 50 or 75 mmHg pressure as a function of perfusion time. (Mean ± SEM, 8 observations per point).

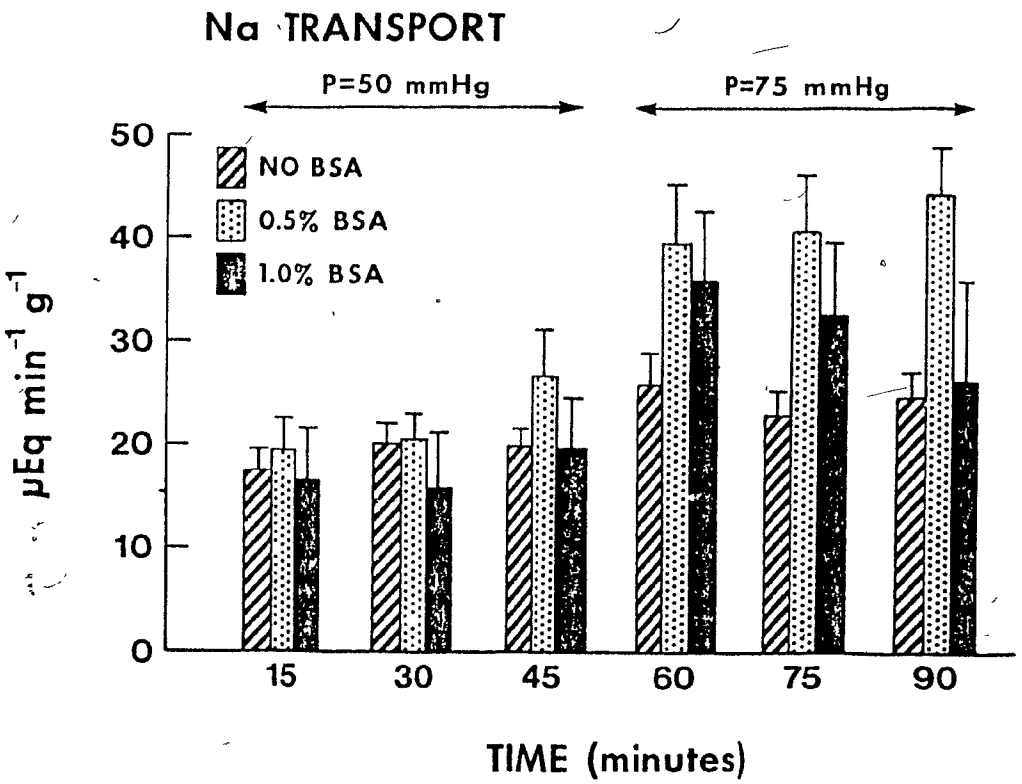

Figure 21. Sodium transport by kidneys perfused with perfusate containing 0,0.5 or 1.0% BSA, at 50 or 75 mmHg pressure as a function of perfusion time. (Mean ± SEM, 8 observations per point).

pressure.

The percentage of filtered glucose reabsorbed by the tubules (Figure 22) exhibited a similar pattern as sodium reabsorption-the 0.5% BSA group (91.11 ± 0.85%) and 1.0% (89.80 ± 1.03%) exhibited higher rates of reabsorption than the group without BSA (84.83 ± 1.21%, $p < .005$). These differences were not apparent during perfusion at 50 mmHg pressure; the reduction in glucose reabsorption in the BSA free group was greatest ($p < .005$), from 89.31 ± 1.04% at 50 mmHg to 80.36 ± 1.79% at 75 mmHg.

The ratio of the clearance of phenol red to the inulin clearance is shown in Figure 23. A ratio greater than 1.0 indicates active secretion of phenol red by the tubules. All three groups were capable of secretion; the 0.5% BSA group showed the highest variability, but was significantly better than both BSA free and 1.0% BSA groups ($p < .005$). The decline of the ratio during the final 30 minutes of perfusion in this group may not indicate a loss of secretory activity, since the high rates of secretion at the beginning exhausted the perfusate phenol red, reducing the amount of substrate available for transport. The disappearance of the phenol red from the recirculating perfusate was readily detectable and served as a visible indication of secretory function during the perfusion period.

3). Histology

Light microscopic evaluation of kidney tissue following perfusion showed that glomerular and tubular ultrastructure remained intact in

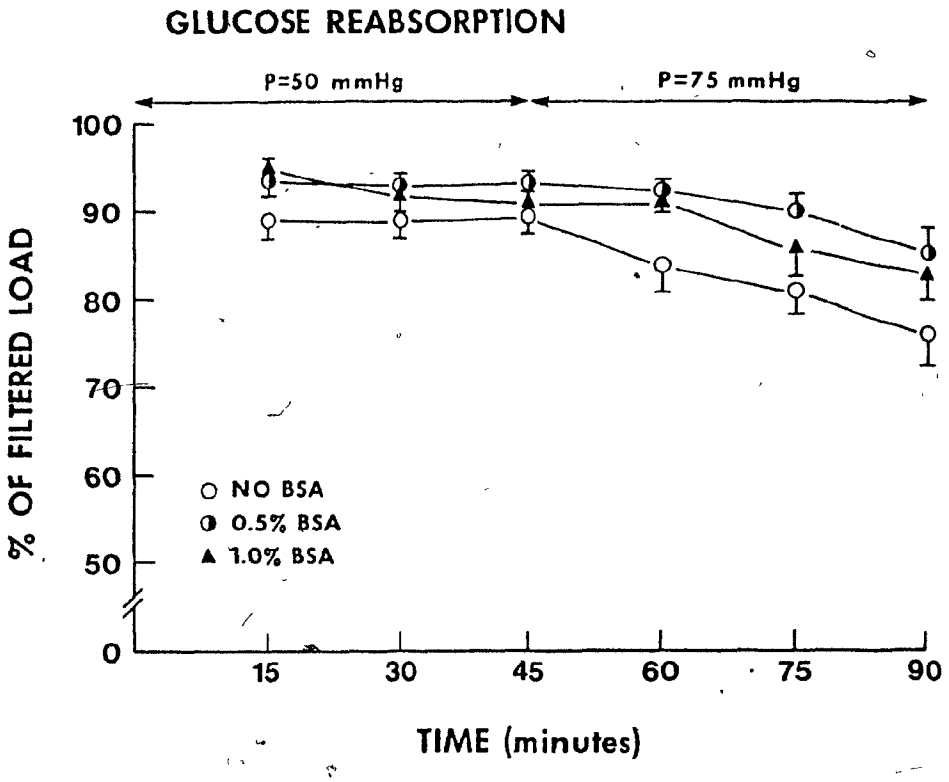

Figure 22. The percentage of filtered glucose reabsorbed by kidneys perfused with 0, 0.5 or 1.0% BSA at 50 or 75 mmHg pressure (Mean ± SEM, 8 observations per point).

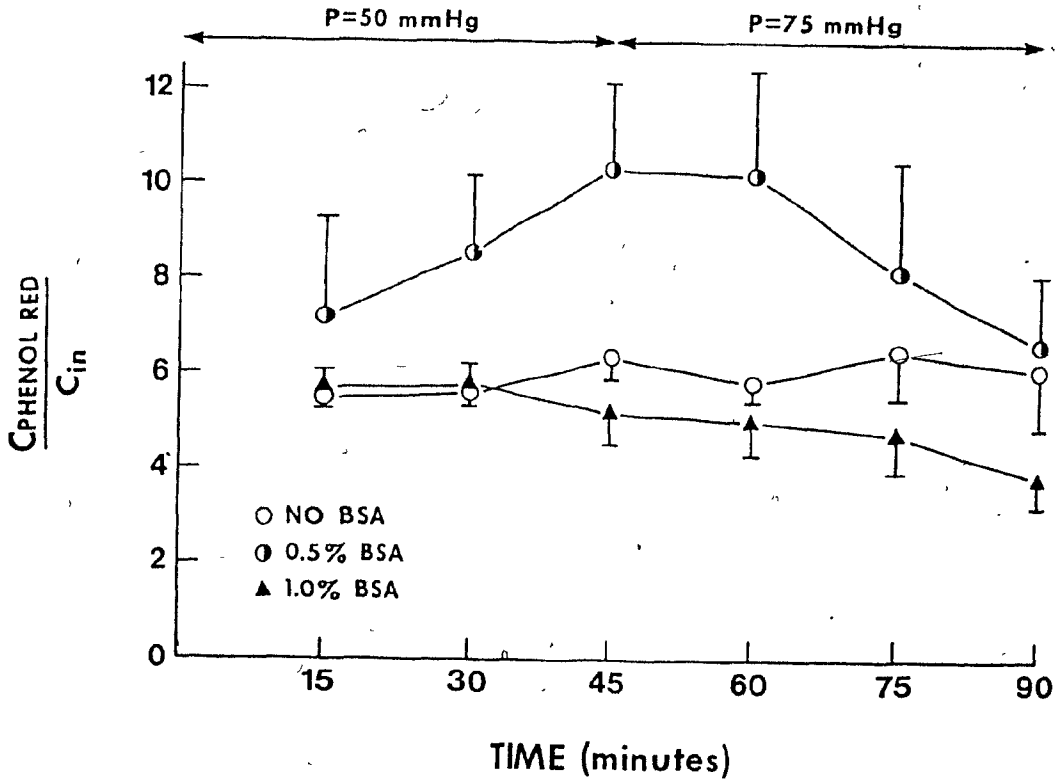

Figure 23. The ratio of the clearance of phenol red ($C_{phenol\ red}$) to inulin clearance (C_{in}) in kidneys perfused with 0, 0.5 or 1.0% BSA. (Mean ± SEM, 8 observations per point).

all treatment groups (Figure 24). Only slight differences were discerned between groups. Proximal tubules of kidneys perfused without BSA (Figure 24 A and B) appeared more dilated than kidneys perfused with BSA . Kidneys perfused with 0.5% BSA showed less dilation (Figure 24 C and D). Perfusion with 1.0% BSA (Figure 24 E and F) were similar in ultrastructure to the other groups; however Bowman's space appeared slightly more distended.

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Figure 24

Photomicrographs of kidney sections following normothermic perfusion with 0, 0.5 or 1.0% BSA. Perfusion pressure was maintained at 50 mmHg during the first three clearance periods, and increased to 75 mmHg for the following three clearance periods (H & E).

A. Kidney perfused without BSA (64X)

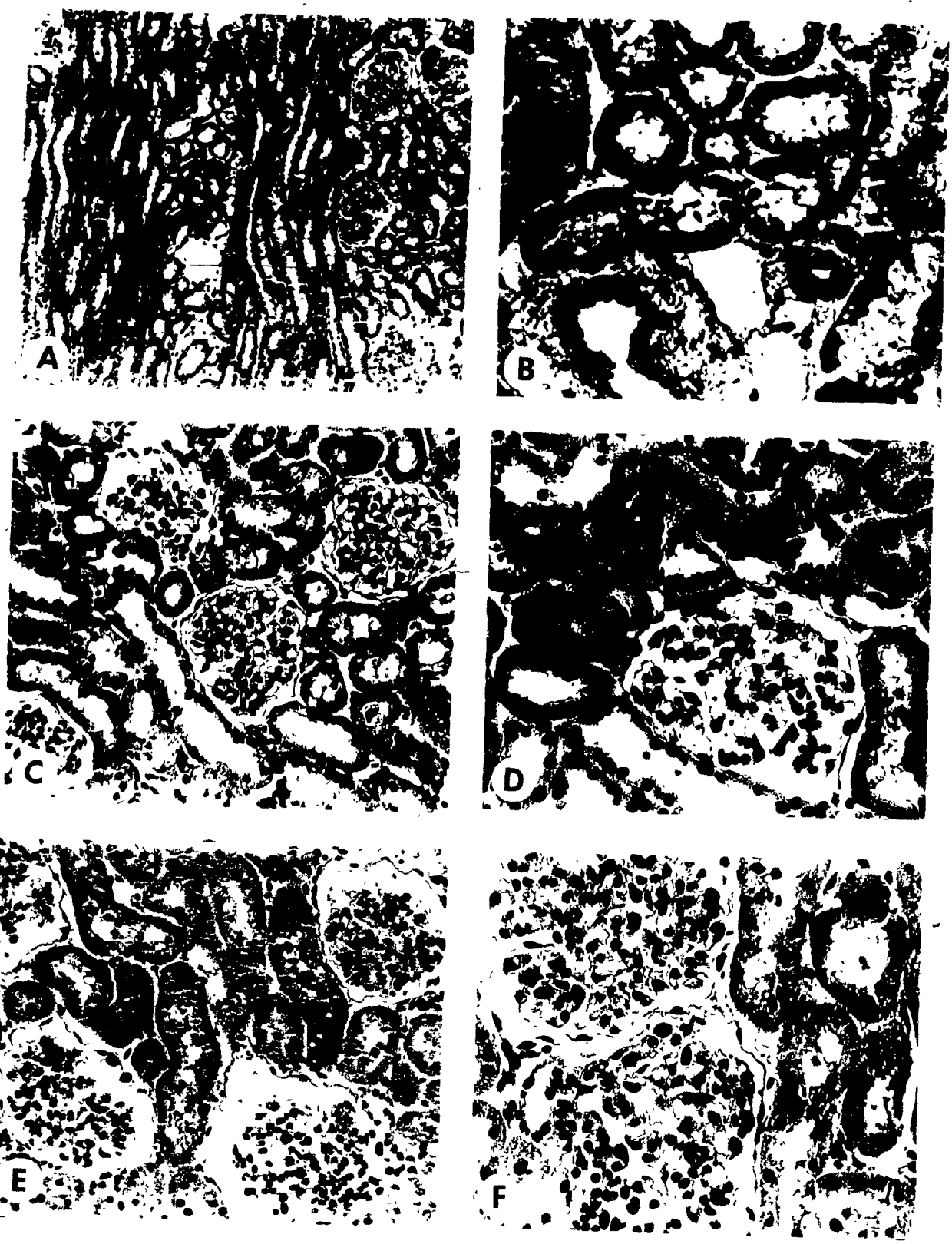
B. Kidney perfused without BSA (256X)

C. Kidney perfused with 0.5% BSA (160X)

D. Kidney perfused with 0.5% BSA (256X)

E. Kidney perfused with 1.0% BSA (160X)

F. Kidney perfused with 1.0% BSA (256X)


A
B
C
D
E
F

Discussion

The addition of isoxsuprine to the initial flushing solution did not increase the perfusate flow rate during perfusion at 50 mmHg pressure with BSA-free medium when compared to the previous work. It did prevent the slight transient increase in renal vascular resistance in comparison to the previous experiment. This effect has been reported in rabbit (Fonteles et. al. , 1973) and rat (Shurek et. al. , 1975 ; Bullivant , 1978a) kidney perfusion. The onset of vasoconstriction is thought to occur via an adrenergic mechanism, which is prevented by a beta-adrenergic agonist. The vasoconstriction may have been initiated during surgical manipulation prior to excision of the kidney, or during blood washout by a release of vasoactive substances from blood cells (Kane and Ewards , 1966). In our experiments, the infiltration of the perihilar tissue with papaverine prior to manipulation should have prevented vasoconstriction during kidney removal. Including isoxsuprine in the flush solution may have facilitated blood washout, allowing a more complete removal of blood prior to attachment to the perfusion circuit. In this way, a more uniform washout was obtained, minimizing areas of vascular occlusion and subsequent ischemia which only became apparent after perfusion progressed when the vasodilator was not included . During perfusion at 75 mmHg , the flow rate progressively increased and this is probably the result of a progressive opening of closed blood vessels, and dilatation of already opened vessels, resulting in a decreased resistance to flow

(Pegg and Farrant , 1969 ; Pegg , 1971). Although, not directly related to the vasodilator, it may have been facilitated by it. A more complete initial blood washout would prevent clotting and permanent obstruction of capillaries.

When BSA was included in the medium, renal vascular resistance increased during perfusion at 50 mmHg, but decreased during perfusion at 75 mmHg. The effect was similar in both the 0.5% and 1.0% BSA groups. It is possible that the BSA contained a substance which caused an increased renal vasoconstriction, or that the low concentrations of BSA had a facilatatory effect on the reactivity of the vascular smooth muscle to vasoconstrictors released by the kidney (Wurzel et. al. , 1964).

Despite this increase, kidneys perfused with perfusate supplemented with BSA exhibited substantially increased perfusate flow rates over kidneys perfused without BSA, at both 50 and 75 mmHg perfusion pressure. This increase may have been due to the increased viscosity of the perfusate (Pegg and Farrant , 1969). This would then have manifested as an increased flow rate in the 1.0% BSA group over the 0.5% BSA group, which was not observed in this study. Little and Cohen (1974) reported an increase in perfusate flow rate when rat kidneys were perfused with increasing BSA concentrations. They proposed that at low albumin concentrations, due to absence of colloid osmotic pressure, leakage of water and solute from the vascular bed resulted in increased interstitial and intratubular hydrostatic pressures which occluded blood vessels. The perfusion pressure was not reduced at low BSA concentrations,

which would promote excessive edema. The contribution of BSA to COP in our study was small: 1.44 mmHg at 0.5% and 2.99 mmHg at 1.0% BSA concentrations (calculated according to Brenner et. al., (1972) for BSA saline solutions). Furthermore, if the effect of BSA was oncotic, the increased extravascular pressure should have decreased the GFR in BSA free perfusions due to increased intratubular hydrostatic pressure (Cohen and Little, 1974). In this study, GFR in kidneys perfused without BSA increased steadily and was increased over the 1.0% BSA group.

The effectiveness of BSA on perfusate flow rate cannot be attributed solely to an increase in colloid osmotic pressure or viscosity. Beneficial effects of small amounts of BSA have been reported in rabbit kidney perfusion (Pegg, 1970; Fonteles et. al., 1973; Fuller and Pegg, 1976). In these studies, dextran was included. Pegg (1970) hypothesized that the BSA exerted its effects by complexing with the dextran and preventing leakage into the interstitium. In the present study, dextran was not included, yet the effect was still apparent, Another suggestion is that BSA exerts its action on the capillary membrane. Landis and Pappenheimer (1963) suggested that protein adsorption to capillary membranes could regulate pore diameter. During perfusion without protein, progressive removal of adsorbed protein could result in a sevenfold increase in pore diameter. Including small amounts of protein would therefore reduce pore size, retain a much greater intravascular volume and perhaps increase vascular elasticity. The observed increase in flow rates with BSA are consistent with these

assumptions. The permeability of the glomerulus to perfusate albumin was low, with no difference between the BSA containing groups. The extent of leakage was considerably less than reported for rabbit kidneys perfused with synthetic colloid or BSA (5%) (Fuller et. al. , 1977) and similar to values found by Wusteman (1977) who reported that the ratio, C_{alb}/C_{in}, was a sensitive indicator of ischemic damage.

The increase in perfusion pressure increased the GFR in all three groups. Slight differences in the groups at each pressure are consistent with the calculated BSA contribution to colloid osmotic pressure and membrane permeability. GFR was higher in the BSA free group due to a lack of colloid osmotic pressure and an increased glomerular membrane permeability. The GFR in the 0.5% BSA perfused kidneys was higher than the 1.0% BSA group, a manifestation of a lower oncotic effect. In addition, the albumin leakage in kidneys perfused with 1.0% BSA remained stable, in contrast to the general decrease observed in the 0.5% group. This may indicate that as the BSA concentration is increased, additional problems, as seen by Fuller et. al.(1977) begin to manifest, peculiar to rabbit kidneys.

By increasing the perfusion pressure, the results of kidney perfusion in our system approach the net filtration pressure achieved in experiments utilizing a colloid source. The corresponding rise in flow rate increases oxygen delivery to the kidney. If the oxygen supply at lower perfusate flow rates was limiting, the increased flow should have allowed the kidney to handle the increase in filtered load. Kidneys perfused without BSA did not maintain the fractional re-

absorption of sodium or glucose, but maintained the same level of sodium uptake . In this group, the oxygen supply at 50 mmHg and reduced flow rate was not limiting function. Pegg and Green (1973) estimated that a flow rate of 4 ml $min^{-1}g^{-1}$ would provide sufficient dissolved O_2 to supply the requirements of a kidney under in vivo conditions. Kidneys operating in vitro do not perform all the functions demanded in vivo and consequently require less oxygen.

When BSA was included, the kidney was capable of responding to the increase in filtered load. Sodium uptake increased and the kidney was able to maintain the proportion of filtered sodium and glucose.

The enhancement of tubular reabsorption by including albumin for peritubular colloid osmotic pressure has been shown for rat kidney (Little and Cohen , 1974 ; Bowman and Maack , 1974), isolated rat tubules (Brenner et. al. , 1969 ; Spitzer and Windhager , 1970 ; Green et. al. , 1974) and rabbit proximal tubules (Grantham et. al. , 1972 ; Imai and Kokko , 1974). Although the mechanism of action of albumin is not completely understood, it is generally accepted that its role in Na^+ reabsorption is oncotic in nature. The process of sodium reabsorption appears to be a two-step process : solute enters tubular cells from the lumen and is actively transported into the lateral channels, followed passively by water. Protein in peritubular capillaries exerts an oncotic effect which contributes to the movement of intercellular space fluid across the tubular basement membrane. If little protein is present, a buildup of solute and water occurs at the lateral cell surface

causing expansion, and lumenal backflux increases through the tight junctions (Imai and Kokko , 1972). Evidence in support of this hypothesis has been reported by Quinn and Marsh (1979) who showed that interstitial pressure is influenced by changes in hydrostatic and oncotic forces within the peritubular capillaries. It seems that the effects of albumin in our case was due to (1) a slight oncotic effect which was sufficient to prevent the backflux observed in BSA free groups; (2) a possible effect on the capillary permeability and (3) provision of some alternate energy source (Galaske et. al. , 1979).

Determination of the ratio of clearance of phenol red to C_{in} indicated that these kidneys retain the ability to secrete organic anions. Secretion with 0.5% BSA was greater than with 1.0% BSA. This may be due to increased binding to protein at the higher concentration (Weiner , 1973).

In conclusion, the protocol was designed to look at the need for a vasodilator, the effect of increased pressure and of BSA. Under the conditions of our experiment, initial flushing with the adrenergic blocking agent, isoxsuprine, did provide some benefits to subsequent function. In all parameters measured, the addition of small amounts of BSA to the perfusate resulted in better kidney function, enabling the kidney to handle an increased work load when pressure was increased.

Experiment 4: The Sensitivity of the In Vitro Perfused Rabbit Kidney Preparation to One Hour Ischemia and Treatment with an Antidiuretic Preparation.

Introduction

To study the effects of cryobiological manipulation of isolated rabbit kidneys, we have developed an *in vitro* normothermic perfusion system. The previous experiments delineated several requirements for maintenance of an adequate level of *in vitro* function during colloid-free reduced pressure perfusion.

The following experiment was performed to study the sensitivity of this system, by comparing function of kidneys perfused with minimal ischemic time prior to perfusion to kidneys subjected to one hour of warm ischemia by in situ clamping of the renal artery. The capacity of control kidneys to respond to an antidiuretic preparation was also examined.

Methods

1). Perfusate

The perfusate was prepared as described in section IV (A) of materials and methods and consisted of the basic salt solution supplemented with glucose, 5.56 mM, lactate, 5.0 mM and BSA (0.5% w/v, Table 1). One litre of perfusate was added to the perfusion circuit.

2). Perfusion

The kidneys were harvested as described in section I of materials and methods. The kidneys were flushed by gravity with 150 ml of perfusate supplemented with insulin (0.1 U/ml), heparin (10 IU/ml) and isoxsuprine-HCL (0.05 mg/ml), after the solution had been gassed with 95% O_2: 5% CO_2 at 25 ^{0}C. The kidneys were attached to the perfusion circuit and the pressure adjusted to 75 mmHg. Perfusate was recirculated through the kidney for a 10 minute stabilization period. Following this, the kidneys were perfused for four consecutive 15 minute collection periods.

3). Treatment Groups

A. Minimal Ischemia: Four kidneys were cannulated, flushed and attached to the perfusion circuit immediately after interruption of blood flow. The ischemic time was less

than 2 minutes. Two consecutive clearance periods were performed. At the completion of the second clearance period, vasopressin (ADH, 50 mU/l Pitressin, Parke Davis) was added to the reservoir and urine was collected for two further clearance periods.

B. One Hour Ischemia: Four kidneys were rendered ischemic for one hour following dissection of the kidney prior to removal. The renal artery was clamped in situ, and the abdomen was closed with towel clips for 60 minutes. The kidneys were removed, flushed and then perfused for 60 minutes at 37°C.

Arterial and urine samples were collected and kidney function assessed. Arterial and urine osmolality was determined. The free water clearance in group I kidneys was calculated according to the formula below:

$C_{H_2O} = V - C_{osm}$ (Pitts, 1971)

where V = urine volume (ml min^{-1})

$C_{osm} = U_{osm} \times V / P_{osm}$

where: C_{H_2O} is the free water clearance

C_{osm} osmotic clearance

Results

1). Perfusion Characteristics

The initial flush period prior to attachment to the perfusion circuit was prolonged in the group of kidneys subjected to sixty minutes of warm ischemia because blood washout was not complete after 150 ml of washout media had flowed through the kidney. Blood continued to be washed from the kidney after attachment to the circuit. The perfusate was not recirculated until it appeared clear of blood. One hour of ischemia depressed the perfusate flow rates in comparison to the controls (Figure 25). Flow rates over 60 minutes averaged 3.17 ± 0.52 ml $min^{-1}g^{-1}$ for ischemic kidneys, significantly lower than the control average of 5.70 ± 0.34 ml $min^{-1}g^{-1}$ ($p < .005$). When ADH was added to non-ischemic kidneys, renal vascular resistance was increased momentarily. The pressure increased by 5 mmHg. This effect was transient and for the remaining 30 minutes of perfusion the resistance decreased.

The effect of ischemia on GFR is seen in Figure 26. GFR was variable in the ischemic group, but was considerably lower than controls at all times. The mean over 60 minutes was 0.103 ± 0.029 ml $min^{-1}g^{-1}$; the control group GFR averaged 0.467 ± 0.031 ml $min^{-1}g^{-1}$ ($p < .001$). Glomerular retention of albumin was impaired in ischemic kidneys (Figure 27). Leakage of albumin decreased with time; however the ratio after 60 minutes of perfusion remained high in comparison to very low levels of leakage seen in controls.

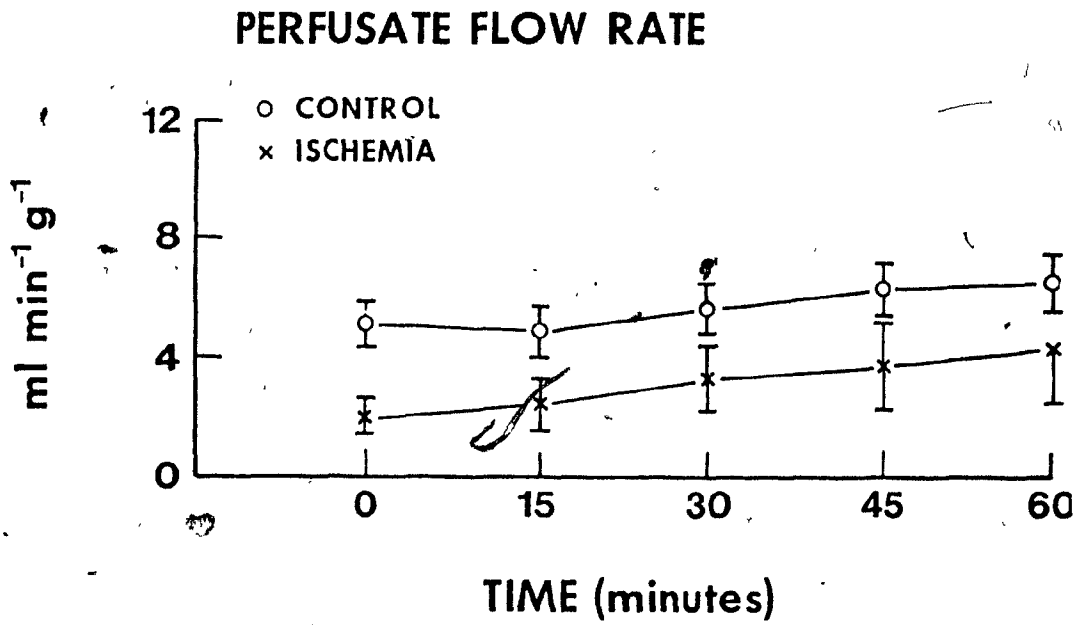


Figure 25. Perfusate flow rates of control and one-hour ischemic kidneys as a function of perfusion time (Mean ± SEM, 4 observations at each point).

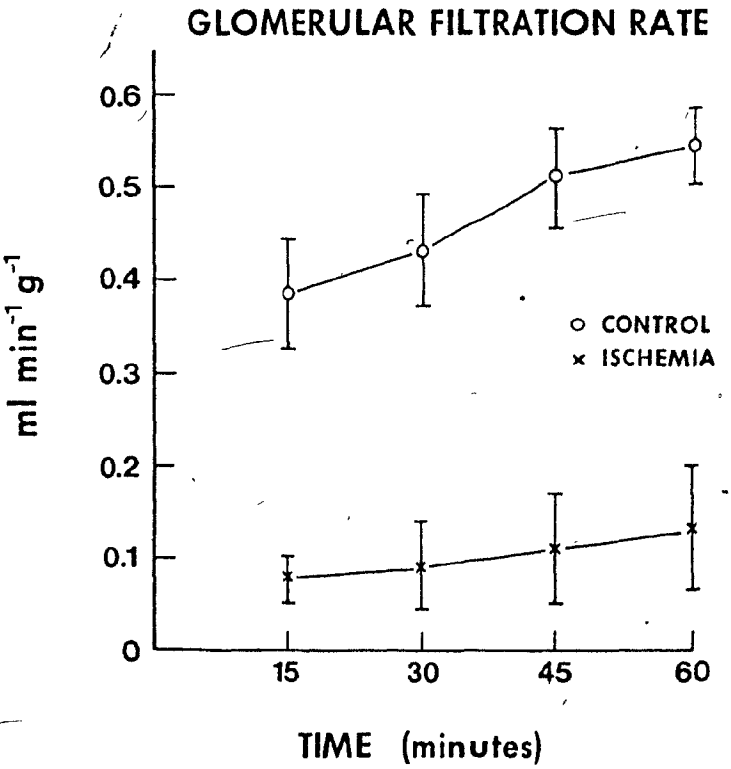


Figure 26. The Glomerular filtration rates of control and one-hour ischemic kidneys as a function of perfusion time. (Mean ± SEM, 4 observations at each point).

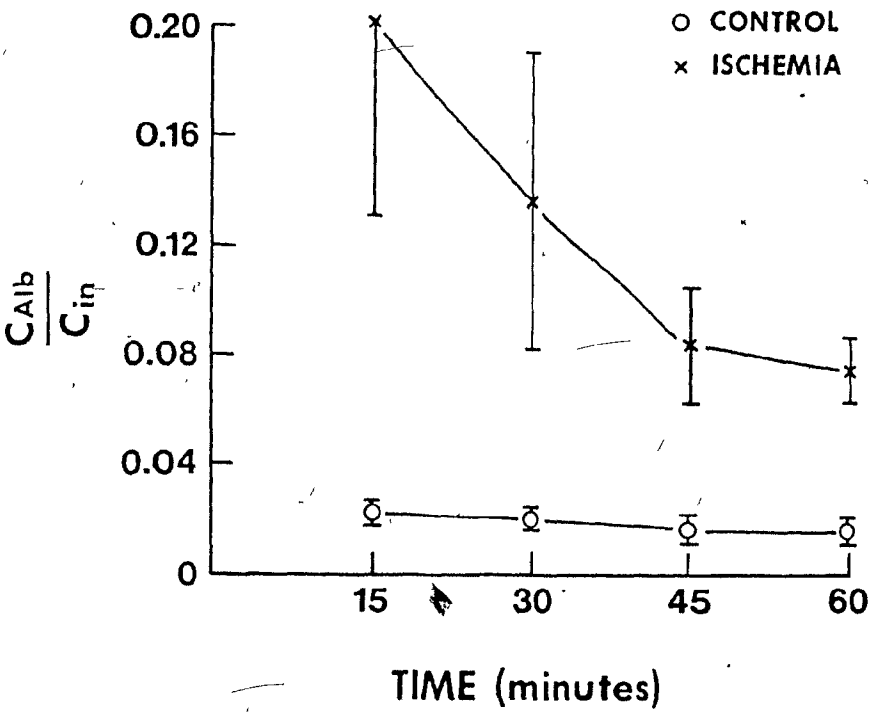


Figure 27. The ratio of the albumin to inulin clearance in control and one-hour ischemic kidneys as a function of perfusion time. (Mean ± SEM, 4 observations at each point).

2). Tubular Function

Figures 28 and 29 indicate the capacity of the kidneys to reabsorb sodium. One hour of ischemia severely impaired sodium reabsorption. Control kidneys averaged 61.22 ± 1.85% reabsorption of filtered sodium, significantly higher than the ischemia kidneys which averaged 16.8 ± 2.48% ($p < .005$). Sodium uptake by tubules of ischemic kidneys was only one tenth (3.8 ± 1.25 μEq $min^{-1}g^{-1}$) of controls (43.02 ± 2.68 μEq $min^{-1}g^{-1}$).

The proportion of filtered glucose reabsorbed in both groups is shown in Figure 30. Controls maintained an average reabsorption of 95.09 ± 0.23% during the 60 minutes. In ischemic kidneys, it was depressed and averaged 51.14 ± 6.82% ($p < .005$). Impairment of secretory activity with ischemia was also significant (Figure 31). Control $C_{phenol\ red}/C_{in}$ ratios were much higher (7.27 ± 0.30) than ischemic kidneys (3.47 ± 0.28 , $p < .005$).

Table 11 shows the effect of addition of ADH to the perfusate after the second clearance period. The values for free water clearance (C_{H_2C}) are expressed as a ratio of the first clearance period. The C_{H_2O} increased in all kidneys in the second clearance periods; addition of ADH reversed this pattern during the third and fourth clearance periods, although a negative value was never obtained.

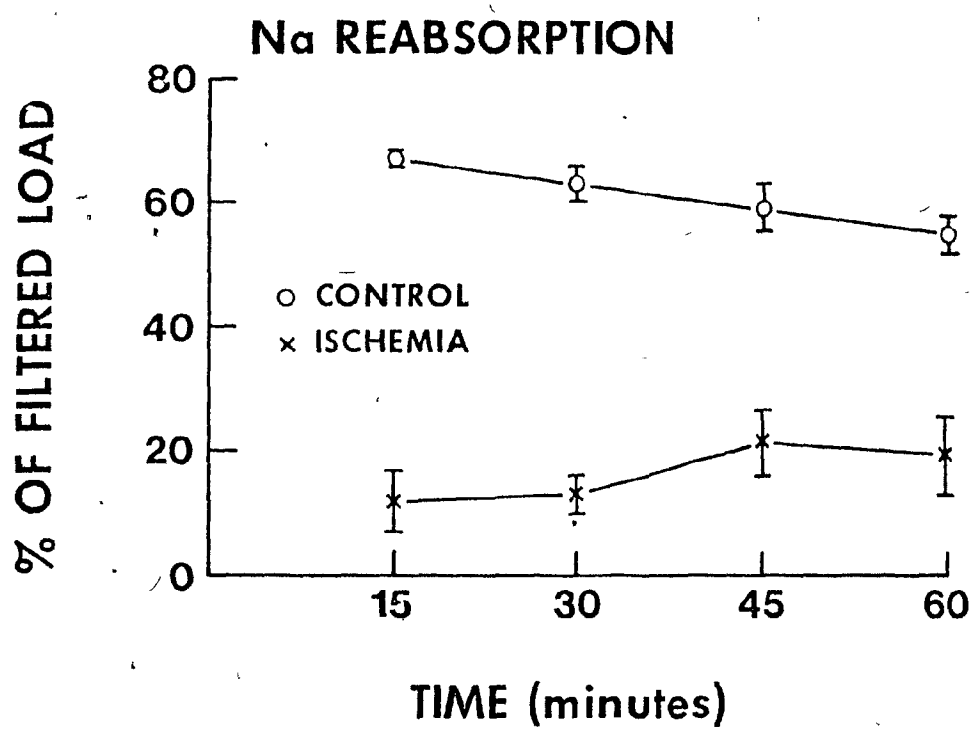


Figure 28. The percentage of filtered sodium reabsorbed by control and one-hour ischemic kidneys as a function of perfusion time. (Mean ± SEM, 4 observations at each point).

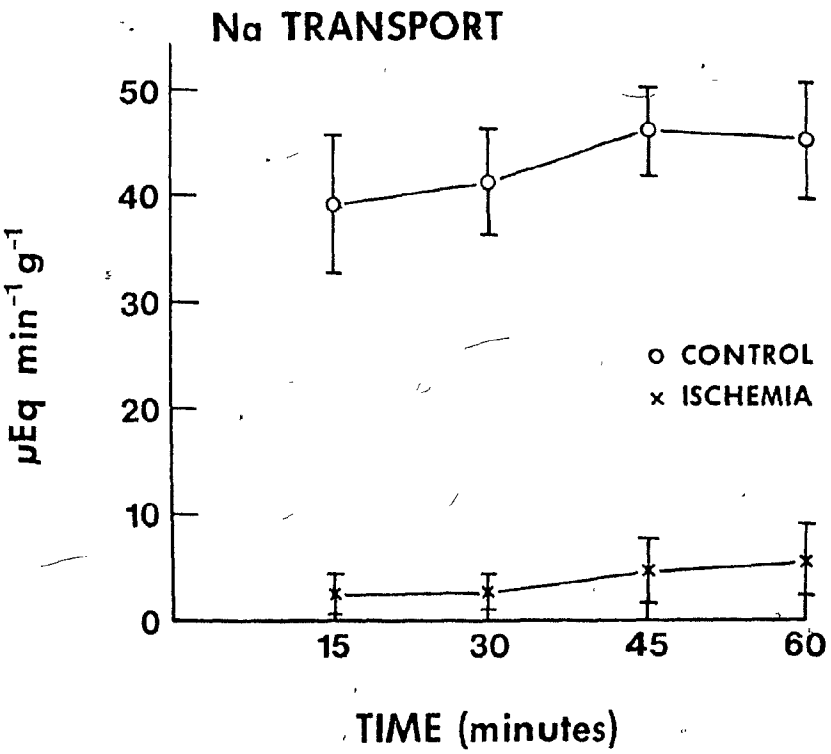


Figure 29. Sodium transport by control and one-hour ischemic kidneys as a function of perfusion time (Mean ± SEM, 4 observations at each point).

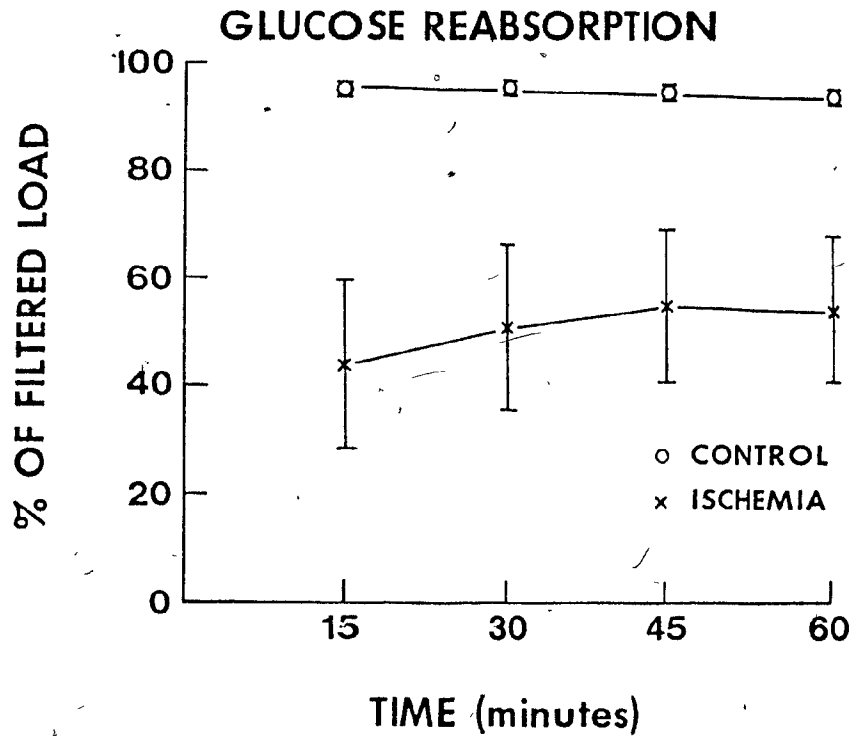


Figure 30. The percentage of filtered glucose reabsorbed by control and one hour ischemic kidneys as a function of perfusion time. (Mean ± SEM, 4 observations at each point).

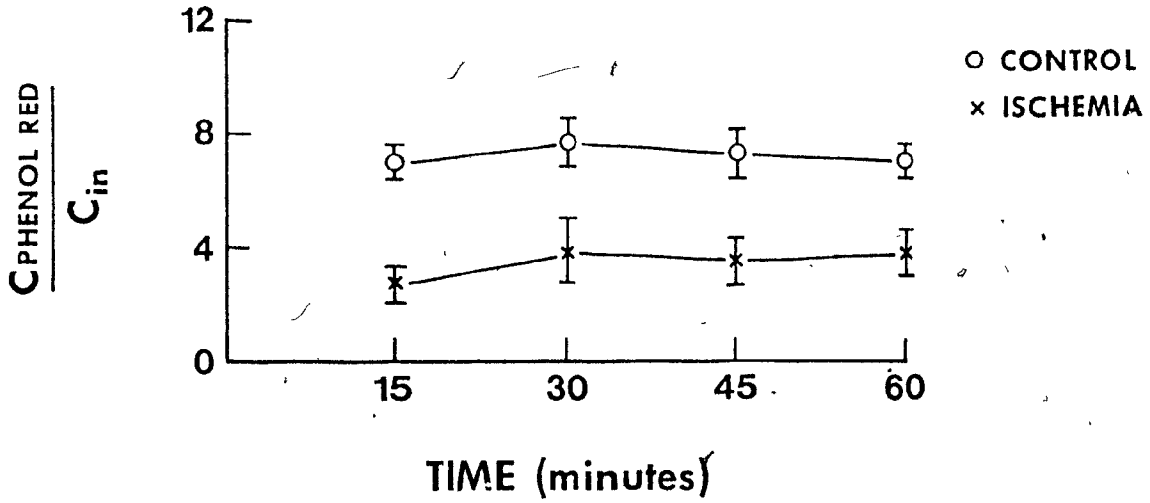


Figure 31. The ratio of phenol red to inulin clearance in control and one-hour ischemic kidneys as a function of perfusion time (Mean ± SEM, 4 observations at each point).

Table 11: Free water clearance in kidneys perfused before and after addition of ADH 50 mU/l. Expressed as the ratio to initial free water clearance.

	Perfusion Time (min)		
15	30	45	60
1	1.773* ± 0.233	0.981 ± 0.206	0.612 ± 0.224

*Mean ± SEM, (Four observations at each point)

Discussion

The freshly isolated controls in this study exhibited a significant amount of function. Table 12 was formulated to enable a comparison of this function to other studies which used the in vitro kidney model. In all cases, the apparatus, perfusate, colloid and time interval were variable, but the values were estimated from the treatment which exhibited the greatest degree of function. Several interesting points are illustrated. In the groups using the rabbit kidney, two studies are presented in which the rabbit kidney was perfused without colloid (Fonteles et. al. , 1973 ; Karow and Jeske, 1976). The first report used Tyrode solution, the second an intracellular ionic composition. Both studies reported low levels of flow rate, GFR and sodium transport in comparison to our system at 37 ^{0}C. The remaining studies using the rabbit kidney contained a source of colloid osmotic pressure. In these studies the flow rate was higher than in our study; however the GFR, sodium reabsorption and albumin retention was slightly better in our system despite the lack of colloid osmotic pressure. Glucose reabsorption was increased over that reported at 60 minutes for these studies. In our system the resumption of function after removal was rapid; equilibrium was established very quickly. In the studies using colloid, initial function was depressed, reaching optima after one hour of perfusion, followed by stabilization and/or reduction of function. In rat perfusion studies, the source of colloid osmotic pressure was BSA

Table 12: Summary of perfusion results of rat and rabbit kidneys with and without colloid.

Species	Colloid	Flow ($ml\ min^{-1}g^{-1}$)	GFR ($ml\ min^{-1}g^{-1}$)	R_{Na} %	R_{Na} $\mu Eq\ min^{-1}g^{-1}$	$R_{Glucose}$ %	C_{alb}/C_{in}	Reference
Rabbit	✓	16.0	0.30	–	32.0	–	0.2	Fuller and Pegg, 1976
	✓	14.0	0.30	60.0	33.0	80.0	0.06	Fuller et. al. , 1977
	✓	9.9	0.50	50.0	–	80.0	0.05	Wusteman, 1977
	✓	–	0.41	53.1	36.1	81.7	0.05	Pegg and Wusteman, 1977
	x	0.7	0.09	50.0	7.0	–	–	Fonteles et. al.,1973
	x	0.7	0.10	70.0	2.14	–	–	Karow and Jeske, 1976
Rat	✓	12.0	0.50	82.0	55.7	–	–	Bullivant, 1978
	✓	33.0	0.54	97.0	–	98.0	–	Maack, 1980
	✓	27.0	0.58	86.0	–	–	–	Merkens et. al., 1978
	✓	–	0.66	87.8	84.1	–	–	Cohen et. al., 1980
	x	5.0	0.25	55.0	18.1	–	–	Little and Cohen, 1974
	x	–	0.49	59.2	40.96	–	–	Bowman and Maack, 1974
	x	5.7	0.47	61.2	43.02	95.1	0.02	Present study

(6.0-7.7%). It is obvious that the rat kidney model exhibits superior Na^+ reabsorption and higher GFR than in rabbit kidneys perfused with or without colloid; perfusion of rabbit kidneys with 5% BSA did not maintain good function (Fuller et. al. , 1977). The table also presents values obtained in rat kidneys perfused without colloid (Little and Cohen , 1974 ; Bowman and Maack , 1974). In these studies, the perfusion pressure was not reduced to compensate for a lack of colloid. Function in these kidneys was very similar to our own studies with rabbit kidneys perfused without colloid at reduced pressure. In one study (Bowman and Maack , 1974), a gradual increase in the BSA concentration returned Na reabsorption to values obtained with kidneys perfused with 7.5% BSA.

The perfusion system which is presented in this study is not capable of mimicking in vivo function; however, it is not subject to the same environment, and seems to function optimally under the given conditions. The sensitivity of the kidney under these conditions is sufficient to determine deleterious effects. Renal ischemia has been used to study events of hemodynamically-mediated acute renal failure, and elicits changes which manifest in vivo -an increased vascular resistance, low GFR, and increased sodium excretion (Stern et. al. , 1978). In this study the effect of ischemic insult was clearly demonstrated in kidneys perfused after one hour ischemia. The differences between control kidneys and ischemic kidneys were significant in all aspects of function considered. Ischemic kidneys demonstrated depressed function very similar to rabbit kidneys perfused with colloid after the same ischemic insult (Wusteman , 1977).

The inability of the perfused kidney to concentrate urine has been

recognized in rabbit (Fuller et. al. , 1977) and rat (Maack , 1980) experiments. It is considered to be a consequence of high perfusate flow rate in the vasa recta, eliminating the concentration gradient in the inner medulla, and due to abnormal transport functions of the distal nephron (De Mello and Maack , 1976). In this study, addition of ADH to the perfusate depressed free water clearance. This indicates that the action of ADH, which involves hormone-receptor complexing, activation of adenyl cyclase and c-AMP mediated changes in membrane permeability (Handler and Orloff , 1973), is still demonstrable under these perfusion conditions.

Experiment 5: Function of Rabbit Kidneys Following Perfusion at 10^0 C with an Intracellular Electrolyte Solution.

Introduction

Most commonly, extracellular type solutions with synthetic or plasma-derived colloid are used for kidney preservation by hypothermic perfusion. For hypothermic storage, flushing without perfusion, formulations, with high potassium and reduced sodium concentrations are used to maintain intracellular potassium and retard sodium influx at temperatures where the ion pump is inhibited.

When a cryoprotectant is included in the perfusate it is essential that the vehicle for cryoprotectant administration is compatible with the cryoprotectant, and that the combination of the two are not deleterious to the kidney. When Me_2SO is chosen as cryoprotectant, the choice of perfusate is especially critical because the effects of Me_2SO on cells may be rendered less harmful if the proper perfusate is used (Keeler et. al. , 1966 ; Karow et. al. , 1979). The literature suggests that the perfusate for addition of Me_2SO should contain high amounts of potassium, and decreased amounts of sodium.

The inclusion of a non-permeating solute to control cell swelling is also an important substituent in hypothermic treatment of kidneys (Pegg and Wusteman , 1977). Fahy et. al. (1979) reported an improved perfusate for hypothermic renal preservation which maintaned tissue slice viability for 4 days at hypothermia. This medium included high potassium, low sodium, glucose as the major

impermeant anion, and contained glutamine and adenine which were important in extension of the storage time of the slices. It has not as yet been tested for whole organ preservation, but offers promise especially with the inclusion of Me_2SO. The following experiment was performed to test a modified version of this perfusate as a potential vehicle for Me_2SO administration.

Methods

1). Perfusate

(i) Hypothermic perfusate: The perfusate for 10 ^{0}C kidney perfusion was described in section IV (B) of the materials and methods. For these experiments, 400 ml of perfusate (1X., 400 mos/kg, Table 3) was used to prime the cold perfusion circuit.

(ii) Normothermic perfusate: The composition of the perfusate for 37 ^{0}C perfusion was identical to that described in experiment 4.

2). Perfusion

The kidneys were removed and flushed in the manner previously described. Kidneys which served as freshly isolated controls were treated in the same manner as in the previous experiment. Kidneys which were perfused at 10 ^{0}C were transfered to the 10 ^{0}C circuit after the initial flush. The kidney was attached and the screw clamp above the artery was opened fully to allow maximum flow. The organ chamber cover was lowered and the kidney was perfused for 75 minutes. The kidneys were then removed and immediately transferred to the 37 ^{0}C circuit. The temperature of perfusate was 27-30 ^{0}C at the time of attachment. By increasing the rate of water

circulation through the heat exchanger, the temperature was increased to 37 ^{0}C during the first 5 minutes. Thereafter, the kidneys were perfused in an identical manner to the freshly isolated controls.

3). Treatment Groups

A. Freshly isolated controls: Five kidneys were transferred to the 37 ^{0}C and 75 mmHg pressure for four consecutive clearance periods of 15 minutes each.

B. Hypothermic perfusion: Five kidneys were perfused at 10 ^{0}C, 60 mmHg pressure for 75 minutes followed by assessment of function at 37 ^{0}C. Samples and calculations were similar to those described in previous experiments.

Results

1). Perfusion Characteristics

The perfusate flow rates are shown in Figure 32. Although in both groups the initial flow rates were similar, the hypothermic-perfused kidneys showed significantly increasing flow rates with perfusion time ($p < .05$), whereas the control group flow rates did not increase to the same extent. The average perfusate flow rates in control kidneys over the 60 minute perfusion period was 5.71 ± 0.28 ml $min^{-1}g^{-1}$, lower than the hypothermic-perfused kidneys average of 9.07 ± 0.56 ml $min^{-1}g^{-1}$ at 37^{0}C ($p < .001$).

The glomerular filtration rates were similar in both groups, and exhibited a significant increase over initial values ($p < .01$, Figure 33). The albumin leakage at the glomerulus was low for both groups (Figure 34). In kidneys perfused at 10^{0}C, the amount of leakage was higher than controls at the beginning of the 37^{0}C perfusion; however protein retention improved in this group with time ($p < .05$) and approached control values by 60 minutes of perfusion at 37^{0}C.

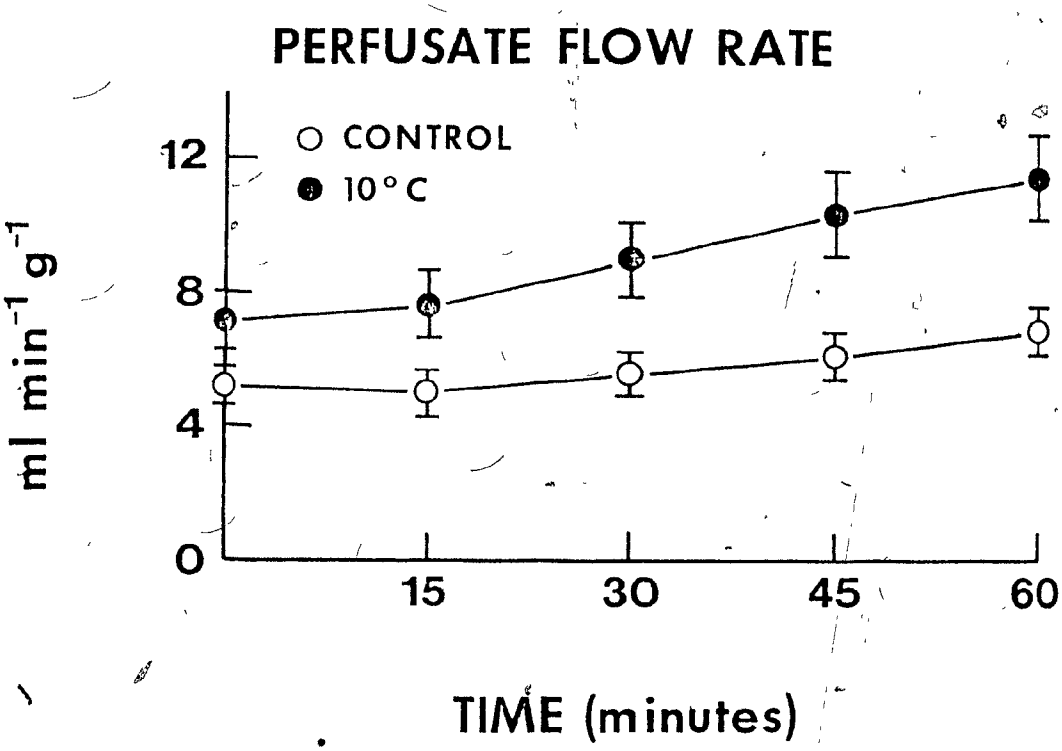


Figure 32. Perfusate flow rates during normothermic perfusion of control and hypothermic-perfused kidneys. Expressed as a function of perfusion time (Mean ± SEM, 5 observations at each point).

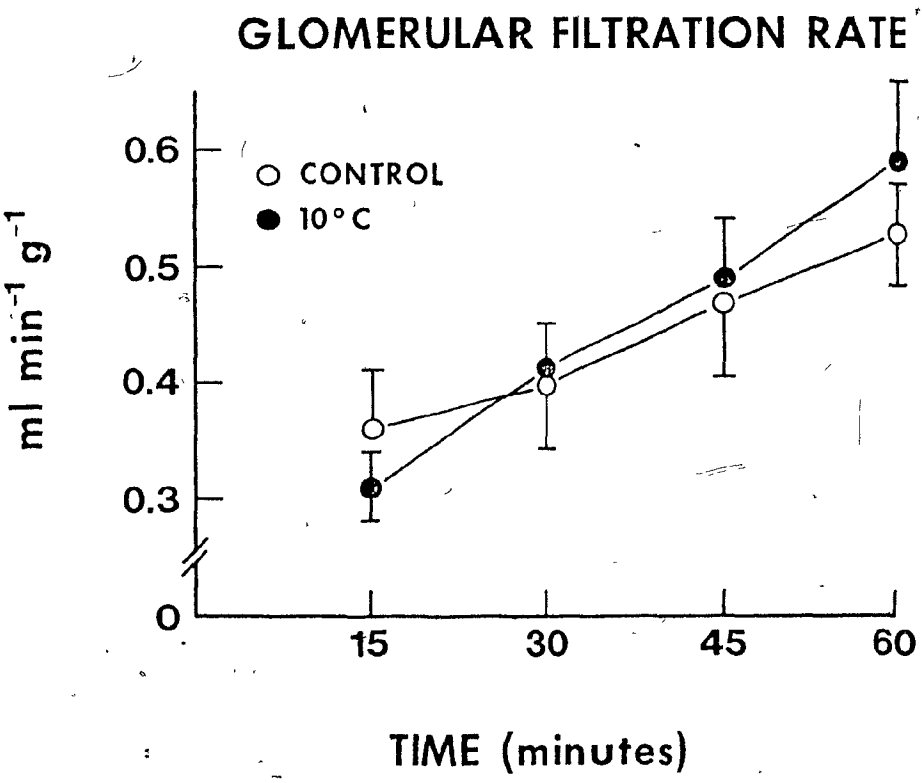


Figure 33. Glomerular filtration rates during normothermic perfusion of control and hypothermic-perfused kidneys. Expressed as a function of perfusion time (Mean ± SEM, 5 observations at each point).

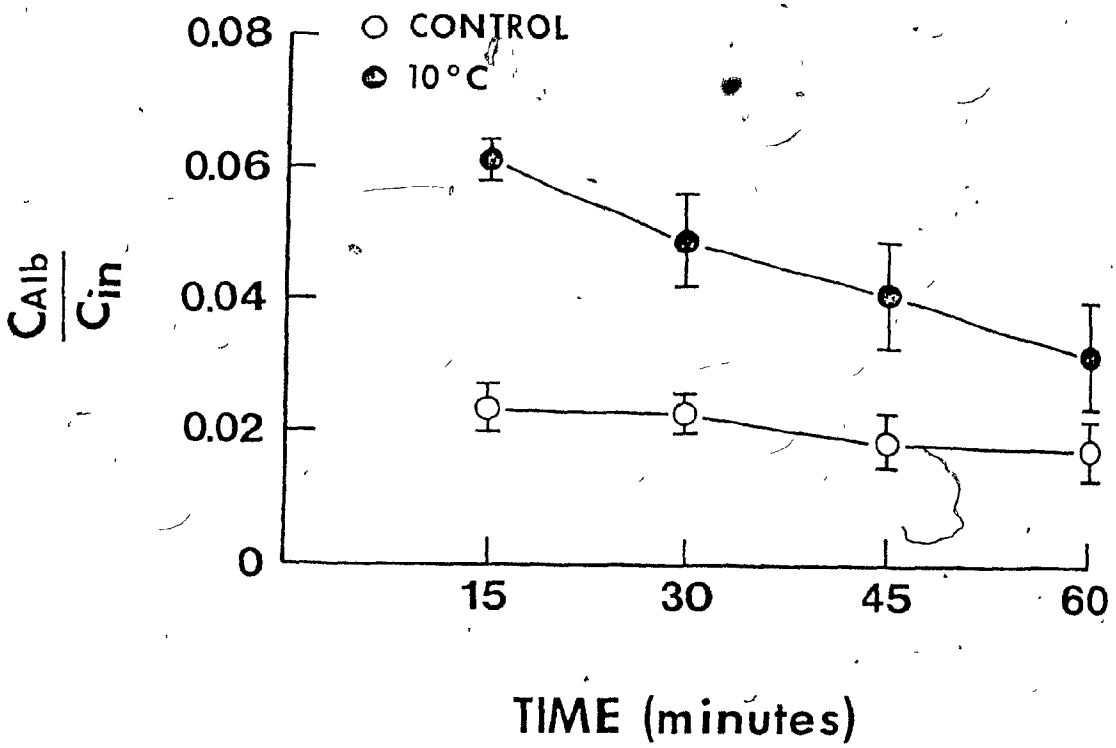


Figure 34. The ratio of albumin to inulin clearance during normothermic perfusion of control and hypothermic perfused kidneys as a function of perfusion time (Mean ± SEM, 5 observations at each point).

2) Tubular Function

The percentage of filtered sodium reabsorbed averaged 63.57 ± 1.95% and 56.88 ± 0.96% in control and 10°C kidneys respectively over the 4 clearance periods. This difference was significant ($p < .01$). The difference between the two groups was greatest at the onset of perfusion (Figure 35) but diminished gradually; at 60 minutes, they were quite similar. Sodium transport by tubules exhibited a similar pattern (Figure 36). Initial rates were lower in the experimental group, but increased rapidly with time ($p < .01$) and actually exceeded the control values by 60 minutes of perfusion at 37°C.

Glucose reabsorption was depressed in hypothermic-perfused kidneys (Figure 37). Average values for the four clearance periods (88.23 ± 1.31%) were significantly lower than controls (94.95 ± 0.20% $p < .001$). The difference may have been due to a lag in washout of the hypothermic perfusate which contained high glucose concentration (180 mM).

Secretory activity of the kidney was well preserved and actually enhanced by the period of perfusion at 10°C (Figure 38). The ratio of phenol red to inulin clearance averaged 7.60 ± 0.30 in controls, and 12.65 ± 0.80 in hypothermically perfused kidneys ($p < .001$).

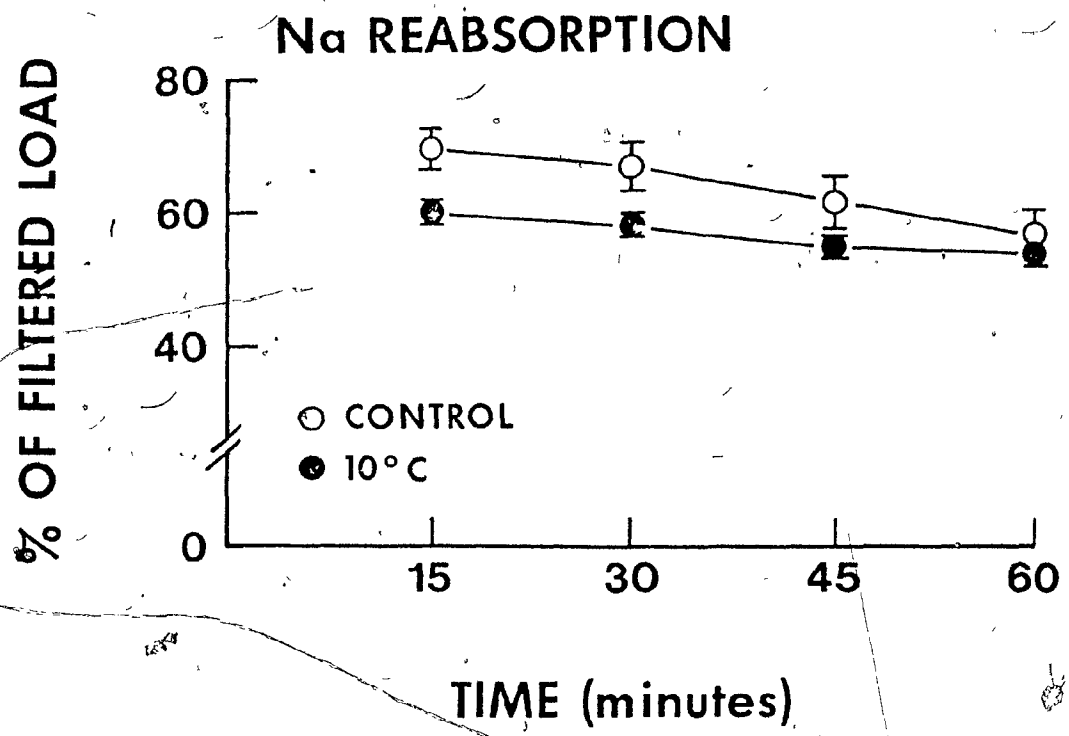


Figure 35. The percentage of filtered sodium reabsorbed during normothermic perfusion of control and hypothermic-perfused kidneys as a function of perfusion time. (Mean ± SEM, 5 observations at each point).

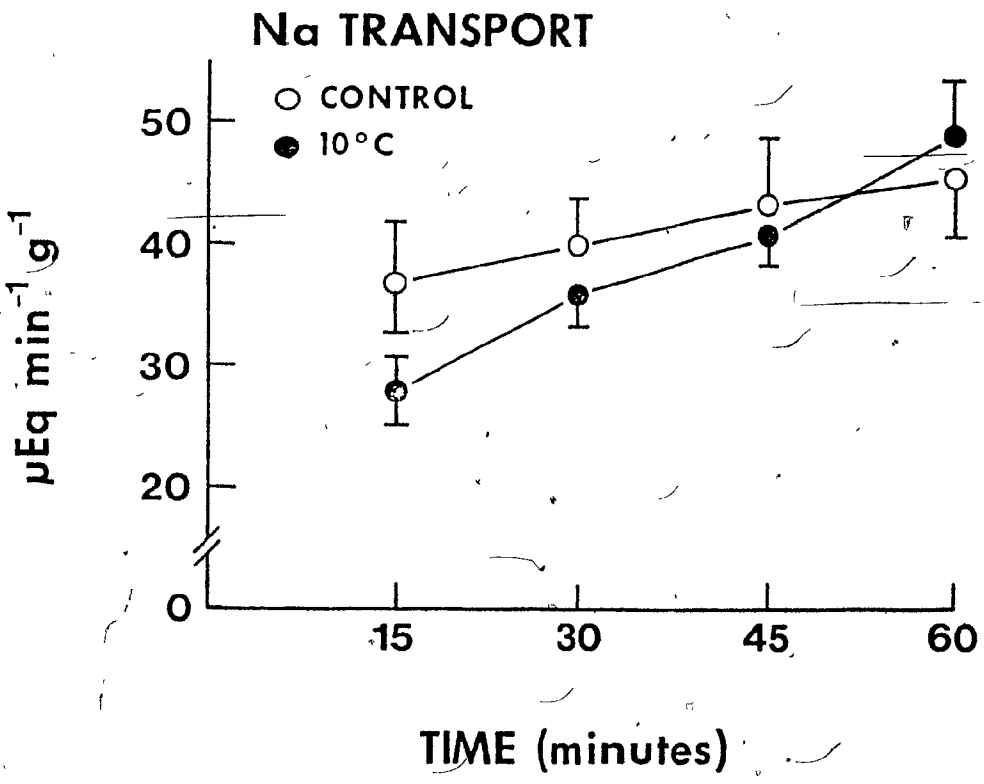


Figure 36. Sodium transport during normothermic perfusion of control and hypothermic-perfused kidneys as function of perfusion time (Mean ± SEM, 5 observations at each point).

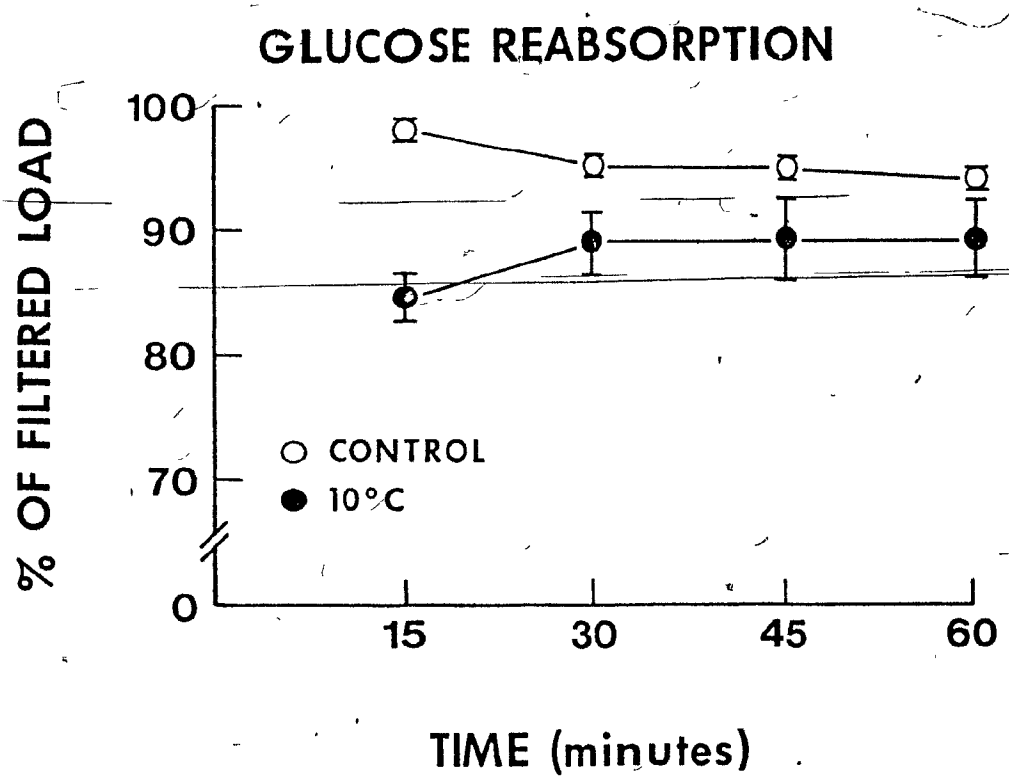


Figure 37. The percentage of filtered glucose reabsorbed during normothermic perfusion of control and hypothermic-perfused kidneys as a function of perfusion time (Mean ± SEM, 5 observations at each point).

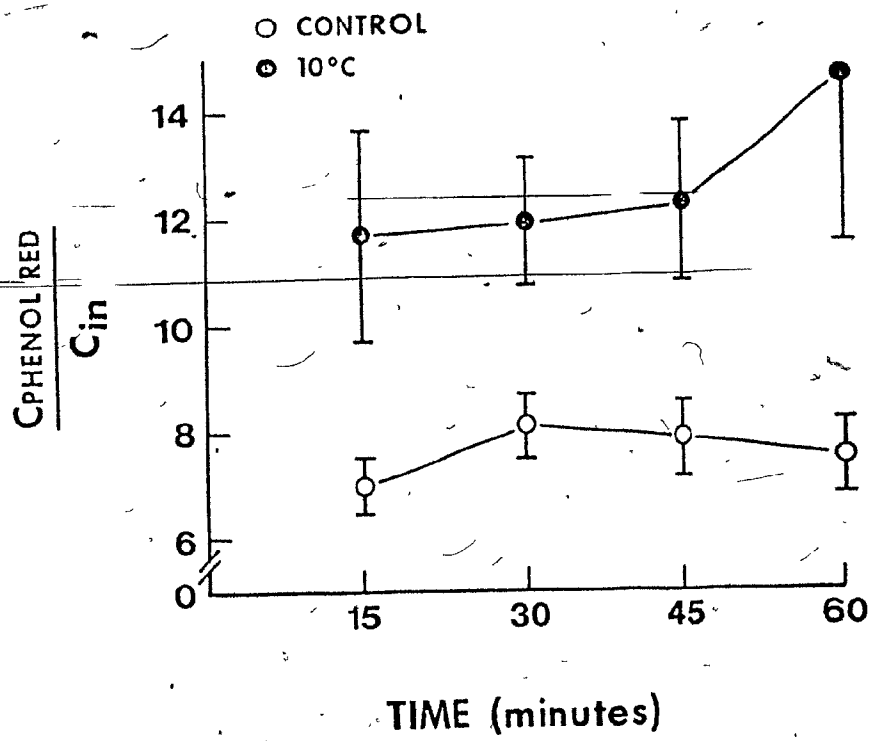


Figure 38: The ratio of phenol red to inulin clearance during normothermic perfusion of control and hypothermic-perfused kidneys. Expressed as a function of perfusion time. (Mean ± SEM, 5 observations at each point).

Discussion

The purpose of the hypothermic perfusion apparatus described is to add and remove the cryoprotectant necessary in freeze-preservation of the kidney. This contrasts with studies aimed at prolonging the interval between kidney retrieval and subsequent reimplantation using methods of hypothermic preservation above freezing temperatures. In this case simple flushing of kidneys will maintain functional viability for up to 72 hours; and other reports of longer periods have been described, (Pegg, 1978).

Hypothermic perfusion is also frequently used for this purpose. The information available from these studies delineates certain parameters which require consideration during short term perfusion for cryoprotectant addition and removal. The circuit described above allowed recirculation of perfusate at 10 ^{0}C. Karow et. al. (1979) found that kidney vascular resistance following Me_2SO treatment at 25 ^{0}C was lower in kidneys when perfusate was re-circulated and suggested that the kidney "conditions" the perfusate similar to cells in tissue culture. It is equally possible that the perfusates used to date lack important unidentified components which are rapidly depleted by open circuit systems, but maintained in sufficient amounts by recirculation. Accumulation of toxic metabolites during hypothermic perfusion does not become a significant factor unless perfusion periods are prolonged (Abouna et. al. , 1972 ; Grundmann et. al. , 1974).

There has not been much work done to establish an optimum

temperature for hypothermic perfusion. Certain studies indicate that 10 ^{0}C is better than lower temperatures (Grundman et. al. , 1972 ; Pegg et. al. , 1977). Belzer and Southard (1980) suggested that higher temperatures should be examined.

The pressure in our system was maintained by establishment of a constant pressure head above the kidney, resulting in a constant flow. Pulsatile flow did not offer any advantages during hypothermic perfusion (Toledo Pereyra et. al. , 1973 ; Pegg and Green , 1976). The perfusion pressure chosen was within the range found satisfactory for hypothermic perfusion by other researchers (Belzer and Southard , 1980 ; Toledo Pereyra , 1980).

An oxygenator was not included in the circuit. Oxygenation during perfusion at 10 ^{0}C has been previously studied and found to be of little value (Pegg et. al. , 1974 ; Claes et. al. , 1974). Johnson et. al. (1979) obtained 100% survival of dog kidneys after 72 hours of hypothermic perfusion without an oxygenator. Belzer and Southard (1980) indicated that high oxygen tensions may be deleterious to membrane-linked functions.

The choice of perfusate for hypothermic introduction of cryoprotectant is difficult. The preservation process is actually a combination of storage and perfusion techniques; the perfusate chosen for non-freezing preservation differs depending on which technique is being used. Another consideration is the choice of the cryoprotectant; its effects on cells dictate the composition of the perfusate. Pegg (1978) presented an excellent review of different preservation techniques and the controversies associated

with them. The most important parameters to be considered in defining a suitable perfusate for hypothermic storage were calcium levels, which should be reduced, magnesium levels, which should be increased, and the inclusion of glucose, mannitol, a steroid, and adenosine. The resultant effects of these constituents are decreased cell swelling, decreased ATP depletion, and prevention of calcium binding to membranes.

The sodium and potassium levels which are most beneficial are equally confusing. Low temperature inhibits the ionic transport system. As a result cellular potassium exits the cell by diffusion down the concentration gradient, and the membrane potential decreases. Sodium and chloride ions enter the cell, and influx exceeds potassium efflux, resulting in water influx and cell swelling (Flores et. al. , 1972). Keeler et. al. (1966) demonstrated that rat kidneys perfused with 0.9% sodium chloride at 0 ^{0}C lost 50% of total potassium in 30 minutes. Dog kidneys similarly perfused with Tyrode solution could not support life, whereas perfusion with high potassium, low sodium solutions preserved function. Collins et. al. (1969) reported that storage of kidneys at 4 ^{0}C after flushing with a potassium rich , sodium poor solution containing elevated glucose levels preserved viability. Aquatella et. al. (1972) observed that these solutions maintained tissue potassium levels, and if an impermeant substance was included, reduced cell swelling . The need for high potassium has been challenged by other workers (Downes et. al. , 1973 ; Green and Pegg , 1979).

Although the benefits of high potassium solutions are debatable

for simple storage, if the cryoprotectant used is Me_2SO evidence supports the use of solutions of high potassium and low sodium content. Keeler et. al. (1966) observed that Me_2SO perfusion promotes excessive loss of potassium from cells of rat kidneys. Karow et. al. (1979) support this view and have used high potassium solutions in conjunction with Me_2SO.

Fahy et. al. (1979) and Collins et. al. (1980) reported a new formulation developed as a hypothermic perfusate for storage of rabbit renal cortex slices and whole kidneys. This perfusate was developed using a rabbit cortical slice assay system, and was suggested as a possible vehicle for Me_2SO administration (Fahy, personal communication). The medium was particularly attractive since it contained increased potassium and low sodium, but the potassium was decreased in comparison to other intracellular type solutions. It also included adenine, glutathione as a reducing agent (Codd et. al. , 1977), and elevated glucose levels.

We adopted a variation of this composition for study. Chlorpromazine was added because of its beneficial effects on vascular resistance (Lokkegaard et. al. , 1979) and membrane fluidity (Kwant and Seeman , 1969 ; Seeman , 1972). The solution was buffered with HEPES , and the osmolality increased by addition of mannitol.

The results of our experiments confirmed the usefulness of the solution for short term hypothermic perfusion. When assayed for function at 37 ^{0}C , the period of hypothermic perfusion was beneficial to the kidney. Perfusate flow rates and tubular secretion was

enhanced over freshly isolated controls. The increased flows may be attributed to a more complete removal of blood from the kidney. Wesson et. al. , (1979) reported incomplete removal of red cells from kidneys after flushing with 100 ml of cold Collins solution. The kidneys were capable of a rapid return to control levels of sodium reabsorption. Although glucose reabsorption appeared depressed, this may have been artifactual, due to incomplete removal of the hypothermic perfusate prior to attachment to the 37 ^{0}C circuit. A period of perfusion without recirculation of the 37 ^{0}C perfusate should identify if the depressed glucose reabsorption is indeed due to functional impairment. The hypothermic perfusion period did not exhibit any deleterious effects on the kidney. In a similar study using an extracellular based perfusate, hypothermically perfused rabbit kidneys did not function as well as freshly isolated controls at 37 ^{0}C and the perfusion itself was thought to be damaging (Pegg and Wusteman , 1977).

In conclusion, the system for hypothermic perfusion which we have described can be used in further studies of kidney preservation. The absence of impaired renal function should allow separation of the effects of Me_2SO on the kidney from effects of hypothermic perfusion alone.

Experiment 6: Kinetics of Permeation and Intracellular Events Associated with Me_2SO Permeation of Rabbit Kidneys during Perfusion at 10°C.

Introduction

Whole organ preservation at subzero temperatures is confined to slow rates of cooling due to the large mass and small surface to volume ratio (Filo et. al. , 1976 ; Jacobsen , 1979). The most recent approach has been to perfuse kidneys with high concentrations of cryoprotective agent to delay concentration of solutes to lower temperatures, permitting slower rates of cooling, and minimizing the main factors contributing to freezing damage. Rabbit kidneys have been successfully permeated with high concentrations of glycerol (Jacobsen , 1978 ; 1979 ; Jacobsen et. al. , 1978), but do not yield viable kidneys after freezing.

Dimethylsulfoxide (Me_2SO) at lower concentrations, has been reported to successfully protect kidneys from freezing damage to a limited extent (Dietzman et. al. , 1973 ; Guttman et. al. , 1977; Toleydo-Pereyra , 1980). A few attempts to treat kidneys with higher concentrations have been reported (Pegg , 1972 ; Jeske et. al. , 1974 ; Karow and Jeske , 1976). Information on the kinetics of permeation and related cellular events above 1.4 M has not been reported. The purpose of the following experiment was to investigate the alterations which occur in the extracellular and intracellular fluid during perfusion with 3 M Me_2SO after slow introduction of the cryoprotectant by examination of total water, inulin and Me_2SO spaces.

Methods

1). Perfusate

(i). Hypothermic perfusate: The 10°C perfusion circuit was primed with 200 ml of 1X, 400 mOs/kg medium (Table 3, section 1V B, materials and methods) to which C^{14}-Inulin (5 µCi, carboxyl-^{14}C inulin, New England Nuclear, Lachine, Quebec) had been added.

(ii). Me_2SO perfusate: 200 ml of the 6 M Me_2SO in 1X perfusion medium containing 25 µCi H^3-Me_2SO (New England Nuclear, Lachine , Quebec) was attached as described in section III Bc of the materials and methods section.

2). Perfusion

The kidneys were removed from the rabbits and flushed in the manner previously described. The renal arterial cannula was attached to the circuit and the 10°C cold perfusate was recirculated through the kidney for 15 minutes prior to the addition of Me_2SO. The Me_2SO solution was pumped into the main circuit at the rate of 5 ml min^{-1}. Perfusate samples were taken every 15 minutes, up to the point when the kidney was removed, for measurement of perfusate concentration of C^{14}- inulin and/or H^3-Me_2SO by liquid scintillation counting in triton toluene-based cocktail on an Intertechnique

SL-30 LS spectrometer. Thus, by 40 minutes the final total circulating volume, neglecting sampling was 400 ml, with a Me_2SO concentration of 3 M. Due to sampling, the final Me_2SO concentration was 3.06 M. The venous/ureter effluent was recorded at 15 minute intervals by timed collections in the return column.

3). Treatment Groups

A total of 18 kidneys were perfused in this study.

A. Controls: Nine kidneys were perfused with medium containing C^{14}- Inulin . Me_2SO-free perfusate was pumped into the circuit at the rate of 5 $ml\ min^{-1}$. Kidneys were perfused for 60, 75 or 90 minutes (3 kidneys in each group) from the time of activation of the accessory reservoir pump. The kidneys were removed from the circuit and the total water and inulin space determined.

B. Me_2SO perfused kidneys: Nine kidneys were perfused as were the controls; but with Me_2SO-containing perfusate pumped into the circuit. Kidneys were perfused for 60, 75 or 90 minutes from the time of introduction of Me_2SO into the circuit (3 kidneys for each time period). The kidneys were removed and the total H_2O/Me_2SO, Me_2SO and inulin spaces determined.

4). Determination of Total H_2O (and Me_2SO), Me_2SO, and Inulin Space

Kidneys were removed from the circuit after 60, 75 or 90 minutes of perfusion with or without Me_2SO (3 kidneys per group). When Me_2SO was added, the time of exposure to the maximum concentration was 20, 35 or 50 minutes respectively. The kidneys were placed in a petri dish, blotted and the capsule slit dorsally and pulled back to the pelvis. The capsule, ureter and blood vessels were removed by cutting a "V" with a #23 scalpel blade into the pelvis. The kidney was transferred to a new dish. Thin slices of cortex were cut from the upper and lower poles and two sections of approximately 100 mg each were placed into pre-weighed liquid scintillation counting vials and tightly capped. Two transverse slices approximately 2-4 mm thick were taken at the midpoints between the middle and upper and lower poles. The cortex was trimmed off and placed in vials as before. Medullary sections of 100 mg approximate weight were treated similarly. The remaining segments were divided into cortex and medullary portions of approximately 300 to 800 mg each and placed in vials for dry weight determinations.

The vials containing larger sections were weighed, uncapped, and placed in a drying oven at 90^{0}C for 120 hours. After removal, they were capped and reweighed.

The tissue in the remaining vials was minced finely with scissors. The vials were weighed to determine the exact weight of wet tissue. One ml of Protosol (New England Nuclear , Lachine, Quebec) was added to each vial. The vial was capped and placed in a shaking water bath at 37^{0}C for 24 hours until all the tissue

was solubilized. Liquid scintillation cocktail and 1 drop of glacial acetic acid was dispensed into each vial. The vials were stored in the dark for 24 hours prior to counting to reduce chemiluminescence. The vials were counted in an Intertechnique model SL -30 Liquid Scintillation Spectrometer.

To estimate the error associated with incomplete drying of samples containing Me_2SO, tissue sections were placed in pre-weighed vials. Sufficient Me_2SO was added to half of the vials to approximate the concentration in the perfusate. The vials were capped and left at room temperature for 60 minutes. They were then dried as described above. The total fluid space was calculated; the difference due to the presence of Me_2SO was ascertained.

The following formulae illustrate the method for computing the respective spaces occupied by water (Me_2SO), Me_2SO and inulin. The concentration of C^{14}-inulin and H^3-Me_2SO in the perfusate at the time of removal was used to calculate the dpm/µl. Counts from digested tissue were corrected to 100 mg wet weight.

Total H_2O (Me_2SO) space: Wet weight-Dry weight

$$\text{Inulin Space} = \frac{\text{dpm } C^{14}\text{-Inulin/100 mg wet tissue}}{\text{dpm } C^{14}\text{-Inulin/ µl perfusate}}$$

$$Me_2SO \text{ Space} = \frac{\text{dpm } H^3\text{-}Me_2SO\text{/100 mg wet tissue}}{\text{dpm } H^3\text{-}Me_2SO \text{ / µl perfusate}}$$

The spaces were expressed as µl/100 mg wet tissue.

Results

The concentration of Me_2SO and the perfusate flow rates in the kidneys perfused with and without Me_2SO addition are shown in Figure 39. The most rapid addition of Me_2SO occurred during the first 5 minutes of addition, at a rate of 136.24 mM min^{-1}. The rate of increase of Me_2SO concentration in the recirculating perfusate decreased with time such that by 20 minutes the total concentration of Me_2SO was 2.04 M; the rate of addition between 20 and 25 minutes was 62.85 mM min^{-1}. Between 35 and 40 minutes of addition, the rate decreased to 40.82 mM min^{-1} to a final concentration of 3.061 M Me_2SO.

Control kidneys, perfused with Me_2SO-free perfusate exhibited stable perfusate flow rates for the entire perfusion period. After 30 minutes of perfusion, with Me_2SO being added at 5 ml min^{-1}, the perfusate flow rates decreased but stabilized after 45 minutes.

The total water (for control kidneys), water and Me_2SO (for Me_2SO-perfused kidneys), Me_2SO and inulin spaces after perfusion at 10 ^{0}C for 60, 75, and 90 minutes are shown in Figure 40 for sections of cortex, and in Figure 41 for sections of medulla. The total fluid space (water and Me_2SO) was slightly higher in Me_2SO-free perfused controls (82.0 ± 2.4 µl 100 mg^{-1}) than Me_2SO-perfused kidneys (79.15 ± 0.87 µl 100 mg^{-1}). However, the values obtained by drying sections of kidney containing Me_2SO indicate that these values were low. This difference or error of 2.35 ± 0.82 µl 100 mg^{-1}, explains the discrepancy between groups. In both control and Me_2SO

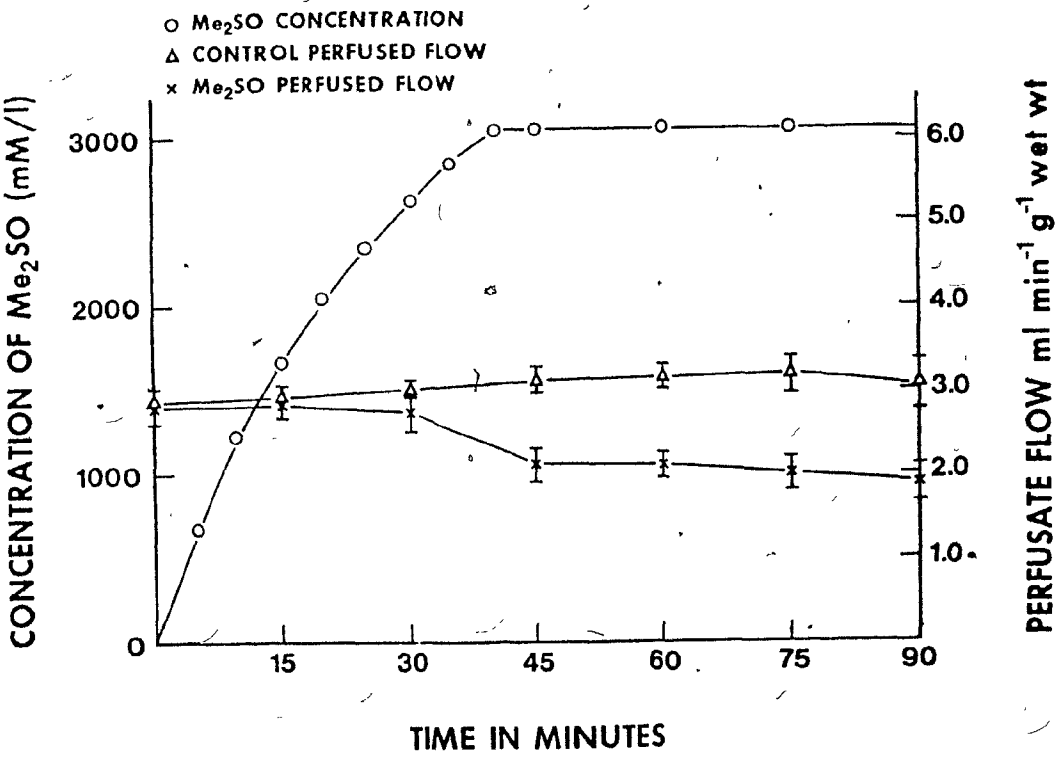


Figure 39. The perfusate flow rates as a function of perfusion time in kidneys perfused at 10°C with and without Me_2SO in the perfusate is also shown as function of time (0).

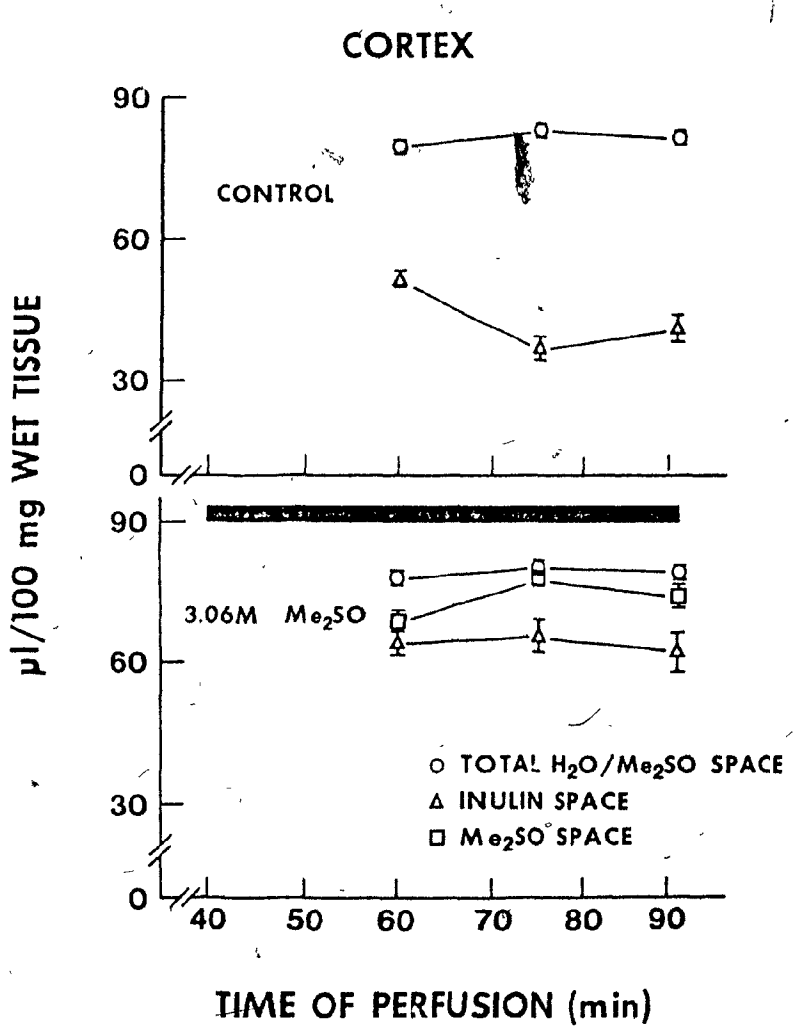


Figure 40. Top: Total H_2O and inulin spaces in cortical sections of kidneys perfused at $10^{\circ}C$ at 60,75 and 90 minutes of perfusion.

Bottom: Total fluid (H_2O and Me_2SO), inulin and Me_2SO spaces in cortical sections of kidneys perfused with 3.06 M Me_2SO at 60,75 and 90 minutes of perfusion. The shaded bar indicates the interval in which perfusate Me_2SO concentration was 3.06 M.

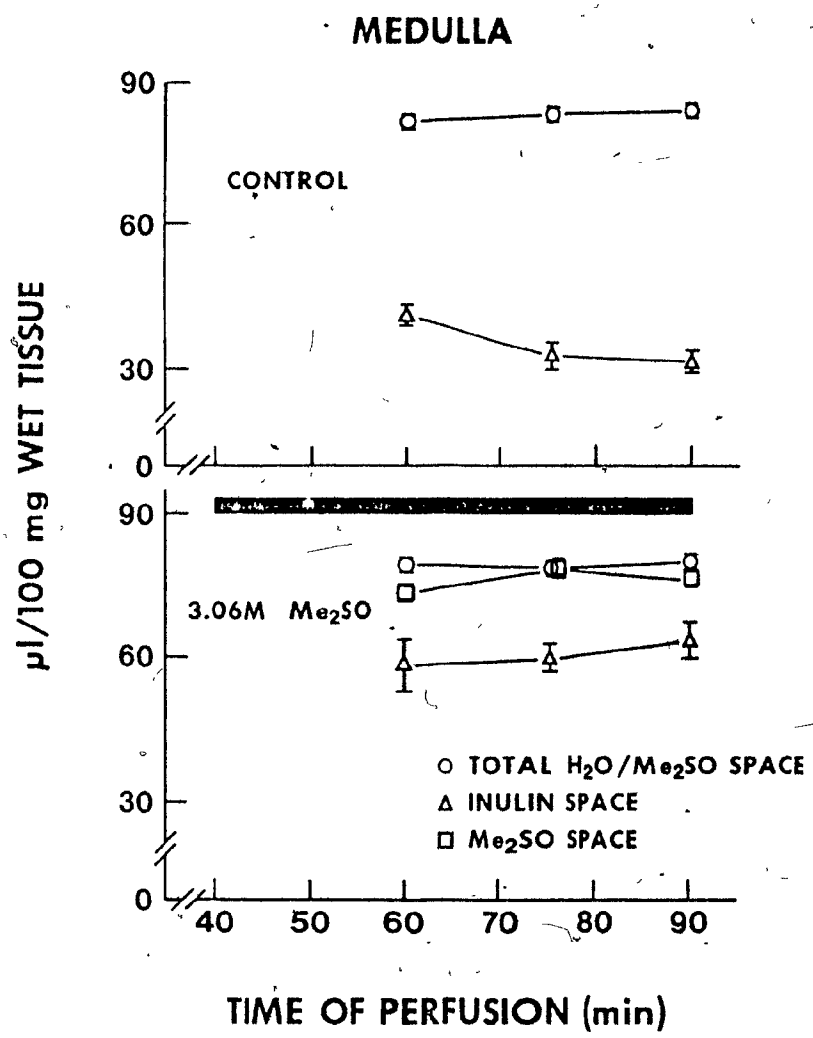


Figure 41. Top: Total H_2O and inulin spaces in medullary sections of kidneys perfused at 10°C at 60, 75 and 90 minutes of perfusion.

Bottom: Total fluid (H_2O and Me_2SO), inulin and Me_2SO spaces in medullary sections of kidneys perfused with 3.06 M Me_2SO at 60, 75 and 90 minutes of perfusion. The shaded bar indicates the interval in which perfusate Me_2SO concentration was 3.06 M.

perfused kidneys, the total fluid space in medullary tissue segments (81.25 ± 0.46) was greater than cortical segments (79.90 ± 0.44, $p < .01$).

The inulin space in cortical segments (53.72 ± 2.20 µl 100 mg^{-1}) of both Me_2SO and Me_2SO-free kidneys was larger than medullary tissue inulin space (47.89 ± 6.11, $p < .01$). Kidneys perfused without Me_2SO exhibited a decrease in inulin space in both cortex and medulla ($p < .05$) as perfusion time increased. This decrease was predominant between 60 and 75 minutes of perfusion. When Me_2SO was included in the perfusate, the inulin space did not change with perfusion time as in controls. The most striking observation was the increase in inulin space in Me_2SO-perfused kidneys in comparison to controls. Inulin space in cortical tissue was more than 50% larger than controls, 64.76 ± 2.46 and 42.70 ± 1.77 µl 100 mg^{-1} respectively , $p < .001$. In medullary tissue, the presence of Me_2SO increased inulin space by 74% over control values (60.81 ± 2.36 and 34.98 ± 1.40 µl 100 mg^{-1} respectively, $p < .001$).

The extent of permeation of cryoprotectant is often described as a ratio of tissue to medium (T/M)Me_2SO concentration, corrected for the amount of total kidney water. In this study, the ratio of Me_2SO space to total kidney fluid is a similar calculation. In the medullary tissue, by 60 minutes of perfusion, which translates to 20 minutes of exposure to 3 M Me_2SO, equilibration was essentially complete, a T/M ratio of 95%, which increased to 100% by 75 minutes, or 35 minutes of exposure to 3 M Me_2SO. In sections of cortical tissue, this ratio was 80% at 60 minutes, and increased to 100%

by 75 minutes.

In cortex and medulla, extracellular equilibration of Me_2SO had been achieved by 60 minutes of perfusion. In the medulla, the Me_2SO had penetrated the cell by 60 minutes, the Me_2SO space was increased significantly over the inulin space at 60 minutes ($p < .05$), 75 minutes ($p < .01$) and 90 minutes ($p < .025$). In the cortex the Me_2SO space did not differ significantly from the inulin space at 60 minutes. By 75 minutes an exposure time to 3 M Me_2SO of 35 minutes, the Me_2SO space had increased significantly over the corresponding inulin space ($p < .005$). Histological examination of the cortex and medulla showed evidence of a reduced cell volume in both sections of cortex and medulla (Figure 42). In these sections, the tubular epithelial cells were contracted, the brush border was flattened. The glomeruli appeared densely packed. The tubular lumen were dilated.

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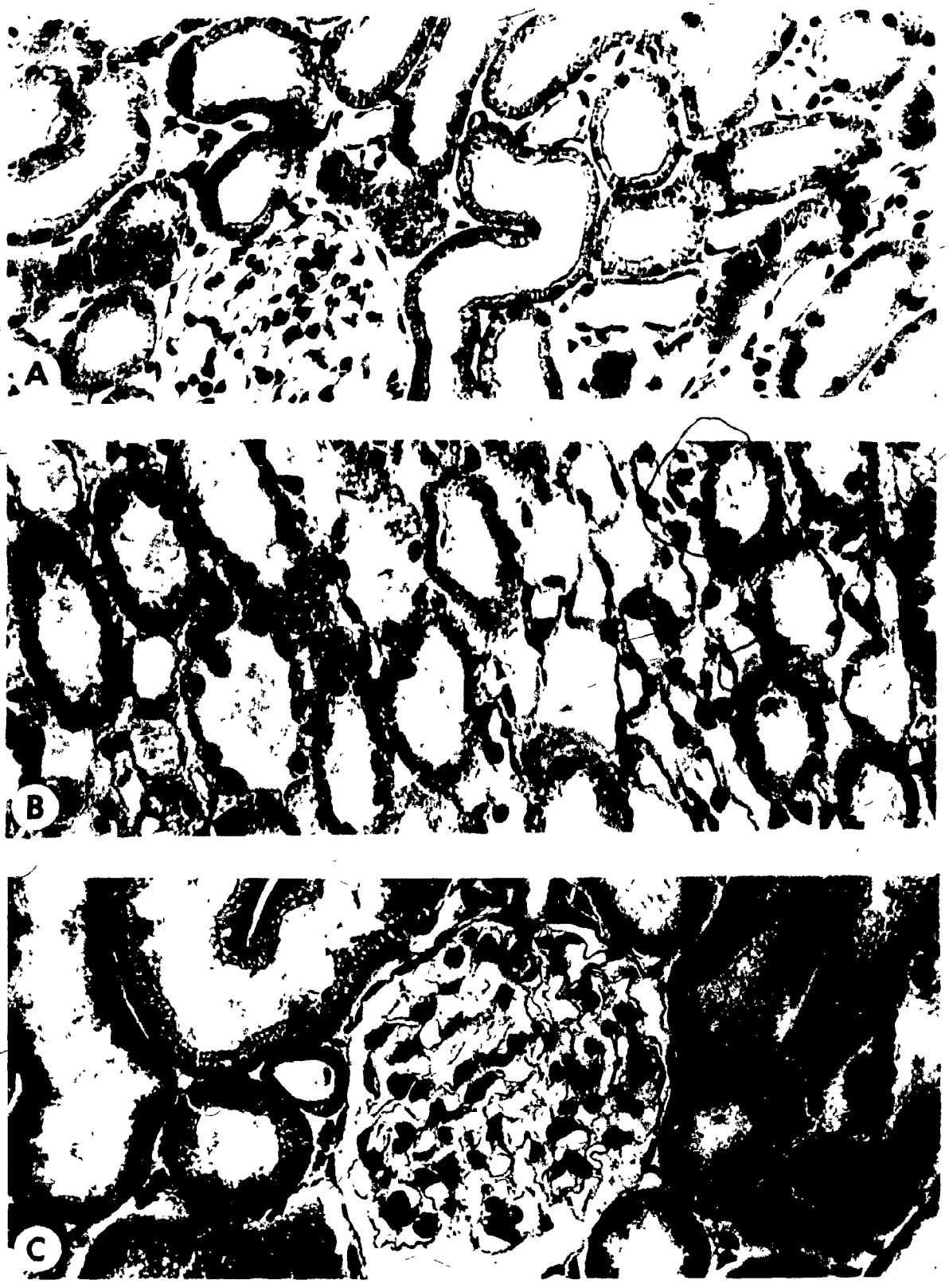
Figure 42

A. Photomicrograph of a section of kidney following perfusion with 3.06 M Me_2SO for 35 minutes at 10°C (H & E, 380X)

B. Kidney section following perfusion with 3.06 M Me_2SO for 50 minutes at 10°C (H & E, 608X)

C. Kidney section following perfusion with 3.06 M Me_2SO for 50 minutes at 10°C (H & E, 608X)


A
B
C

Discussion

Complete permeation of rabbit kidneys was obtained with 3 M Me_2SO at 10 ^{0}C after perfusion with the final concentration for 35 minutes. This time agrees with other reports of dog (Small et. al.,1977) and rabbit kidneys (Karow et. al., 1979) perfused with 1.4 M Me_2SO at 10 ^{0}C, and with reports of 3 M glycerol permeation at 10 ^{0}C (Pegg and Robinson, 1978). The equilibration in this study was complete in the medulla before the cortex, and before tissue sampling. It is therefore not possible to estimate the time required for medullary equilibration from the results. This contrasts with the data reported for 1.4 M Me_2SO where, in the dog experiment, maximum equilibration in the medulla was only 60%. This discrepancy may be explained by the difference in flow rates of our kidneys, which were quite high in comparison to Small et. al. (1977), Karow et. al. (1979) and Karow and Jeske (1976). Pegg and Robinson (1978) indicated that in rabbit kidneys equilibration with glycerol at 10 ^{0}C was more complete in the medulla and corticomedulla regions of the kidney than in the cortex. The reason for this is not clear.

The estimation of inulin space and total (water and Me_2SO) space has not been studied previously in kidneys perfused with cryoprotectant. In this study some evidence of cellular permeation of Me_2SO was obtained. An interesting point is the kinetics of permeation at 60 minutes (20 minutes of exposure to 3 M Me_2SO). Although at this point the percent equilibration of cortical tissue

with perfusate Me_2SO was 80%, very little had penetrated the cell. By 75 minutes (35 minute exposure to 3 M Me_2SO), the Me_2SO had equilibrated intracellularly to a much greater extent. Farrant (1972) and Elford (1970) have suggested that not all intracellular water is available as a solvent for Me_2SO . In this study, the total water space was slightly underestimated by drying tissue with Me_2SO. After 90 minutes of perfusion (50 minute exposure to 3 M Me_2SO), the total water space was still larger than that occupied by the Me_2SO. This suggests that the phenomenon is also apparent in kidney tissue, and should be brought into consideration when attempting to correlate cryoprotectant concentration and the point at which intracellular freezing is likely to occur.

The results clearly demonstrated an osmotic effect of Me_2SO on kidney cells at 10^{0}C, despite the slow addition of Me_2SO. By 60 minutes of perfusion, the Me_2SO had equilibrated extracellularly in both cortex and medulla; but as shown in the cortex, the Me_2SO had hardly penetrated the cell after 20 minutes of exposure to the 3 M concentration.

A striking difference in the inulin space occurred upon addition of Me_2SO to the perfusate. In control kidneys, the inulin space decreased during perfusion, an indication of cell swelling. Kidneys perfused with Me_2SO exhibited a large increase in inulin space which did not decrease when Me_2SO had entered the cell. There are several likely explanations. Cells treated with Me_2SO have demonstrated an increase in permeability of various biological

membranes (Pegg, 1972). Alternately, Farrant (1972) demonstrated that leakage of cations from red cells did not occur in presence of Me_2SO up to 5 M. In our study, the Me_2SO equlibrated more rapidly in the medulla than in the cortex, yet the inulin space of the medulla was less than the cortex. If inulin leakage into cells was the predominant factor, it is likely that the inulin space should have increased steadily in proportion to increased intracellular Me_2SO in the cortex, which was not so.

A second alternative is the increased inulin space is a reflection of increased inulin concentration in the tubules, creating a false, high value for the extracellular space. This would have manifested to the same extent in Me_2SO-free perfused kidneys. Although inulin space in these kidneys was increased over normal values, it was significantly less than Me_2SO-perfused kidneys.

Another explanation could be the expansion of tubular lumen volume. Me_2SO has been shown to increase glomerular filtration (Pegg and Farrant, 1968; Karow and Jeske, 1976). This phenomenon would increase perfusate delivery to tubules and increase intratubular volume which would subsequently manifest as an increase in the inulin space. This would seem more feasible, and the histological evidence supports this hypothesis.

The results reported here define important factors in the treatment of organs with cryoprotectants which are not applicable to cell suspensions or tissue slices. They help to elucidate the reason for unsuccessful extrapolation of freezing protocols from the single cell to the organ. The accumulation of large amounts of extracellular fluid would contribute significantly to freezing damage by extracellular ice formation. Attempts to decrease the Me_2SO

concentration in the perfusate would need to be accomplished extremely gradually to prevent a lag in the concentration of Me_2SO intracellularly, or by control of osmotic flux by other means while removing the Me_2SO.

Experiment 7: Function of Rabbit Kidneys In Vitro at Normothermia following Equilibration with 3 M Me_2SO and Removal Hypertonic Washout at 10^0C.

Introduction

It was demonstrated in experiment 6 that permeation of rabbit kidneys with Me_2SO by perfusion at 10^0 C was complete after 35 minutes of exposure to 3 M Me_2SO. Previous attempts to perfuse rabbit kidneys with Me_2SO concentrations above 1.4 M produced marked structural alterations (Pegg , 1972 ; Jeske et. al. ,1974 ; Karow and Jeske , 1976). No attempts have been made to remove high concentrations of Me_2SO or to examine kidney function following its removal.

The following study examined the ability of rabbit kidneys to function in vitro at 37^0 C following treatment with Me_2SO. This study differed from previous reports in several aspects: 1)The final concentration of Me_2SO was 3 M; 2) The Me_2SO was introduced by slow addition to the perfusate; and 3) The removal of Me_2SO from the perfusion circuit was accomplished quickly while counteracting the osmotic stress of the rapid dilution by the inclusion of an impermeant solute, mannitol, in the washout solution.

Methods

1). Perfusate

(i) Hypothermic perfusate: The 10 °C perfusion circuit was primed with 200 ml of 1X., 400 mOs/kg medium (Table 3, materials and methods, section 1VB.).

(ii) Me_2SO perfusate: 200 ml of the 6 M Me_2SO in 1ℓ perfusion medium was attached to the accessory reservoir of the 10°C perfusion circuit.

(iii) Washout Solutions: The 2X hypothermic perfusion medium was diluted to single strength with a mannitol solution and/or ddH_2O to yield solutions of 500,600,700 or 800 mOs/kg (Table 4, materials and methods, section 1VB).

(iv) Normothermic perfusate: The composition of the perfusate for 37 °C perfusion was identical to that described for experiments 4 and 5 (Table 1, materials and methods, section 1VA).

2). Introduction of Me_2SO

The 10 °C perfusion circuit was primed with 200 ml of 1X hypothermic perfusion medium. The perfusate was recirculated to fill the condenser and bubble trap and equilibrate at 10 °C. Following the

initial flush at 25°C, the kidney was attached to the circuit via the arterial cannula and perfused for a 15 minute equilibration period. After 15 minutes, the pump attached to the Me_2SO-containing reservoir was activated and the media at 6 M Me_2SO was pumped into the circuit at the desired rate. After the Me_2SO reservoir had emptied, the kidney was perfused with the resultant 3 M Me_2SO perfusion medium for an additional 35 minutes prior to the start of the washout procedures. During perfusion the venous and ureter effluent was recorded at 15 minute intervals.

3). Washout of Me_2SO

Following the equilibration with 3 M Me_2SO, the washout phase was started. Two different washout procedures were performed. The first consisted of perfusion with media adjusted to 600 mOs/kg, followed by 500 mOs/kg and 400 mOs/kg. At each step the kidney was perfused for 20 minutes. The second procedure consisted of 5 solutions adjusted to 800, 700, 600, 500 and 400 mOs/kg, 15 minutes at each step. Four hundred ml of perfusate used for the first step (800 or 600 mOs/kg) was attached to the circuit. The outlet to the pump was opened, but the return system remained connected to the reservoir containing the first perfusate. The speed of the pump was adjusted to maintain the normal pressure head without loss of fluid through the overflow. The volume of the circuit, not including the reservoirs, was 80 ml. The first 200 ml of perfusate

was not recirculated, but flowed through the kidney and collected into the first reservoir. After the 200 ml flush, the return system was changed to allow recirculation of the Me_2SO-free perfusate. The same procedure was followed for each successive washout step with the exception that the perfusate volume was 200 ml, 100 ml of which was not recirculated. The venous and ureter effluent was measured at 5 minute intervals.

4). Treatment Groups

A. Freshly isolated controls: Five kidneys were immediately placed on the 37 ^{0}C circuit following the initial flush, perfused, and function assessed.

B. Hypothermic controls: Three kidneys were placed on the 10 ^{0}C circuit following the initial flush. They were perfused for 3 hours at 10 ^{0}C, then connected to the 37 ^{0}C circuit and perfused.

C. Five kidneys were placed on the 10 ^{0}C circuit following the initial flush. The kidneys were perfused at 10 ^{0}C with Me_2SO-free perfusate (400 mOs/kg) for 15 minutes. The Me_2SO concentration was increased by addition of 6 M Me_2SO in perfusate at the rate of 5 ml min^{-1} to the main circuit. When the concentration of Me_2SO in the main circuit was 3 M, the kidneys were perfused for an additional 35 minutes

for equilibration with the 3M Me_2SO. The Me_2SO was removed according to the first washout protocol which consisted of perfusion with 600, 500 and 400 mOs/kg perfusate in successive 20 minute steps. The kidneys were assayed at 37°C as described.

D. Five kidneys were perfused with Me_2SO as in group C. The Me_2SO was removed according to the second washout protocol consisting of perfusion with 800, 700, 600, 500 and 400 mOs/kg perfusate in successive 15 minute steps followed by assay at 37°C.

E. Five kidneys were perfused with Me_2SO in a similar manner to groups C and D; however, the rate of addition of the Me_2SO perfusate was 3 $ml\ min^{-1}$. Removal of the Me_2SO was accomplished in the same manner as group C. Kidney function was assessed by perfusion at 37°C.

Results

1). Perfusion Characteristics at 10°C

The calculated increase in Me_2SO concentration in groups where the addition of Me_2SO perfusate was 5 ml min^{-1} (Groups C and D) and 3 ml min^{-1} (Group E) is shown in Figure 43. The rate of increase of Me_2SO concentration was most rapid during the first 5 minutes, 133.53 mM min^{-1} at 5 ml min^{-1} infusion, and 83.72 mM min^{-1} at 3 ml min^{-1} infusion. After 20 minutes of infusion at 5 ml min^{-1}, the concentration of Me_2SO was 2.0 M, the rate of addition during the next 5 minutes was 61.54 mM min^{-1}; while at 3 ml min^{-1} infusion, the total circulating concentration of Me_2SO reached 2.06 M after 35 minutes with a rate of increase of 36.9 mM min^{-1} during the next 5 minutes. Infusion at 5 ml min^{-1} was complete after 40 minutes with a final concentration of 3.0 M Me_2SO and a rate of increase of 40 mM min^{-1} during the last 5 minutes. After 67 minutes, the 3 ml min^{-1} infusion was complete; a rate of increase during the last 5 minutes of 19 mM min^{-1}.

Flow rates of kidneys perfused at 10°C without Me_2SO (Group B) increased gradually throughout the perfusion period (Figure 43). Perfusate flow rates in kidneys perfused with Me_2SO added at 3 ml min^{-1} (Group E) and a 5 ml min^{-1} (Group C and D) exhibited similar patterns of increasing resistance after 45 minutes of perfusion, but remained stable for the remainder of the Me_2SO perfusion, and lower than controls.

Perfusate flow rates during the stepwise washout of the kidneys

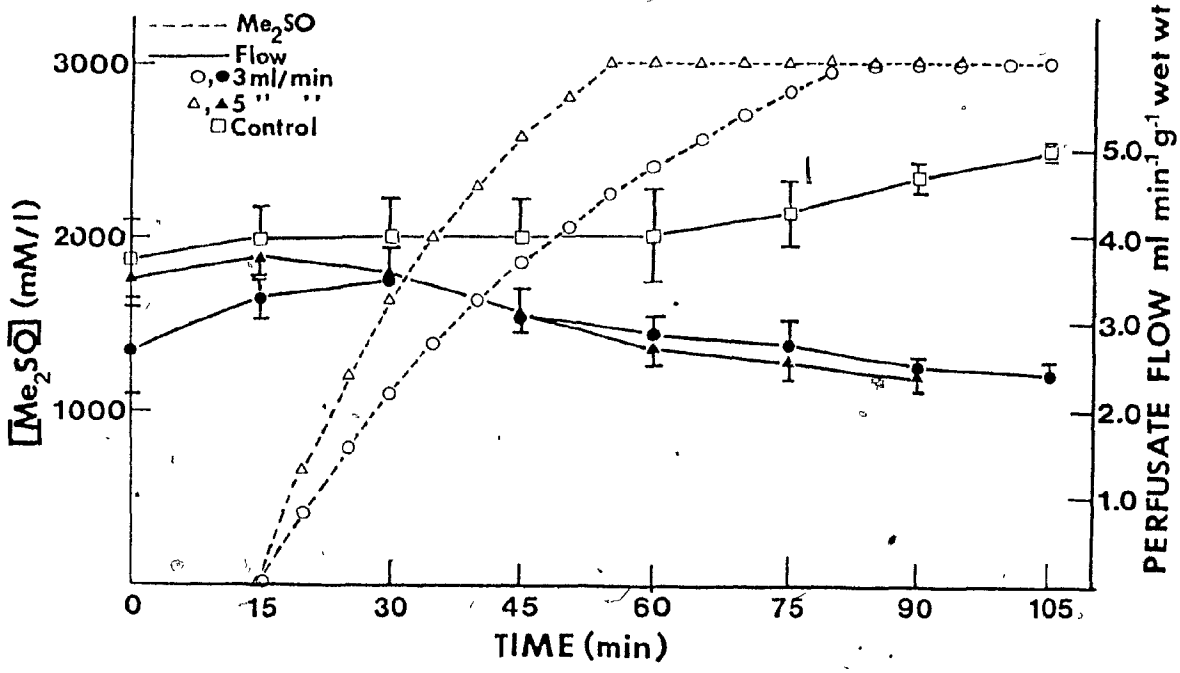


Figure 43. Perfusate flow rates (——) and corresponding perfusate Me_2SO concentration (---) of kidneys perfused at 10°C with Me_2SO introduced at 3 ml min^{-1} (Group E), and 5 ml min^{-1} (Groups C and D). Control kidneys were perfused at 10°C without Me_2SO. Expressed as a function of perfusion time.

with hyperosmolar solutions are shown in Figure 44. Kidneys perfused with the 600-500-400 mOs/kg solutions exhibited very little change in renal vascular resistance during the initial step of perfusion with 600 mOs/kg medium. The resistance decreased (with corresponding increase in flow rate) during the 500 mOs/kg stage and remained stable during the final stage of perfusion with the 400 mOs/kg medium at a flow rate similar to that prior to the introduction of Me_2SO. Kidneys perfused with the 800-700-600-500-400 mOs/kg solutions exhibited a steadily decreasing resistance during perfusion with 800 and 700 mOs/kg solutions, and remained stable over the remainder of the washout steps.

2). Perfusion Characteristics at 37 ^{0}C

The perfusate flow rates are shown in Figure 45. Initially, groups which were subject to Me_2SO addition and removal exhibited depressed flow rates compared to freshly isolated control kidneys, Group A , which averaged 5.93 ± 0.58 ml $min^{-1}g^{-1}$, or compared to kidneys perfused at 10 ^{0}C without Me_2SO addition and removal (Group B), which averaged 6.58 ± 0.54 ml $min^{-1}g^{-1}$ at the beginning of the collection periods. Both Group C, (4.15 ± 0.45 ml $min^{-1}g^{-1}$), and Group E, (3.99 ± 0.58 ml $min^{-1}g^{-1}$) were significantly depressed ($p < .01$); and kidneys exposed to the Me_2SO washout beginning with 800 mOs/kg (Group D) exhibited very low flows (2.67 ± 0.36 ml $min^{-1}g^{-1}$) at the beginning in comparison to controls ($p < .001$). After 60 minutes of perfusion at 37 ^{0}C, flow rates in Groups C and E had

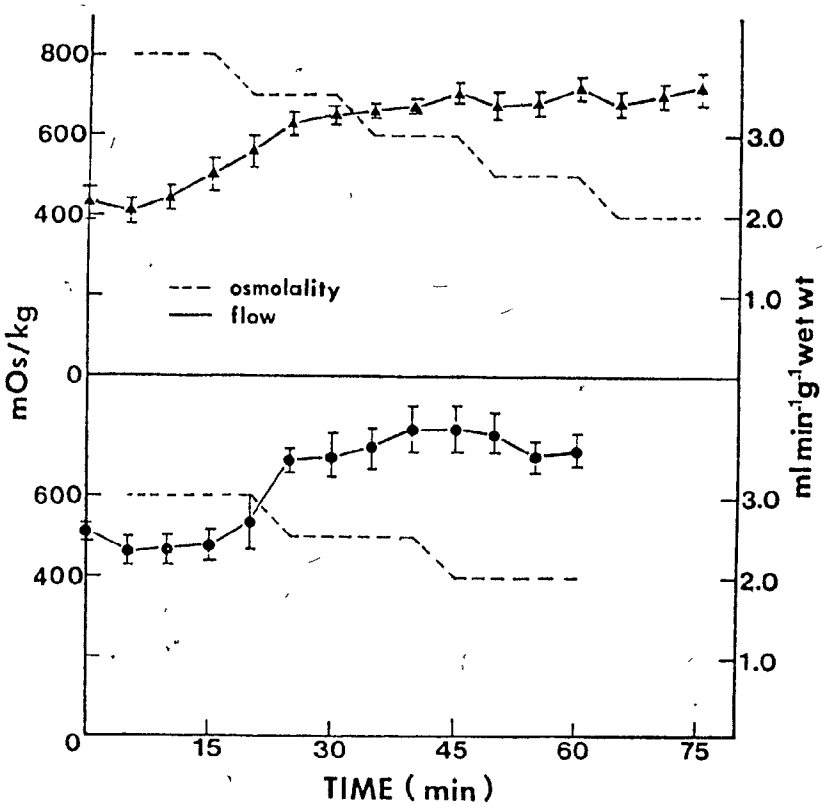


Figure 44. Perfusate flow rates and corresponding perfusate osmolality as a function of perfusion time during Me_2SO washout. Top: Kidneys perfused with 800/700/600/500/400 mOs/kg media (Group D). Mean ± SEM, 5 observations at each point. Bottom: Kidneys perfused with 600/500 400 mOs/kg media (Groups C and E). Mean ± SEM, 10 observations at each point.

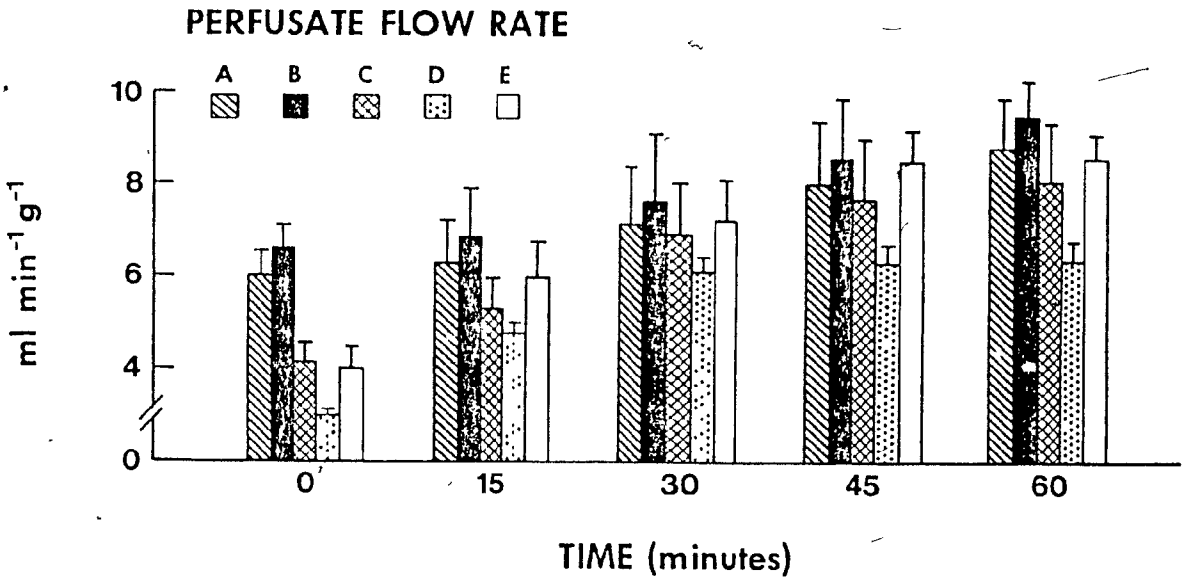


Figure 45. Perfusate flow rates of control and Me_2SO-perfused kidneys during normothermic perfusion. Expressed as a function of perfusion time. (Mean ± SEM. Groups A,C, D and E: 5 observations at each point. Group B, 3 observations at each point).

increased significantly ($p < .05$) over initial rates to values similar to control groups. Group D flow rates also increased, but remained lower than the other four groups ($p < 0.1$).

The glomerular filtration rates (GFR) showed a similar pattern in all groups (Figure 46). Initially GFR in all groups perfused at 10°C were lower than controls; however, as the perfusion period progressed, it increased significantly in all 4 groups ($p < .05$) over the values obtained in the first collection period. The ratio of the clearance of albumin to the clearance of inulin, is shown in Figure 47.

Protein leakage was slight in all groups and retention increased as perfusion progressed. Perfusion at 10°C without Me_2SO (Group B) did not affect albumin retention: the average over the four clearance periods, 0.015 ± 0.004 was lower than freshly isolated controls (Group A) which averaged 0.028 ± 0.003. Leakage was significantly higher in Group D, (0.046 ± 0.004) than in controls, or in Group C (0.029 ± 0.004, $p < .05$).

3). Tubular Function

The ability of the kidneys to handle sodium is illustrated in Figures 48 and 49. Hypothermic perfusion without Me_2SO did not impair sodium reabsorption; Group A kidneys reabsorbed an average of 56.08 ± 2.83% of filtered sodium, Group B averaged 54.06 ± 1.47%. Both groups were very stable, capable of handling the increased load delivered by the increased filtered load at the

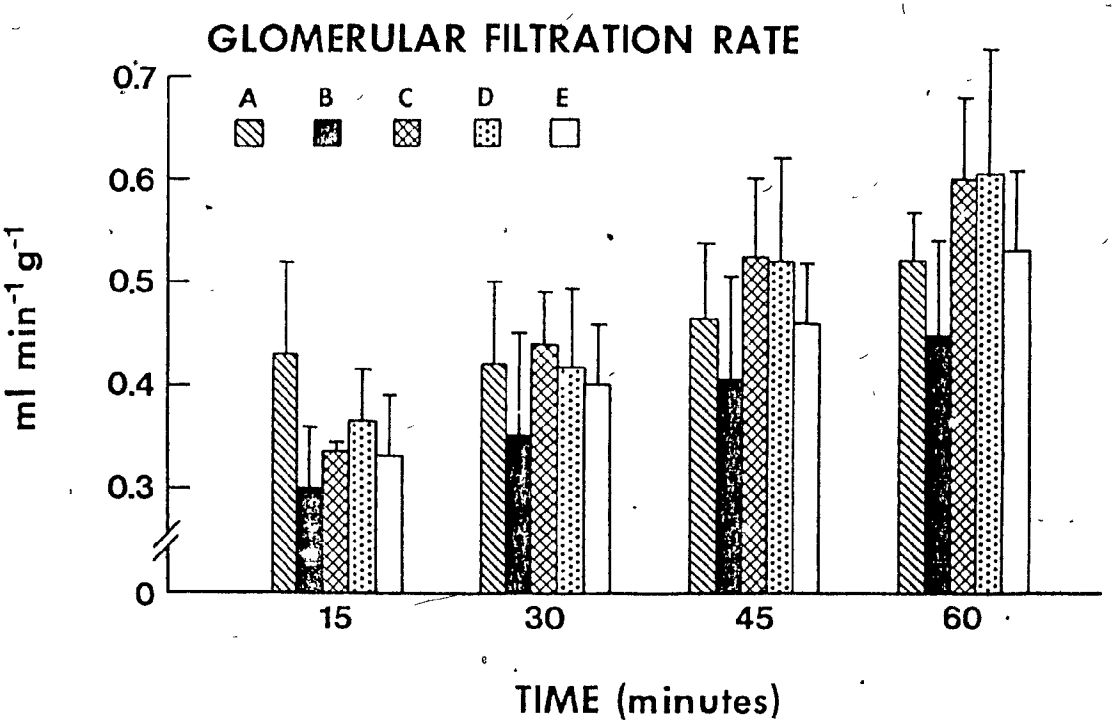


Figure 46. Glomerular filtration rates of control and Me_2SO-perfused kidneys during normothermic perfusion. Expressed as a function of perfusion time. (Mean ± SEM. Groups A,C, D and E: 5 observations at each point. Group B: 3 observations at each point).

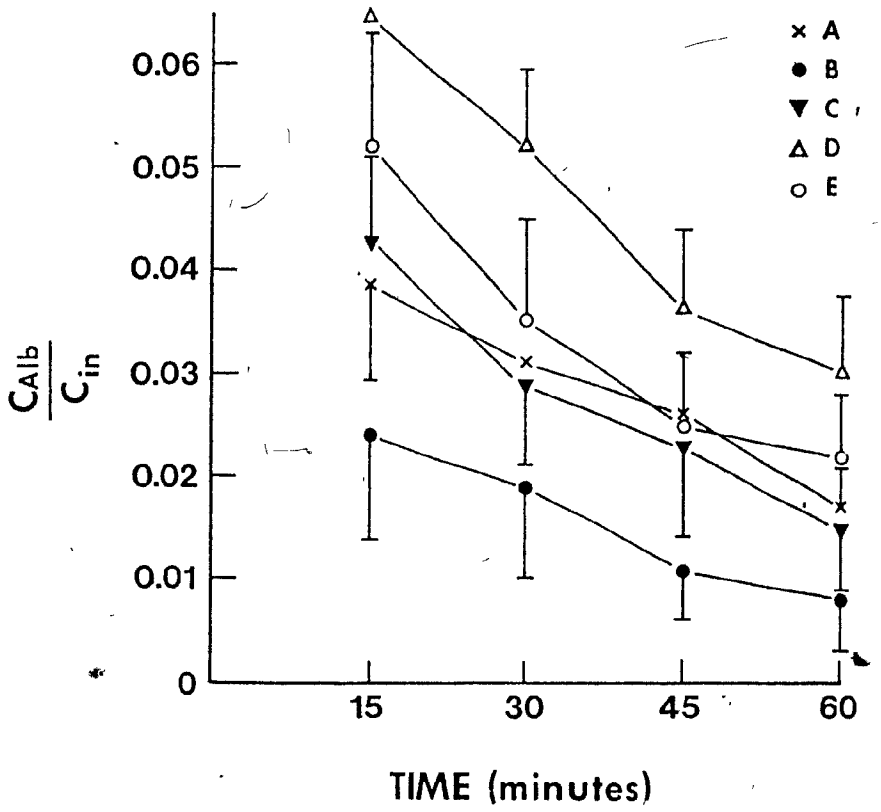


Figure 47. The ratio of albumin to inulin clearance of control and Me_2SO-perfused kidneys during normothermic perfusion. Expressed as a function of perfusion time. (Mean ± SEM. Groups A, C, D and E: 5 observations at each point. Group B: 3 observations at each point.

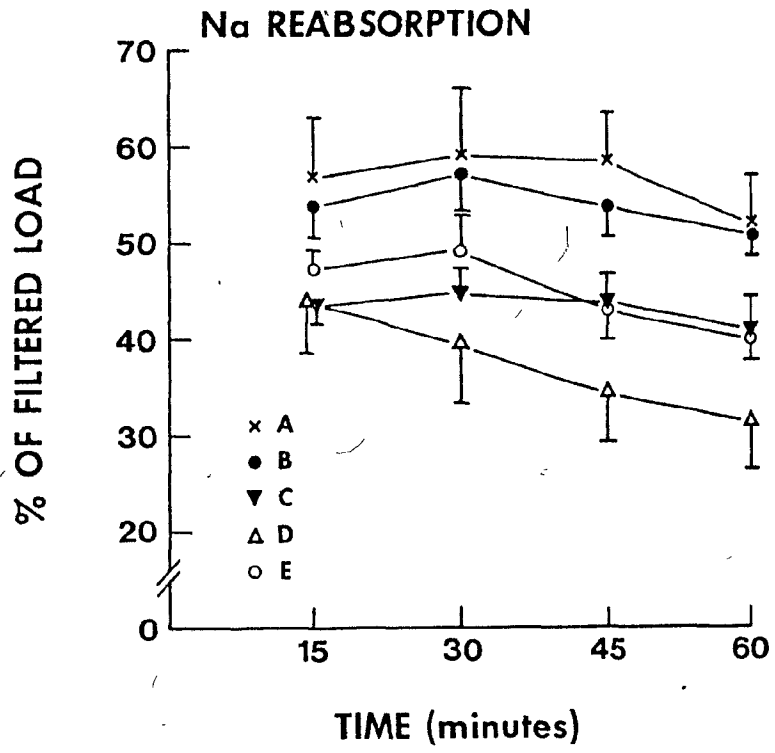


Figure 48. The percentage of filtered sodium reabsorbed by control and Me_2SO-perfused kidneys during normothermic perfusion. Expressed as a function of perfusion time (Mean ± SEM. Groups A, C,D and E: 5 observations at each point. Group B, 3 observations at each point).

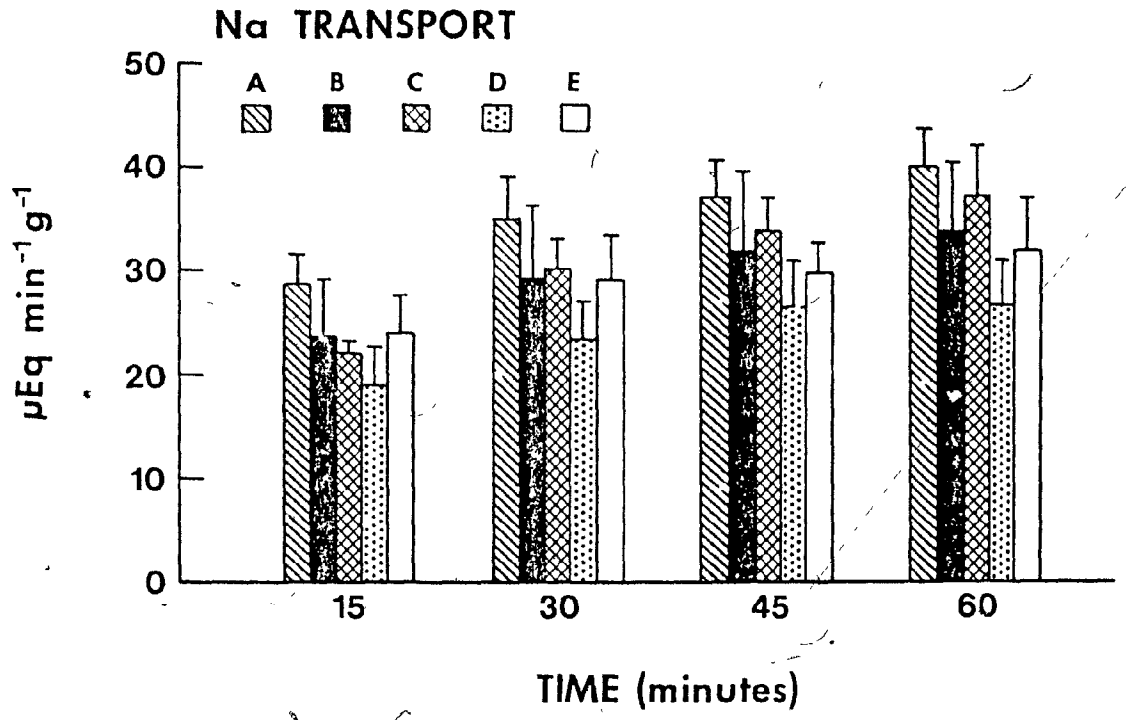


Figure 49. Sodium transport by control and Me_2SO-perfused kidneys during normothermic perfusion. Expressed as a function of perfusion time. (Mean ± SEM. Groups A,C, D and E: 5 observations at each point. Group B, 3 observations at each point).

glomerulus. All three groups treated with 3 M Me_2SO exhibited depressed proportional reabsorption of sodium. Both Group C (43.35 ± 1.27%) and Group E (44.79 ± 1.56%) were similarly depressed over control kidneys ($p < .01$). The decreased sodium reabsorption in Group D, 37.47 ± 2.75% was more pronounced ($p < .005$). While Groups C and E also maintained stable rates of reabsorption, Group D progressively decreased with time. The sodium transport (Figure 49) increased in all groups as perfusion progressed ($p < .01$); only sodium transport in group D was significantly less than freshly isolated controls ($p < .005$).

Glucose reabsorption (Figure 50) did not vary with perfusion time. The percent reabsorption, averaged over the four clearance periods, was greater than 90% in all groups except Group D. In this group, glucose reabsorption (86.30 ± 1.51%) was significantly reduced compared to Groups A (94.12 ± 0.37%) and B (94.83 ± 0.53%) $P < .005$. Group C (91.85 ± 0.69%) and E(91.38 ± 0.93%) were similar to controls and superior to Group D ($p < .01$). The ratio of the clearance of phenol red to the clearance of inulin indicates the secretory activity of the tubules (Figure 51). The higher the ratio, the greater the concentrating ability of the kidney tubules. In kidneys treated with Me_2SO with the 600-500-400 washout treatment (Groups C and E), initial values were lower than controls , ($p < 0.1$ and $p < 0.25$ respectively), but increased during perfusion. Group D kidneys, in which washout was initiated at higher osmolality, were depressed in comparison to the control average ($p < .005$) and continued to decrease.

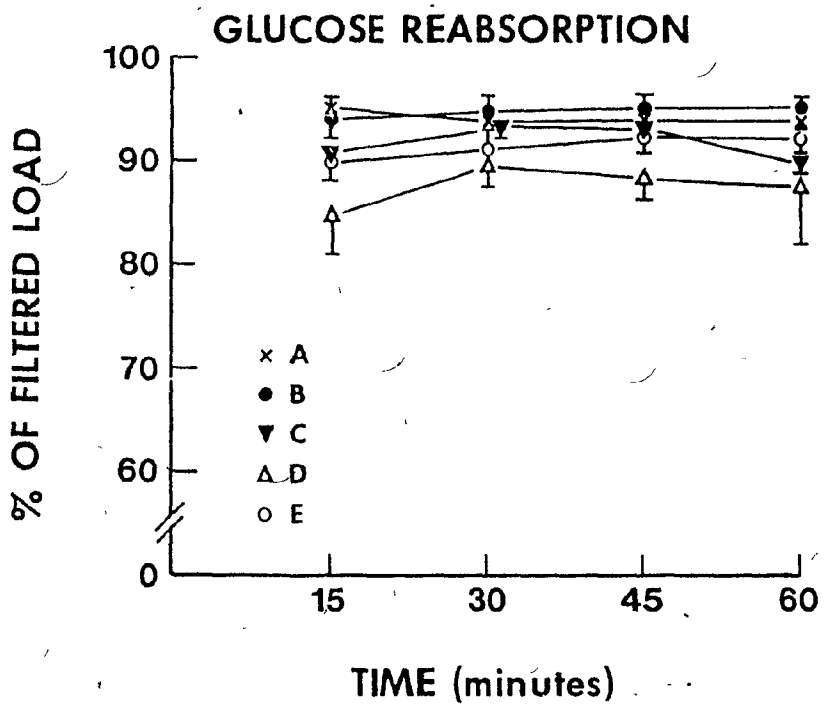


Figure 50. The percentage of filtered glucose reabsorbed by control and Me_2SO-perfused kidneys during normothermic perfusion. Expressed as a function of perfusion time (Mean ± SEM. Groups A,C,D and E: 5 observations at each point, Group B, 3 observations at each point).

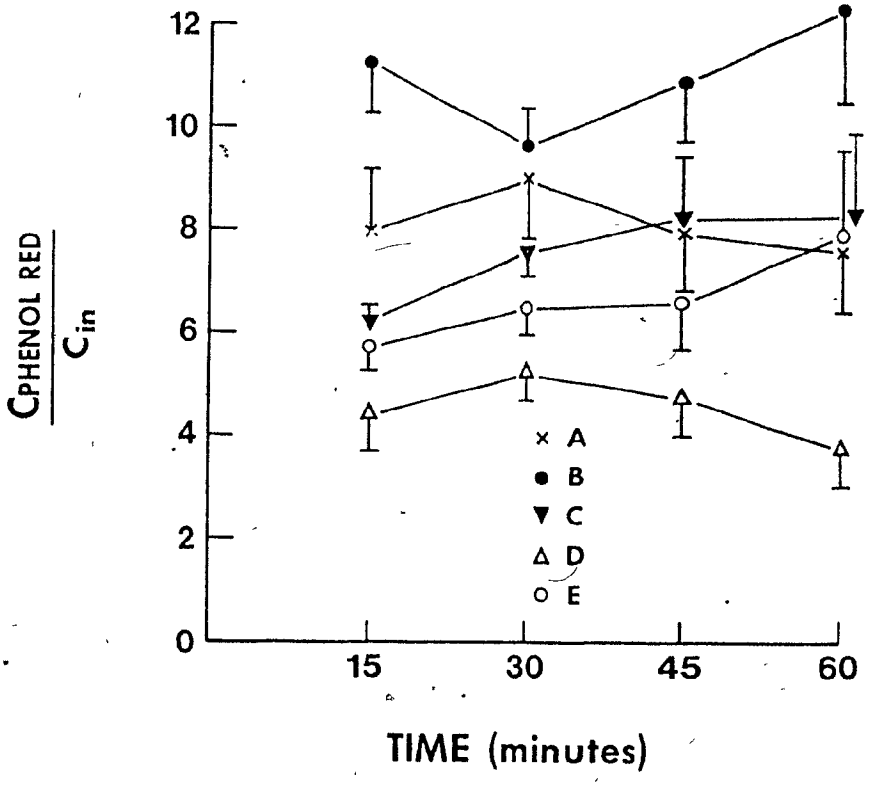


Figure 51. The ratio of phenol red to inulin clearance in kidneys perfused during normothermic perfusion. Expressed as a function of perfusion time (Mean ± SEM. Groups A,C, D and E: 5 observations at each point. Group B, 3 observations at each point).

4). Histology

Histological examination of kidney sections did not reveal severe ultrastructural changes in any of the five groups of kidneys studied (Figure 52). Both Groups C and E exhibited well-preserved ultrastructure similar to control groups A and B. Kidneys in Group D exhibited glomerular alterations (Figure 52, G and H), not observed in the other groups.

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Figure 52

A. Freshly Isolated Control. (Group A) Section following perfusion at 37°C for 60 minutes (H & E, 64X).

B. As above (H & E, 256X)

C. Hypothermic-perfused Control. (Group B) Section following perfusion at 10°C for 3 hours and assessment at 37°C for 60 minutes. (H & E, 64X)

D. As above (H & E, 256X)

E. Me_2SO perfused kidney (Group C). Me_2SO added at 5 ml min^{-1} to 3 M, washout with 600/500/400 mOs/kg media at 10°C and assessed at 37°C for 60 minutes (H & E, 160X)

F. As above (H & E, 256X)

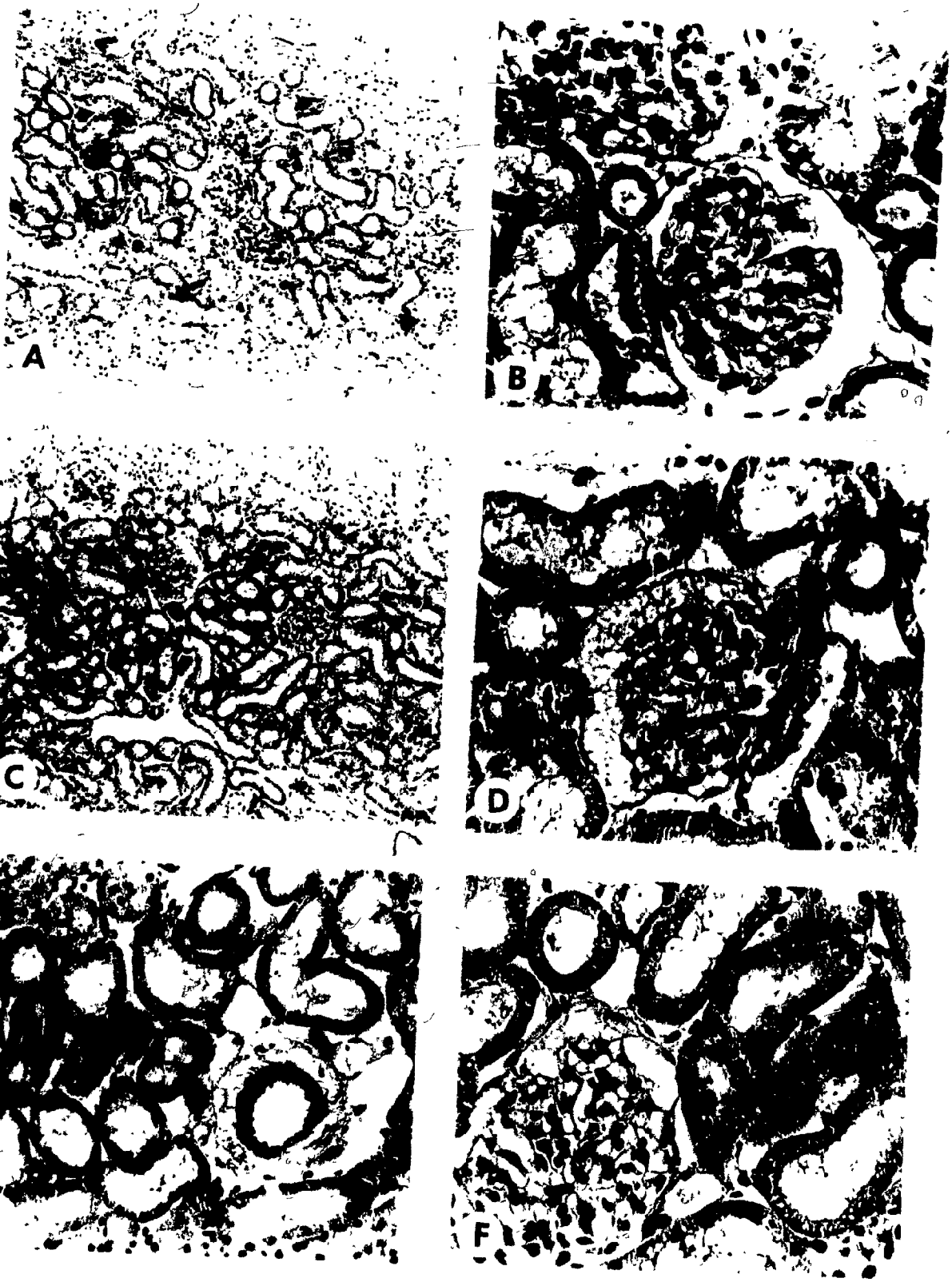
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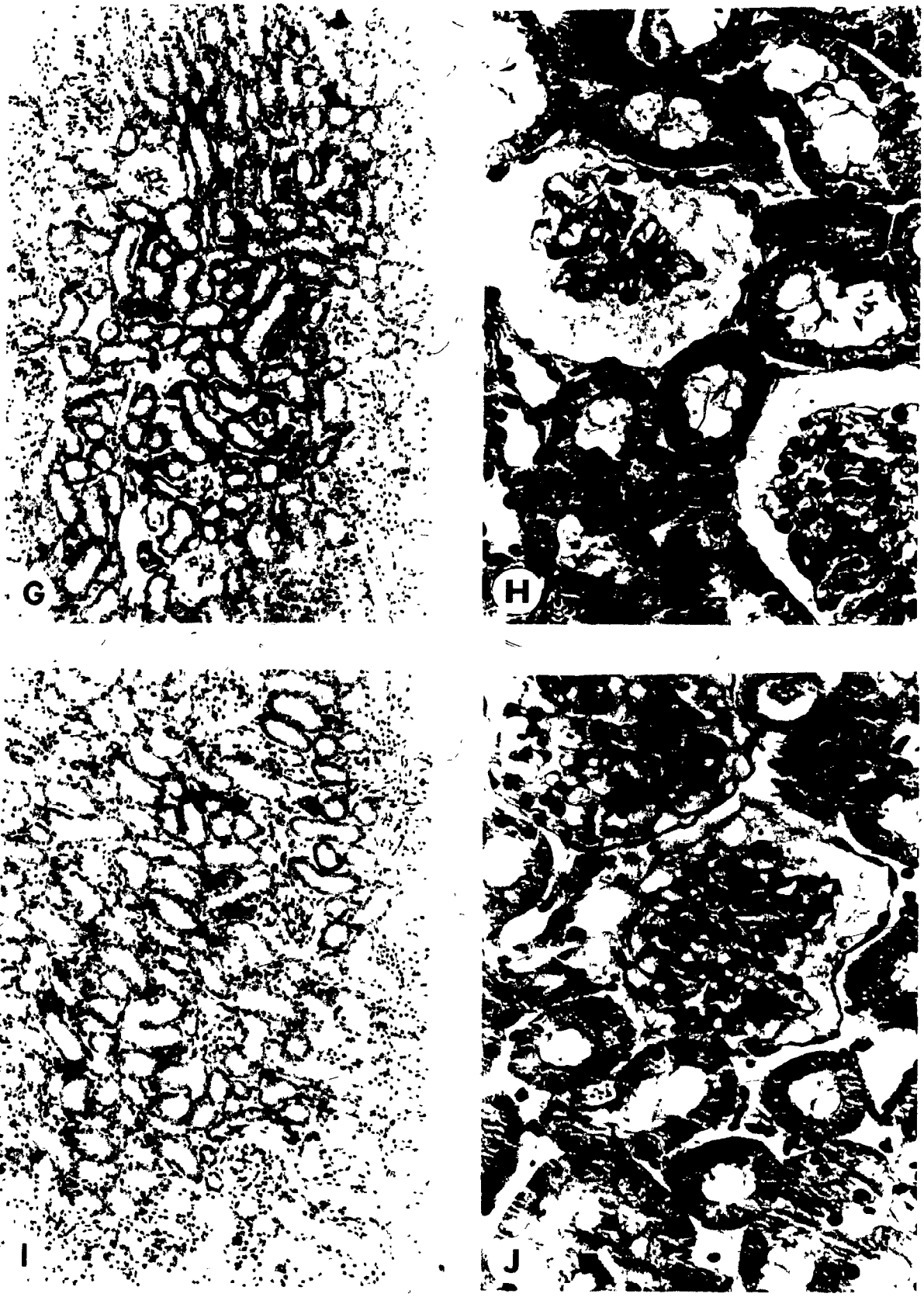
Figure 52 - Continued

G. Me_2SO-perfused kidney (Group D) . Me_2SO added at 5 ml min^{-1} to 3 M, washout with 800/700/600/500/400 mOs/kg media at 10°C, and assessed at 37°C for 60 minutes. (H & E, 100X).

H. As above (H & E, 400X)

I. Me_2SO perfused kidney (Group E). Me_2SO added at 3 ml min^{-1} to 3 M, washout with 600/500/400 mOs/kg media at 10° and assessed at 37°C for 60 minutes (H & E, 100X).

J. As above (H & E, 400X)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H
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J

Discussion

The results obtained in this study provide evidence that kidneys treated with 3 M Me_2SO can function immediately following the removal of the cryoprotectant. The protocol designed for the introduction and removal of the Me_2SO represents what is believed to be a procedure which not only is less harmful to the kidney, but may also be less predisposing to further damage during slow cooling to low temperatures and subsequent thawing.

The solution which served as the vehicle for Me_2SO introduction was a modified Collin's solution, renal preservation solution-2. This solution was reported to maintain a K^+ /Na^+ ratio in tissue slices after four days cold storage equivalent to that of fresh tissue (Fahy et. al. , 1979). The solution was supplemented with mannitol and HEPES buffer to render it useful for perfusion. The use of a solution containing high levels of potassium and low levels of sodium have been applied primarily for organ storage procedures (Collins et. al. , 1969 ; Sacks et. al. , 1973 ; Ross et. al. , 1979);, however the benefits of this type of solution have not been universally accepted for storage and perfusion above freezing (Fuller and Pegg , 1976 ; Green and Pegg , 1979). Keeler et. al. (1966) clearly demonstrated that in dog and rat kidneys, perfusion with Me_2SO in a balanced salt solution resulted in a 50% loss of cellular potassium, which was incompatible with kidney survival. This loss of potassium and subsequent loss of viability was prevented by perfusion with Me_2SO in a potassium rich

solution. Experiments with the isolated rabbit kidney which used potassium rich solutions as carrier of Me_2SO have confirmed its usefulness (Fonteles et. al. , 1973 ; Jeske et. al. , 1974 ; Karow and Jeske , 1976 ; Karow et. al. , 1979).

In a previous experiment (5) the solution employed in this study did not have damaging effects on kidney function when perfused at 10^0 C for a length of time needed to add the Me_2SO and allow equilibration. In this study, perfusion of kidneys with Me_2SO-free perfusate for 3 hours at 10^0 C confirmed that this potassium rich, sodium poor medium had no deleterious effects on subsequent kidney function at 37^0 C. In certain parameters measured, specifically perfusate flow rate and phenol red concentrating ability, the cold - perfused controls proved to be superior to freshly isolated kidneys. These differences may be attributed to a progressive dilation of the renal vasculature during hypothermic perfusion which helped in clearing trapped red cells or other components of blood from capillaries, resulting in a more complete perfusion at 37^0 C. Increased tubule perfusion of peritubular capillaries would then deliver increased amounts of phenol red for transport into the tubular lumen. The low levels of protein leakage into the urine indicated that the albumin retention capability of the glomerulus remained intact, and there was minimal sloughing of damaged tubules. The capacity of the tubules to reabsorb sodium and potassium was very similar to controls. These results are comparable to those reported by Pegg and Wusteman (1977). Freshly isolated controls functioned quite similarly in both studies; however, the detrimental affect of hypothermic perfusion

without cryoprotectant observed in that study was not apparent in the present work.

The introduction of Me_2SO was accomplished at two rates of addition. In both, the rate of addition was decreased as the perfusate concentration of Me_2SO increased. After the perfusate concentration reached 2 M Me_2SO, the rate of addition was less than 100 mM min^{-1} in the more rapid addition. Previous reports by others studying Me_2SO in concentrations above 1.5 M did not increase the concentration slowly (Pegg , 1972 ; Karow and Jeske , 1976). In the first report, the perfusate flow rate increased briefly followed by a dramatic increase in renal vascular resistance at both 37 ^{0}C and 5 ^{0}C. The vascular endothelium was severely damaged with 2 M Me_2SO. Perfusion with 2.8 M Me_2SO at 37 ^{0}C (Karow and Jeske , 1976) did not result in the same phenomenon, however flow rates during perfusion were very low.

Subsequent studies have confirmed that rapid addition of Me_2SO has deleterious, osmotic effects which cause damage and which can be predisposing to damage from subsequent treatment. Guttman et. al. (1979) reported that rapid addition of 1.5 M Me_2SO to dog kidneys perfused at 10 ^{0}C resulted in increased release of LDH. This was reduced if addition was accomplished in graduated steps. Karow et. al. (1979) found that at 25 ^{0}C, if the rate of addition and removal of 1.4 M Me_2SO was decreased, an increased number of dog kidneys survived after reimplantation.

The importance of osmotic effects have been confirmed for rabbit kidneys perfused with glycerol, which require very slow glycerolization

procedures (Pegg and Wusteman, 1977; Jacobsen et. al., 1978; 1979). In this respect glycerol is probably more likely to exert osmotic stress at low temperatures due to a relatively low permeability in comparison to Me_2SO (McGann, 1978). The data obtained during 10 ^{0}C and 37 C perfusions indicate that the rate of addition of Me_2SO is not a critical factor, provided that the rate is sufficiently reduced to prevent osmotic-induced fluctuations in cell volume. Perfusate flow rates at constant pressure were very similar at rates of addition which differed by at least 40%. In vitro function at 37 ^{0}C did not indicate large differences between the two groups in which the Me_2SO had been removed in the same manner (Groups C and E).

Despite the absence of osmotic effects, the kidneys perfused with Me_2SO did not exhibit the pattern of decreasing renal vascular resistance observed in hypothermic perfused kidneys with Me_2SO-free perfusate. Me_2SO may have affected the vascular smooth muscle cells and produced vasoconstriction. The nature of this action is not well documented, but is evident in different cell and tissue slice systems. Leibo et. al. (1974) found that after exposure of eight-celled mouse embryos to Me_2SO for 90 minutes, the embryos did not attain original volume unless diluted back to their original osmolality. Fahy (1980) observed that tissue slices placed in 1.92 M Me_2SO at 0 ^{0}C produced a rapid drop in weight followed by a steady increase over 30 minutes, but never regained initial weight. Effects of Me_2SO have been documented (David, 1972; Pribor, 1975; Williams and Harris, 1977) and in many cases the same effects are attributable to glycerol.

Removal of Me_2SO was accomplished in a manner which has not been previously described for the kidney. This technique offers two advantages over slowly decreasing the Me_2SO concentration . It

immediately introduces a Me_2SO -free perfusate and prevents prolonged exposure to the cryoprotectant.As soon as the perfusate is switched to one without cryoprotectant, the Me_2SO exits the cell down the concentration gradient. However, the Me_2SO trapped in the cell pulls water into the cell presumably at a faster rate than the Me_2SO can leave, which causes cell swelling. If this water influx is too great, the cell will exceed its volume limits and burst. By introducing an impermeant solute, mannitol, the total water flux is reduced since a countering force favouring cell dehydration is applied. As the Me_2SO leaves the cell, the effective osmotic force favouring water entry is reduced. If the concentration of mannitol remained high, the cell would lose water. This dehydration is prevented by reducing the mannitol content of the perfusate in several steps.

The most striking manifestation of the effect of this washout technique was the absence of an increase in the renal vascular resistance which would in our constant pressure system have manifested as a decrease in flow rate. Although attempts to remove Me_2SO in concentrations greater than 1.4 M have not been reported, kidneys which have been deglycerolized at slow rates after equilibration with 2 M or higher concentrations of glycerol invariably exhibited a significant increase in resistance (Pegg and Wusteman, 1977; Pegg and Robinson, 1978; Jacobsen, 1979).

The in vitro assay of kidney function by normothermic perfusion with a cell-free, extracellular based perfusate has been shown to be sensitive to various manipulations by our lab and by others. The freshly isolated controls exhibited a level of function at 37 ^{0}C which compared very closely to controls reported by Pegg and Wusteman (1977) and

which have subsequently been shown to support life following transplantation (Jacobsen et. al.,1978). The kidneys treated with 3 M Me_2SO in this study were capable of maintaining a level of function comparable to kidney function after treatment with 2 M glycerol (Pegg and Wusteman, 1977). Function of kidneys perfused with the Me_2SO-free medium indicated that the high potassium, low sodium perfusate did not in itself incur damage.

The results indicated that kidneys in which the Me_2SO was removed by high osmolality washout (800 mOs/kg initial step) did not function as well as those initially subjected to 600 mOs/kg perfusate. In the first case, kidneys exhibited a more rapid increase in flow rate following Me_2SO removal, which was delayed if washout commenced with 600 mOs/kg perfusate. In group D perfusate flow rates and tubule function at 37 ^{0}C was impaired; histological examination revealed alterations of glomerular structure when compared to controls. The reason for this difference in recovery is not readily apparent. One explanation might be that after prolonged exposure to the 800 mOs/kg solution, the Me_2SO had diffused down its concentration gradient and been diluted by the perfusate. The cell is confronted by an unopposed force favouring dehydration, which might cause cell damage. By initiating washout at a lower osmolality, the dehydrating force which is exerted on the cell is less harmful.

The results may provide some degree of optimism for future attempts of renal preservation. Recent reports of the contribution of high concentrations of cryoprotective agents to freezing damage were carried out without the above precautions of addition and removal (Armitage and Pegg, 1979; Fahy, 1980). The results of exposure of organized

tissues or organs to to cryoprotective agents is actually the result of three or more treatments: the addition of the agent, exposure to the agent, and its removal. Each of these manipulations is difficult to separate. Suboptimal procedures for introduction and removal may not have severe deleterious effects, but they can predispose to freezing damage from manipulations such as concentration of solutes or high cryoprotectant concentrations. We have demonstrated that kidneys are able to function after addition, equilibration and removal of 3 M Me_2SO at 10 ^{0}C. The removal of Me_2SO can be accomplished without deleterious effects if the osmotic stresses of the cryoprotectant are counteracted with increased amounts of impermeant solute in the perfusate. Excessive amounts of impermeant solute may be deleterious to the kidney.

Summary and Conclusions

This study was designed with two main objectives. The first dealt with the hypothesis that a simple method of *in vitro* normothermic rabbit kidney perfusion could be developed to serve as a suitable test of the functional integrity of the kidney prior to reimplantation. The second objective was to employ the system to investigate the possibility of treating rabbit kidneys with multimolar concentrations of Me_2SO if addition, permeation and removal of the cryoprotectant were performed in a non-deleterious manner.

The initial subject of investigation, following the design and construction of the perfusion circuit, was the use of an oncotic agent. The effects of dextrans of molecular weight 70,000, 80,000 or 180,000 were compared to a colloid-free perfusate at reduced perfusion pressure. Inclusion of dextrans of different molecular weights, and from two different suppliers, did not result in satisfactory perfusion characteristics. Kidney performance *in vitro* was quite variable. Kidneys exhibited steadily increasing renal vascular resistance, low rates of glomerular filtration, and depressed tubular reabsorption.

Microscopic evaluation indicated ultrastructural changes. Glomerulae of dextran-perfused kidneys remained intact, but expansion of Bowman's space was evident, with compression of the glomerular capillary lumen. In certain areas, the endothelial lining of the peritubular capillaries was stripped from the basement

membrane. Proximal tubules exhibited mitochondrial swelling, cytoplasmic vacuolization with disruption of the brush border and loss of cell cytoplasm.

Rabbit kidney perfusion without dextran, at a reduced perfusion pressure of 50 mmHg resulted in a more suitable preparation with rapid onset of function and maintenance for two hours. Renal vascular resistance decreased (and perfusate flow rate increased at constant pressure) as perfusion progressed. The glomerular filtration rates in this group were significantly increased over dextran-perfused kidneys. Renal tubules exhibited stable sodium and glucose reabsorption.

The dextrans used in this study proved to be unsuitable as a source of colloid osmotic pressure. They did not control edema, and it was concluded that the dextrans were not completely retained within the vascular system. It was not possible to ascertain whether the deleterious action of the dextran was due to a direct toxic effect on proximal tubules or to a secondary effect of ischemia. The changes in the vascular system and the obvious edema caused significant reduction of perfusate flow rate. This may have resulted in decreased oxygen delivery to certain areas of the kidney. In studies of rat kidney ultrastructure following ischemic periods (Latta et. al. , 1965 ; Reimer et. al. , 1972), similar ultrastructural changes were described. Other reports have implicated dextran as being directly toxic to proximal tubules (Imai and Kokko , 1974), precipitating epithelial disintegration, which is consistent with

the findings of this experiment.

On the basis of morphological appearance and texture of the kidney during perfusion, and on histological evidence, edema was more effectively controlled by perfusion at reduced pressure without dextran. Other advantages of the colloid-free system were apparent. The rapid onset of function, and the stability of the preparation were two features important for an *in vitro* assay of integrated function. Reabsorption of sodium and glucose, despite the absence of an oncotic agent, provided evidence that the reabsorption rates measured active transport by the kidney. The high cost of suitable albumin preparations, the apparent similarity in function to rabbit kidneys perfused *in vitro* with some colloid source (Fuller *et. al.* , 1977 ; Wusteman , 1978), and the superior function compared to reports of rabbit kidney perfusion *in vitro* with Tyrode solution at low flow rates (Fonteles *et. al.* , 1973) support the claim that the colloid-free reduced pressure system of rabbit kidney perfusion could prove to be of great potential value.

This system was chosen for further investigation and development. Limited information is available concerning the utilization of exogenous energy substrate by the rabbit kidney *in vitro* (Fuller *et. al.* , 1977). Lactate, butyrate and glucose utilization have been studied in the isolated perfused rat kidney (Ross *et. al.* , 1973 ; Gregg *et. al.* , 1978 ; Cohen *et. al.* , 1980). In the second experiment of this study, the sensitivity of the kidney to available exogenous energy substrate was determined by

monitering its ability to reabsorb sodium during in vitro perfusion.

With substrate-free perfusate, sodium reabsorption decreased steadily with perfusion time. Supplementation with glucose resulted in slightly increased levels of sodium reabsorption and prevented the rapid decline observed during the final thirty minutes of perfusion with substrate-free perfusate. When the glucose-containing perfusate was supplemented with lactate or butyrate, sodium reabsorption increased. Lactate supplementation was the most beneficial, resulting in significantly higher rates of sodium and glucose reabsorption. The net glucose utilization for sodium transport was reduced when lactate or butyrate was added to the glucose-containing perfusate.

The rabbit kidney exhibited dependence on exogenous energy sources for reabsorption. Endogenous energy sources were steadily depleted during perfusion and unless exogenous substrate was available, capacity to handle filtered sodium or glucose decreased. Under the perfusion conditions described, the combination of lactate with glucose best supported reabsorption .

Not only did this experiment identify the appropriate energy sources; of equal significance was the demonstration that this system was sensitive enough to detect this change. This supports the potential of the system for assessment of the effects of preservation treatments. The system is sensitive to changes which are modest in comparison to those anticipated following long term storage procedures.

Kidneys perfused in the first experiment were attached to the

circuit immediately following removal. In the second experiment, the kidney was flushed with perfusate prior to attachment to the circuit. Perfusate flow rates during the first hour of perfusion were slightly increased in the second experiment. By 90 minutes of perfusion, the flow rates were similar. A vasodilator, isoxsuprine HCl, was included in the flushing of kidneys used in experiment 3. The kidneys were perfused in a similar manner to those in experiment 2 for the first 3 clearance periods. Flow rates were similar at the onset of perfusion, but by 30 minutes had increased in the third experiment, in contrast to the transient decrease observed in the second experiment. The addition of the vasodilator was beneficial, although not striking at the onset of perfusion. Including isoxsuprine may have aided in clearing the kidney of blood and blood components, producing a more uniform washout, and preventing the delayed release of vasoactive blood components trapped in the kidney micro-circulation and only freed after perfusion had progressed. The increase in flow rate and higher glomerular filtration rates in experiment 3 support this conclusion.

Increasing the perfusion pressure from 50 mmHg to 75 mmHg increased the perfusate flow rates and the amount filtered at the glomerulus. The kidneys did not respond to the increased filtered load. Although the amount of sodium reabsorbed by the tubules remained constant, the proportion of filtered sodium and glucose reabsorbed decreased. Under these conditions, the kidney was operating at capacity, and increased oxygen delivery

did not enhance function. It is concluded that oxygen availability in this system is not limiting function.

Including small amounts of bovine serum albumin (BSA) in the perfusate significantly increased perfusate flow rates at both 50 and 75 mmHg perfusion pressure. Measurement of the albumin leakage at the glomerulus indicated that the kidneys retained albumin at the glomerulus. Kidneys perfused with 0.5% or 1.0% BSA exhibited increased reabsorption of filtered glucose and sodium. When the perfusion pressure was increased, the tubular sodium transport increased significantly and the kidneys perfused with 0.5% BSA maintained a higher proportion of sodium and glucose reabsorption. Perfusion with 1.0% BSA, although exhibiting increased function over BSA-free medium, resulted in function below that obtained with 0.5% BSA.

The beneficial effects of the BSA could not be attributed solely to the provision of oncotic pressure. Increasing concentration from 0.5 to 1.0% decreased sodium transport, and did not increase perfusate flow rates over 0.5% BSA. However the GFR in the 1.0% BSA group was significantly lower than the 0 or 0.5% groups. It was concluded that the effects of BSA were due to an oncotic effect, to regulation of pore diameter of blood vessels and/or the possible supply of an unidentified substance which increased function. A slight oncotic effect would aid in tubular reabsorption by the prevention of tubular backflux of reabsorbate through tight junctions between proximal tubule cells. BSA adherence to vessel walls would regulate pore diameter, and prevent excessive

filtration into the interstitium. These effects would be manifest at both concentrations of BSA, but in this system 0.5% BSA was optimal. It is suggested that these beneficial effects are overshadowed by deleterious effects which manifest as the concentration is increased, as reported by Fuller et. al. (1977). It is also possible that the BSA exerts effects which are peculiar to rabbit kidneys and not to rat kidneys perfused in vitro. In kidneys perfused with 1.0% BSA, protein leakage at the glomerulus remained constant and Bowman's space was distended. Kidneys perfused with 0.5% exhibited a decreasing ratio of albumin to inulin clearance, with no evidence of distension of Bowman's space.

In the final step concerned with development of the normothermic perfusion system, kidneys were perfused for one hour at 37 ^{0}C with perfusate containing 0.5% BSA and 5 mM lactate. The GFR was initially high and remained so for the four clearance periods, exhibiting a slight increase with time. The amount of sodium transported from lumen to peritubular capillaries exhibited a similar pattern. The proportion of filtered sodium and glucose was stable; glucose reabsorption remained higher than 90%. Albumin leakage was minimal and kidneys were capable of secretion of phenol red.

In vitro function was comparable to rabbit kidneys perfused at 37 ^{0}C with colloid (Fuller and Pegg , 1976 ; Fuller et. al. , 1977 ; Pegg and Wusteman , 1977 ; Wusteman , 1977), and superior to kidney function reported without colloid (Fonteles et.

al. , 1973). Perhaps most interesting is the comparison with rat kidney function without BSA (Bowman and Maack , 1974 ; Little and Cohen , 1974). Function was very similar for both species and were shown for the rat to return to control levels if the BSA (7.5%) was added to the perfusate (Bowman and Maack , 1974). It is conceivable that should a suitable colloid source be identified, function of the rabbit kidney using the present system could equal that obtained with optimal procedures described for the rat.

This perfusion model was able to delineate the effects of hemodynamically-mediated acute renal failure. Function obtained with freshly isolated controls was significantly better than kidneys exposed to one hour of warm ischemia prior to perfusion. Although the kidney perfused under these conditions is liable to the same concentration defect as other systems using the rat (Maack , 1980) or rabbit (Fuller et. al. , 1977) kidney, addition of ADH indicated that the capability of hormonal interaction and associated cellular events altering tubular permeability remained intact.

The isolated rabbit kidney perfused in vitro with a colloid-free reduced pressure system is not capable of mimicking in vivo function; however it is not subjected to the same milieu, and seems to function optimally under the given conditions. The sensitivity is sufficient to recognize deleterious effects. Early onset and stabilization of function permits rapid evaluation of the ability to perform integrated functions which are necessary

to support life *in vivo*. It is concluded that this system is a valuable *in vitro* method of assessment of kidney function which can be used as an alternative to reimplantation. The system is economical, capable of producing immediate results and applicable for studies of renal preservation.

Although the model is a functional assay, the full potential is not realized. Several aspects merit future investigation. The search for a suitable oncotic agent should continue. Purification of economically feasible albumin preparations, combinations of other synthetic colloids with albumin or small amounts of rabbit serum might prove beneficial. Consideration might be given to the composition of the perfusion medium. Supplementation with amino acids, or substitution with a more complete medium formulation could provide components to further enhance kidney function.

The spectrum of renal function during *in vitro* perfusion could be investigated further. Evaluation of the ability to handle other filtered solutes phosphate, potassium, amino acids, bicarbonate would identify areas of efficient and deficient function. The response to hormonal or pharmacological stimuli should be investigated to determine if the kidney will respond as *in vivo*.

Reimplantation of the kidney following perfusion would provide important information. Correlation between *in vitro* function and ability to support life would enable the system to

predict viability as well as functional integrity.

The normothermic perfusion system was employed to assess the functional integrity of the rabbit kidney following perfusion at 10^{0}C with the cryoprotectant Me_2SO. A simple perfusion circuit for hypothermic perfusion at constant pressure was constructed. The perfusate was a high potassium, low sodium formulation. The perfusate was chosen based on reports of Me_2SO-perfused dog, rat and rabbit kidneys which suggested that an intracellular formulation was superior to an extracellular formulation when Me_2SO was included. The perfusate employed in this study was not identical to these reports, but had proven effective for storage of renal cortical slices at 4^{0}C. It was modified for this study to render it suitable for hypothermic perfusion.

To evaluate the effects of perfusion at 10^{0}C with the potassium-rich, sodium poor medium, kidneys were perfused for 75 minutes with Me_2SO-free hypothermic perfusate. The kidneys were transferred to the 37^{0}C circuit and function was assessed for a one-hour period. The hypothermic perfusate, and the perfusion circuit proved to be acceptable for the period of perfusion necessary for introduction of cryoprotectant. The 10^{0}C perfusion resulted in increased perfusate flow rates and increased phenol red secretory ability in comparison to freshly isolated controls. Rapid recovery to control levels of sodium reabsorption was observed . The innocuous effects of this perfusion were in sharp contrast to the deleterious effects of cold-perfusion of rabbit kidneys with an extracellular formulation containing haemaccel

and similar amounts of mannitol (Pegg and Wusteman , 1977). The system was considered acceptable for introduction of Me_2SO. It was concluded that, provided it did not interact with Me_2SO to produce deleterious effects, the system would allow assessment of treatment of kidneys with Me_2SO.

The permeation kinetics of the cryoprotectant Me_2SO at concentrations above 3 M were investigated by measurement of total fluid (water and/or Me_2SO), inulin and Me_2SO spaces using radio - labelled inulin and Me_2SO. The concentration of Me_2SO in the recirculating perfusate was increased gradually to 3.06 M over 40 minutes. Through study of the change in the Me_2SO space and inulin space, evidence of cellular permeation of Me_2SO was obtained. Control kidneys exhibited a decreased inulin space during perfusion, an indication of cell swelling. Kidneys perfused with Me_2SO demonstrated a doubling of the inulin space which did not decrease during perfusion. The proposed explanation was marked increase in tubular lumen volume which was evident upon histological examination.

Although maximum permeation of medullary sections was achieved prior to tissue sampling, the permeation of the kidney cortex was maximal only after exposure to 3.06 M Me_2SO for 35 minutes. It was concluded that a period of exposure of 35 minutes to 3 M Me_2SO was necessary for permeation. The interval between extracellular and intracellular equilibration of Me_2SO provided direct evidence of an osmotic effect of Me_2SO on kidney cells, and substantiated the need to introduce the

cryoprotectant slowly to reduce osmotic effects.

Rabbit kidneys were assayed for function in vitro at 37°C following introduction, equilibration and removal of 3 M Me_2SO. The concentration of Me_2SO in the recirculating perfusate was increased at two different rates; one similar to the previous study for permeation kinetics, and a second rate 40% slower. Me_2SO was washed from the kidney by perfusion with Me_2SO-free perfusate which contained different concentrations of mannitol.

During perfusion at 10°C, flow rate increased gradually over time in control (Me_2SO-free) perfused kidneys. After 45 minutes, in groups perfused with Me_2SO, flow decreased and then stabilized. During the washout stage, kidneys in which washout commenced at 800 mOs/kg exhibited a steadily increasing flow rate from onset of the washout procedure, then stabilized. Where washout commenced at 600 mOs/kg, flow rate remained constant for 20 minutes followed by an increase and stabilization.

At 37°C, in all groups perfused with Me_2SO, function was depressed at the beginning of perfusion. As perfusion progressed function in groups in which the washout procedure began at 600 mOs/kg increased to levels similar to controls; the proportion of filtered sodium reabsorbed was depressed. In kidneys where washout began at 800 mOs/kg, perfusate flow rate, sodium and glucose reabsorption and phenol red secretion remained lower than controls, and lower than groups in which washout began at 600 mOs/kg.

Regardless of the method of Me_2SO introduction and removal, kidneys could function following treatment with 3 M Me_2SO. The

rate of addition of Me_2SO was not critical if added slowly to prevent osmotic effects. The method of Me_2SO removal has not been previously described for the kidney. It demonstrated that Me_2SO can be removed rapidly if the osmotic flux of water is controlled. Furthermore, washout with excessively hypertonic medium may be deleterious. In this experiment the rapid increase in flow rate upon institution of washout with 800 mOs/kg medium can be interpreted as the result of overestimation of the time necessary for Me_2SO to leave the cell. This would subject the cell to a large dehydrating force (800 mOs/kg) when the concentration of intracellular Me_2SO had diminished. This dehydration would increase the radius of blood vessels and result in increased flow rates under constant pressure. Initiation of washout with 600 mOs/kg medium was sufficient to prevent excessive cell swelling due to the Me_2SO initially trapped in the cell. The dehydrating force of this solution was reduced, and subsequently caused less damage.

A dramatic increase in resistance during washout of cryoprotectant has been observed when attempting to remove glycerol by gradual reduction of perfusate concentration over an extended period of time (Pegg and Wusteman, 1977; Pegg and Robinson, 1978; Jacobsen, 1979). The washout method described here eliminated this problem and prevented prolonged exposure to the cryoprotectant.

The evidence presented supports the hypothesis that kidneys can tolerate high concentrations of Me_2SO if certain conditions are controlled. It is possible that higher concentrations of Me_2SO could be used. As the concentration increases, the osmolality of the washout solutions and the time of exposure to them could be adjusted to prevent excessive dehydration.

A specific toxic effect of Me_2SO has not been substantiated in this study. Studies which have implicated intrinsic Me_2SO toxicity (Pegg, 1972; Fahy and Karow, 1977; Fahy, 1980) are inconclusive due to deleterious effects precipitated by inappropriate procedures of addition and removal of the cryoprotectant at high concentrations. Although the importance of osmotic damage to kidneys has been recognized and surmounted when using glycerol, Me_2SO has not, until now, received equal attention. This may be due in part to the assumption that Me_2SO is a rapid cell permeator (Taylor et. al., 1974) exhibiting little osmotic effect. Glycerol, in contrast, has been accepted as a poor permeator at low temperatures (Dooley, 1980). The present study indicates that Me_2SO exerts a significant osmotic effect which must be considered when interpreting studies which utilized a single step addition or removal.

The methodology developed in this study for cryoprotection and functional assessment of rabbit kidneys permitted a more complete understanding of the effects of Me_2SO on the kidney. Further application of these techniques should aid in the elucidation of unresolved problems and controversies associated with kidney cryopreservation. Increasing the Me_2SO concentration, and replacement of the perfusate with solutions which resemble salt concentrations developed during cooling, would aid in the identification of potentially harmful factors. The effects of these factors may be ameliorated by altering the perfusate composition before or during the early phases of cooling. Coupled with the evaluation of different cooling and thawing protocols, a freeze-thaw process which retains kidney function could be developed.

Contribution to Knowledge

This study was undertaken to develop a simple method of *in vitro* normothermic perfusion of rabbit kidneys, to be used as an assay of renal functional integrity. This assay served to elucidate the effects of hypothermic perfusion with 3.0 Me_2SO.

A. Kidney function was assessed by normothermic *in vitro* perfusion. The effect of including dextran of M.W. 180,000, 80,000 or 70,000 was compared to a colloid-free reduced pressure system.

1). Including dextran as an oncotic agent resulted in deleterious effects on kidneys. Loss of kidney function correlated with histological findings of vascular impairment, edema formation and proximal tubule damage.

2). These effects were present and not specific to one molecular weight range, nor to one source.

3). Normothermic perfusion without colloid at reduced pressure was more effective, kidneys exhibited stable function with acceptable perfusion characteristics.

4). With colloid-free reduced pressure perfusion, onset of function was rapid and remained stable for 2 hours.

B. Kidney utilization of exogenous energy substrate was investigated in terms of glucose utilization and sodium reabsorption.

1). Kidneys perfused *in vitro* with a colloid-free reduced pressure system were sensitive to exogenous supply of energy substrate,

2). Perfusion with glucose as sole energy substrate maintained stable rates of sodium reabsorption in comparison to a fall off during substrate-free perfusion.

3). Supplementation of glucose-containing perfusate with lactate or butyrate enhanced tubule sodium and glucose reabsorption.

4). Lactate was the preferred energy substrate over butyrate, and significantly decreased net glucose utilization per unit of sodium reabsorbed.

C. Kidneys were perfused at 50 and 75 mmHg perfusion pressure. The concentration of BSA in the perfusate was 0, 0.5 or 1.0% BSA.

1). When pressure was increased, kidneys perfused without BSA were not capable of responding to the increased filtered load.

2). Including 0.5% BSA in the perfusate enhanced perfusion characteristics, sodium reabsorption, glucose reabsorption and phenol red secretion.

Kidneys were able to compensate for the increased filtered load when the pressure was increased.

3). Increasing the concentration of BSA to 1.0% did not improve kidney function *in vitro* over 0.5% BSA. Deleterious effects were found at the higher BSA concentration.

D. The rabbit kidney perfused *in vitro* at normothermia with the colloid-free-reduced pressure system was sensitive to one-hour ischemia and to an exogenous antidiuretic preparation.

1). The function of freshly isolated controls was significantly improved over kidneys perfused after one hour warm ischemia; which indicated that the sensitivity of the preparation was adequate for use as an assay system.

2). Kidneys perfused under these conditions were not able to concentrate urine to a significant degree; however urine hypotonicity is ameliorated with antidiuretic hormone. The concentration defect is related to the high perfusate flow rates. Ability to respond to a hormonal stimulus was preserved.

3). Rabbit kidney perfusion without colloid at reduced pressure resulted in function which was equal to or better than reports of rabbit kidney function *in vitro* utilizing a synthetic colloid, or BSA.

4). Kidney function in this system was equal to rat kidney function during perfusion under similar conditions.

E. The function of rabbit kidneys was assayed at 37 ^{0}C following hypothermic (10 ^{0}C) perfusion with a low sodium, high potassium solution made hypertonic with glucose and mannitol.

1). Perfusion for 75 minutes at 10 ^{0}C had no deleterious effects on rabbit kidneys.

2). The hypothermic perfusion period resulted in higher perfusate flow rates and increased secretory ability in comparison to freshly isolated controls.

F. The kinetics of permeation of 3.0 M Me_2SO was studied in rabbit kidneys perfused at 10 ^{0}C with a low sodium, high potassium perfusate. The Me_2SO was gradually introduced into the recirculating perfusate.

1). Maximal equilibration of Me_2SO and renal tissue was achieved after 35 minutes of perfusion with 3.0 M Me_2SO.

2). Equilibration of the medulla was complete prior to the cortex.

3). Direct evidence of intracellular Me_2SO permeation was obtained.

4). Me_2SO exhibited dramatic effects on the inulin

space, producing a doubling of the inulin space in comparison to controls perfused without Me_2SO.

5). Following equilibration with 3.0 M Me_2SO, the tubular lumen volume is increased, due to increased filtration.

G. Rabbit kidney function was assessed at 37 ^{0}C after introduction, equilibration and removal of 3.0 M Me_2SO at 10 ^{0}C.

1). Functional integrity of kidneys perfused with 3.0 M Me_2SO was well-preserved.

2). Rate of addition of Me_2SO was not critical provided it was added slowly to prevent osmotic shock.

3). Me_2SO, at 3.0 M concentration did not demonstrate toxicity if added slowly and removed with hypertonic washout solutions.

4). Me_2SO removal by hypertonic washout with solutions of decreasing hypertonicity effectively removed the Me_2SO quickly without initiating high renal vascular resistance.

5). The effective osmotic force exerted by the Me_2SO is less than the molar concentration. Washout with solutions of very high concentrations of impermeant solute can cause further dehydration and kidney damage.

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APPENDIX

Computer programme using BASIC language for calculation of indices of kidney function.

```
19 GOTO50
20 Y=LEN(STR$(INT(A)))
21 FOR=0TOY:PRINT"■";:NEXT
22 A=A+0.0005:PRINTINT(A);".";RIGHT$(STR$(INT(1000*(A+1))),3),
23 RETURN
50 PRINT"■    ■WEIL'S■ ■KIDNEYS■ ■■":PRINT:PRINT
55 INPUT"IS DATA ON FILE (Y/N)";A$
60 IFA$="Y"THENSS=1:GOTO20000
100 PRINT"■"SPC(10)"KIDNEY PERFUSION"
115 PRINT:PRINT:PRINT
116 INPUT"RABBIT";RA$
117 PRINT:PRINT
120 INPUT"KIDNEY DESIGNATION";NA$
125 PRINT:PRINT
130 INPUT"LEFT OR RIGHT KIDNEY";KI$
135 PRINT:PRINT
140 INPUT"WET WEIGHT";WT
145 PRINT:PRINT :
150 INPUT"DRY WEIGHT";DR
200 PRINT"■":DIMEX$(79),TR$(79)
201 IFSS=1THENRETURN
210 PRINT"DESCRIBE EXPERIMENT (2 LINES)":PRINT
220 INPUTEX$:PRINT:PRINT
230 PRINT"DESCRIBE TREATMENT (2 LINES)":PRINT
240 INPUTTR$
300 PRINT"■"
310 INPUT"NUMBER OF SAMPLES";NU
315 PRINT:PRINT
320 INPUT"CLEARANCE PERIOD";MI
400 PRINT"■FLOW":FORJ=1TONU+1:PRINT:PRINTJ;:INPUTFL(J):NEXT
420 PRINT"■PRESSURE":FORJ=1TONU+1:PRINT:PRINTJ;:INPUTPR(J):NEXT
450 P$="PERFUSATE":O$="URINE"
451 P$(1)="INULIN":P$(2)="SODIUM":P$(3)="POTASSIUM":P$(4)="GLUCOSE":S$="
452 P$(5)="ALBUMIN":P$(6)="PHENOL RED":P$(7)="VOLUME"
500 N1=6:Z$=P$:N2=NU:Z=1
510 FORK=1TON1:PRINT"■"Z$;S$;P$(K)
530     FORJ=1TON2:PRINTJ;
540 ONZGOSUB601,602,603,604,605,606,607,608,609,610,611,612,613
550     NEXTJ
```

```
555 Z=Z+1
556 GOSUB35000
559 NEXTK
560 IFN1=7THEN700
565 N1=7:K=1:Z$=0$
570 GOTO510
601 INPUTP1,F2:PI(J)=(P1+P2)/2:RETURN
602 INPUTPN(J):RETURN
603 INPUTPK(J):RETURN
604 INPUTPG(J):RETURN
605 INPUTPA(J):RETURN
606 INPUTPP(J):RETURN
607 INPUTP1,F2:UI(J)=(P1+P2)/2:RETURN
608 INPUTUN(J):RETURN
609 INPUTUK(J):RETURN
610 INPUTUG(J):RETURN
611 INPUTUA(J):RETURN
612 INPUTUP(J):RETURN
613 INPUTUV(J):RETURN
700 REM U:P INULIN RATIO
710 FORJ=1TONU:R(J)=UI(J)/PI(J):NEXT
720 FORJ=1TONU:GFR(J)=R(J)*UV(J)/MI:NEXT
740 REM % SODIUM REABSORPTION
750 FORJ=1TONU
755 RN(J)=(1-((UN(J)*(UV(J)/MI))/(GFR(J)*PN(J))))*100:NEXT
770 REM MICROEQUIV OF SODIUM PER MIN
775 FORJ=1TONU
780 MN(J)=((GFR(J)*PN(J))-((UV(J)/MI)*UN(J))):NEXT
800 REM % GLUCOSE REABSORPTION
810 FORJ=1TONU
820 GLU(J)=(1-(UG(J)*(UV(J)/MI))/GFR(J)*PG(J))*100:NEXT
840 REM U:P PHENOL RED RATIO
850 FORJ=1TONU:PH(J)=UP(J)/PP(J):NEXT
860 REM CLEARANCE OF PHENOL RED
870 FORJ=1TONU:CPR(J)=PH(J)*UV(J)/MI:NEXT
890 FORJ=1TONU:RG(J)=CPR(J)/GFR(J):NEXT
920 FORJ=1TONU:AR(J)=UA(J)/PA(J):NEXT
930 REM CLEARANCE OF ALBUMIN
940 FORJ=1TONU:CA(J)=AR(J)*UV(J)/MI:NEXT
960 FORJ=1TONU:CG(J)=CA(J)/GFR(J):NEXT
1000 REM RESISTANCE
1010 FORJ=1TONU+1:RES(J)=PR(J)/(FL(J)/WT):NEXT
1050 REM DRY WT TO WET WT RATIO
1051 WR=DR/WT
1400 PRINT:INPUT"IS DATA TO BE FILED (Y/N)";A$
1410 IFA$="Y"THEN25000
```

```
1500 PRINT"␣ CHOOSE # TO RECALL CALCULATIONS"
1510 PRINT:PRINT
1515 PRINT" 1. U:P INULIN RATIO"
1516 PRINT" 2. GFR"
1517 PRINT" 3. % REABS. SODIUM"
1518 PRINT" 4. REABS. SODIUM MICPOEQ/MIN"
1519 PRINT" 5. % REABS. GLUCOSE"
1520 PRINT" 6. CLEARANCE OF PHENOL RED"
1521 PRINT" 7. U:P PHENOL RED"
1522 PRINT" 8. CLEAR PHE RED/GFR"
1523 PRINT" 9. U:P. ALBUMIN"
1524 PRINT"10. C ALB/GFR"
1525 PRINT"11. URINE VOLUME"
1526 PRINT"12. DRY WT./WETWT."
1527 PRINT"13. FLOW"
1528 PRINT"14. RESISTANCE"
1530 PRINT:PRINT:INPUT"NUMBER";L
1550 PRINT"␣TIME",,"DPY","WET"
1551 PRINT
1570 Z=0
1571 IFL=12THENGOSUB2012:GOSUB10000:GOTO1500
1575 IFL=13THEN1650
1576 IFL=14THEN1650
1600 FORJ=1TONJ
1610 ONLGOSUB2001,2002,2003,2004,2005,2006,2007,2008,2009,2010,2011
1615 Z=Z+MI:PRINT
1616 NEXT
1617 PRINT:PPINT:GOSUB10000:GOTO1500
1650 FORJ=1TONJ+1
1655 IFL=13THENGOSUB2013
1660 IFL=14THENGOSUB2014
1661 Z=Z+MI:PRINT
1665 NEXT
1670 PRINT:PRINT:GOSUB10000:GOTO1500
2001 PRINTZ,:A=R(J):GOSUB20:RETURN
2002 PPINTZ,:A=GFR(J):GOSUB20:A=GFR(J)/DR:GOSUB20:A=GFR(J)/WT:GOSUB20:RETURN
2003 PRINTZ,:A=RN(J):GOSUB20:RETURN
2004 PRINTZ,:A=MN(J):GOSUB20:A=MN(J)/DR:GOSUB20:A=MN(J)/WT:GOSUB20:RETURN
2005 PRINTZ,:A=GLU(J):GOSUB20:RETURN
2006 PRINTZ,:A=CPR(J):GOSUB20:A=CPR(J)/DR:GOSUB20:A=CPR(J)/WT:GOSUB20:RETURN
2007 PRINTZ,:A=PH(J):GOSUB20:RETURN
2008 PRINTZ,:A=RG(J):GOSUB20:RETURN
2009 PRINTZ,:A=AR(J):GOSUB20:RETURN
2010 PRINTZ,:A=CG(J):GOSUB20:RETURN
2011 PRINTZ,:A=UV(J)/MI:GOSUB20:PRINT,:A=UV(J)/MI/WT:GOSUB20:RETURN
2012 PRINTWR:RETURN
```

```
2013 PRINTZ,:A=FL(J):GOSUB20:A=FL(J)/UR:GOSUB20:A=FL(J)/WT:GOSUB20:RETURN
2014 PRINTZ,:A=RES(J):GOSUB20:RETURN
10000 PRINT:PRINT"TO CONTINUE PRESS ANY LETTER"
10010 GETA$:IFA$=""THEN10010
10020 RETURN
20000 PRINT"IS CASSETTE READY FOR READING?"
20010 PRINT:PRINT"ENTER FILE # & NAME";
20020 INPUTZ,Z$
20030 GOSUB200
20035 OPENZ,1,0,Z$
20040 INPUT#Z,RA$,NA$,EX$,TR$,KI$,WT,DP,NU,MI,UR
21000 FORJ=1TONU
21010 INPUT#Z,PI(J):INPUT#Z,PN(J):INPUT#Z,PK(J):INPUT#Z,PG(J):INPUT#Z,PP(J)
21020 INPUT#Z,PA(J):INPUT#Z,UI(J):INPUT#Z,UN(J):INPUT#Z,UK(J):INPUT#Z,UG(J)
21030 INPUT#Z,UP(J):INPUT#Z,UV(J):INPUT#Z,UA(J)
21040 INPUT#Z,R(J):INPUT#Z,GFR(J):INPUT#Z,RN(J):INPUT#Z,AN(J):INPUT#Z,GLU(J)
21050 INPUT#Z,PH(J):INPUT#Z,CPR(J):INPUT#Z,RG(J):INPUT#Z,AA(J):INPUT#Z,CA(J)
21060 INPUT#Z,CG(J)
21070 NEXTJ
22000 FORJ=1TONU+1
22010 INPUT#Z,RES(J):INPUT#Z,FL(J):INPUT#Z,PR(J)
2202 NEXTJ
2203 CLOSEZ
23000 GOSUB30000:GOTO1500
25000 PRINT"  IS CASSETTE READY FOR FILING"
25010 PRINT:PRINT"ENTER FILE # & NAME";
25030 INPUTZ,Z$
25031 POKE243,122:POKE244,2
25035 OPENZ,1,1,Z$
25040 PRINT#Z,RA$:PRINT#Z,NA$:PRINT#Z,EX$:PRINT#Z,TR$:PRINT#Z,KI$:PRINT#Z,WT
25045 PRINT#Z,DR:PRINT#Z,NU:PRINT#Z,MI:PRINT#Z,UR
26000 FORJ=1TONU
26010 PRINT#Z,PI(J):PRINT#Z,PN(J):PRINT#Z,PK(J):PRINT#Z,PG(J):PRINT#Z,PP(J)
26020 PRINT#Z,PA(J):PRINT#Z,UI(J):PRINT#Z,UN(J):PRINT#Z,UK(J):PRINT#Z,UG(J)
26030 PRINT#Z,UP(J):PRINT#Z,UV(J):PRINT#Z,UA(J)
26040 PRINT#Z,R(J):PRINT#Z,GFR(J):PRINT#Z,RN(J):PRINT#Z,AN(J):PRINT#Z,GLU(J)
26050 PRINT#Z,PH(J):PRINT#Z,CPR(J):PRINT#Z,RG(J):PRINT#Z,AA(J):PRINT#Z,CA(J)
26060 PRINT#Z,CG(J)
26070 NEXTJ
27000 FORJ=1TONU+1
27010 PRINT#Z,RES(J):PRINT#Z,FL(J):PRINT#Z,PR(J)
27020 NEXTJ
27030 CLOSEZ
27031 GOSUB30000:GOTO1500
30000 REM**ALARM**
```

```
30005 POKE59467,PEEK(59467)AND227OR16
30110 POKE59466,15
30140 FORI=1TO8
30150 READC(I)
30160 DATA235,218,187,176,156,140,124,116
30170 FORJ=1TO5
30180 POKE59464,C(I)
30190 NEXTJ,I
30200 RESTORE
30210 POKE59467,0:POKE59464,255:POKE59466,0
30220 FORJ=1TO100:NEXT
30240 RETURN
35000 PRINT:PRINT"IS DATA CORRECT (Y/N) ?";
35010 GETA$:IFA$=""THEN35010
35012 IFA$="Y"THEN RETURN
35015 IFA$<>"N"THEN35010
35020 K=K-1:J=J-1:Z=Z-1
35025 RETURN
```