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Analysis of the Proteoglycan Content in Fresh and Cryopreserved Porcine Cardiovascular Tissues

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ANALYSIS OF THE PROTEOGLYCAN CONTENT IN FRESH AND CRYOPRESERVED PORCINE CARDIOVASCULAR TISSUES

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ABSTRACT

ANALYSIS OF THE PROTEOGLYCAN CONTENT IN FRESH AND CRYOPRESERVED PORCINE CARDIOVASCULAR TISSUES

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The purpose of this research was to study the effects of cryopreservation on the proteoglycan (PG) and mineral content of aorta conduit tissue. Proteoglycans from fresh and cryopreserved porcine aorta conduit tissues were isolated by extraction with 4 M guanidine (Gdn) - hydrochloride (HCl) for 48 hours. This concentration of Gdn-HCl and 48 hours extraction time was demonstrated to be optimal for proteoglycan extraction. The crude proteoglycan extracts were purified by CsCl isopycnic centrifugation. Quantitative analysis of extracted proteoglycans revealed that the content of proteoglycan material from cryopreserved tissue, measured as uronate and

protein per unit weight of wet tissue, was similar to that from fresh tissue (440 ± 30 versus 430 ± 7 μg uronate/g wet tissue and 3139 ± 39 versus 2639 ± 15 μg protein/g wet tissue). Gel permeation column chromatography studies suggested that proteoglycans present in three CsCl fractions from cryopreserved tissues had molecular weights similar to proteoglycans present in similar fractions from fresh tissue; K_{av} = 0.13 and 0.47 (I), 0.20 (II), and 0.43 (III) from cryopreserved tissue and K_{av} = 0.13 and 0.50 (I), 0.23 (II), and 0.40 (III) from fresh tissue.

Cryopreserved and fresh tissues were extracted using Gdn-HCl followed by sequential digestion of the tissues with collagenase, elastase, and papain. Glycosaminoglycans (GAGs) of the PGs were isolated and quantitated. Gdn-HCl extracted about 61 % and 62 % of the total GAG (proteoglycan) material from cryopreserved and fresh tissues, respectively. Collagenase solubilized proteoglycans from Gdn-HCl-extracted tissue represented 20 % and 13 %, respectively, of the total GAGs present in cryopreserved and fresh tissue. Subsequent elastase hydrolysis of collagenase digested tissue released about 11 % of total GAGs from cryopreserved tissue and 16 % from fresh tissue. The remainding 8 %, from cryopreserved tissues, and 9 %, from fresh tissue, of total GAGs were obtained through use of a final papain hydrolysis. There was essentially no difference between fresh and cryopreserved tissues in the relative distribution of proteoglycans in the extracts and digestions except in the initial

digestion step where more proteoglycan was obtained from collagenase solubilization of cryopreserved tissue than fresh tissue ($P < 0.05$).

The histologic status of the fresh and cryopreserved porcine aortic conduit did not differ markedly. The normal tissue architecture was not affected markedly by the cryopreservation procedure as neither alteration of elastic structure, fibrous proteins nor alteration of nuclear distribution or smooth muscle cell morphology was detected. Electron microscopic comparisons demonstrated a retention of proteoglycans in the porcine aortic conduit after cryopreservation, and the relative morphological distribution of proteoglycan content in cryopreserved tissue was similar to that in fresh tissue.

Quantitative tissue mineral studies revealed that the mean calcium content of the cryopreserved aorta conduit tissue (165 ± 3 μg/g wet tissue) was higher than that of the fresh tissue (105 ± 4 μg/g wet tissue) ($P < 0.05$). The mean phosphorus content was 703 ± 35 μg/g wet tissue from cryopreserved tissue and 720 ± 26 μg/g wet tissue from fresh tissue.

The studies indicate that there is no significant alteration in the content, molecular size, or distribution of PGs in properly cryopreserved tissue. However, the total calcium level appears to be increased in tissue cryopreserved by the cryopreservation process used in this study.

DEDICATION

To My Son

David Sang-Gil Lee

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CHAPTER I

INTRODUCTION

A. Cardiovascular System.

The cardiovascular system consists of the heart, which is a muscular pumping device and keeps the blood in motion; a closed system of blood vessels, which circulate blood to and from all parts of the body; and the lymphatic vessels, an ancillary set of vessels, which circulate lymph. The heart is a four-chambered (two atria and two ventricles) muscular organ through which the blood contained in the circulatory system is pumped. The direction of flow of the blood is largely determined by the presence of valves which allows blood to flow from the atria into the ventricles but prevents it from flowing back up into the atria from the ventricles The valves of the heart are fibrous flaps of tissue covered with endothelium. Four sets of valves are of importance to the normal functioning of the heart. Two of these, the atrioventricular valves are located in the heart, guarding the openings between the atria and ventricles. The other two, the semilunar valves are locked inside

the pulmonary artery and the great aorta just as they arise from the right and left ventricles, respectively.

The valve guarding the right atrioventricular orifice consists of three flaps of endocardium and is called the tricuspid valve. The valve that guards the left atrioventricular orifice is similar in structure to the tricuspid except that it has only two flaps and is, therefore, named the bicuspid or, more commonly, the mitral valve. The semilunar valves consist of half-moon-shaped flaps growing out from the lining of the pulmonary artery (pulmonary valve) and great aorta (aortic valve). Whereas the atrioventricular valves prevent blood from flowing back up into the atria from the ventricles; the semilunar valves prevent it from flowing back down into the ventricles from the aorta and pulmonary artery.

The aorta is an elastic artery leaving the heart to supply the systemic circulation. The elastic arteries functionally serve as conduction tubes, but they also facilitate the movement of blood along the tube. During the contraction (systolic) period of the cardiac cycle, the ventricles of the heart pump blood into the elastic arteries. The pressure generated in the elastic arteries by the ventricles moves the blood along the arterial tree, and the pressure also causes the wall of the arteries to distend. The distension is limited by the network of

collagenous fibers in the tunica media and tunica adventitia. During the relaxation (diastolic) period of the cardiac cycle, arterial pressure and the resultant flow of blood within the vessel are maintained by the elastic recoil of the distended arterial wall.

Aorta have three coats, an inner smooth layer (tunica intima), a middle layer (tunica media), and an outer layer of connective tissue (tunica adventitia). The tunica intima of aorta is relatively thick and consists of an endothelial lining with its basal lamina (a subendothelial layer of connective tissue) and a layer of elastic material (the internal elastic membrane). The endothelium is a simple squamous epithelium. The cells are typically flat, but they are elongated and oriented with their log axis parallel to the direction of the artery. The endothelium and the basal lamina serve as a barrier to the passage of substances. The subendothelial layer consists of connective tissue with both collagenous and elastic fibers. The main cell type of this layer is the smooth muscle cell. It is not only contractile, but it also produces the extracellular ground substance and fibers. The tunica media is the thickest of the three layers. It consists of sheets of elastic material with intervening layers comprised of smooth muscle cells, collagenous fibers, and ground substance. The smooth muscle cells in tunica media produce the collagenous, elastic components, and ground substance of this layer. The tunica adventitia is

relatively thin, and a connective tissue layer. The main extracellular component of this layer are collagenous fibers which is relatively inextensible and prevent the expansion of the arterial wall beyond physiological limits during the systolic period of the cardiac cycle. There is also a loose network of elastic fibers in this layer. Cells of the tunica adventitia are fibroblasts.

B. Historical Perspective of Heart Valve Replacements.

Diseased and malfunctioning heart valves have been replaced as a routine procedure for almost three decades. There have been many improvements in heart valve substitutes and surgical techniques during this time.

In the normal heart, there is a virtually nonobstructed flow of blood from the left ventricle to the ascending aorta through the fully open aortic valve during systole. There is a similar nonobstructed flow from the atrium through the mitral valve to the ventricle by retraction of the mitral valve during diastole. The goal of cardiac valve transplantation research has been the search for perfect valve substitutes, namely, mechanical, tissue (bioprosthetic), or allograft valves. There are several major criteria to be considered in a particular valve design. An ideal valve substitute should function efficiently with perfect hemodynamic performance and present a minimum load to the

heart. The substitute should also have superior durability and maintain its efficiency for the life span of the patient. It should not cause damage to molecular or cellular blood components or stimulate thrombus formation so that anticoagulation therapy will be required. The valve substitute should be available in a wide variety of sizes permitting a match for recipients and should require a relatively simple surgical implant.

In the mid 1940's, Hufnagel used an acrylic prosthesis for the replacement of the thoracic aorta of animals, and this work provided the impetus for him to develop and implant prosthetic heart valves in humans (Hufnagel, 1947; Hufnagel and Harvey, 1953). In the early 1950's, Hufnagel et al. (1954) and Campbell (1950) independently designed mechanical valves. This totally mechanical valvular prosthesis, consisting of a lucite tube and a mobile spherical poppet, was successfully implanted into the descending aorta of a patient with severe aortic regurgitation (Hufnagel et al., 1954). One of Hufnagel's patients, who received a prosthetic heart valve, survived over two decades without evidence of wear, thrombosis, or embolism (Hufnagel and Gomes, 1976).

In 1958, the caged-ball valve was implanted in the mitral position in dogs by Ellis and Bulbulian (1958) and in the aortic position by Edwards and

Smith (1958). These accomplishments, together with the development of the technique of extracorporeal circulation, prompted Harken in March 1960 to correct for defective valves with a caged-ball prosthesis in the subcoronary position in patients with aortic regurgitation (Harken et al., 1960). Five months later, Starr implanted the first Starr-Edwards prosthesis in the mitral position (Starr and Edwards, 1961), and the first clinical Starr-Edwards aortic valve implantation was performed one year later (Lefrak and Starr, 1979).

After early design improvement, silastic ball noncloth-covered Starr-Edwards mechanical valves became the standard for mechanical valves. Although a number of similar designs of the caged-ball valves appeared in clinical use (Lefrak and Starr, 1979), all but Starr-Edwards, Smeloff-Sutter, and Magovern-Cromie valves are no longer available for clinical use due to high complication rates and problems with durability and structural design.

The concept of the tilting disk valve arose because most of the caged-ball valves were unnecessarily bulky, and their hemodynamic characteristics were less than ideal. A flat disk instead of a ball was used as an occluder in caged-disk valves to reduce the profile of such valves, but they still suffered from the hemodynamic problem of having an occluder that remained relatively obstructive in the open position. A disk that tilted within the valve ring was

specially attractive since it caused minimal obstruction to blood flow in the open position. Flap valves were the earliest examples of the tilting disk (Kernan et al., 1957; Pierce et al., 1968). Bjork and Shiley in 1969 collaborated to produce a hingeless tilting disk valve, the Bjork-Shiley valve, in which the free-floating disk was restrained by two low-profile M-shaped struts (Bjork, 1969; Bjork, 1970). This valve is now the most commonly used mechanical prosthesis worldwide.

In the mid 1960's, the design of a rigid bileaflet prosthesis was introduced and tested in vitro, in animals, and in clinical usage. Certain elements in this design, the bileaflet principle and a low profile, were used earlier in the Gott-Daggett, Kalke-Lillehei, and Wada prostheses. Clinical experiences with the hinge bileaflet prosthesis were first reported with the introduction of the Gott-Daggett valve (Gott et al., 1964). This valve had two semicircular leaflets retained within the ring by four hinges. Follow-up experiences revealed that an incidence of high thrombogenicity was probably the consequent lack of proper washout on the outflow surface (Young et al., 1969). The Gott-Daggett prosthesis was withdrawn from clinical use because the thrombogenicity of this valve could not be reduced even with treatment of a colloid graphite coating on the synthetic surfaces (Gott et al., 1961). The Kalke-Lillehei and Wada designs have not been introduced into clinical usage.

Although these earlier prostheses failed due to mechanical malfunctions and marked thrombogenicity, the most striking finding from these earlier studies was that their hemodynamics were superior to all other prostheses available at that time.

In 1976, this design was modified and refined for heart valves manufactured entirely from pyrolitic carbon (Emery et al., 1978). Pyrolitic carbon, with its thrombo-resistant properties and great durability, replaced the titanium of the valve ring and the different materials used earlier for the cusps. Experimental and later clinical results demonstrated that the bileaflet design using pyrolitic carbon provided distinct and dramatic improvements in prosthesis performance (Lillehei, 1986). The oldest and most broadly used member of the bileaflet family of valves is the St. Jude Medical valve, a cleverly designed valve made entirely of pyrolitic carbon with two semi-circular discs that open with a new pivot mechanism that eliminates the need for any supporting struts. Postoperative catheterization studies in multicenters revealed good results in terms of survival, low incidence of thromboembolic complications, and negligible transvalvular gradients in the large sizes (Lillehei, 1986; Pass et al., 1984; Wortham et al., 1981).

Although many modern mechanical heart valve substitutes are accepted

as having long-term durability (Silver and Butany, 1988), the question of fatigue or wearing out remains a pertinent issue. The most important disadvantage of a mechanical valve is thrombogenicity, which has been reduced recently by improved microhemodynamics within the hinge mechanism, but is still significantly higher than that of xenograft valves (Magilligan, 1987; Schoen and Hobson, 1987). Therefore, anticoagulant therapy is required for most mechanical prostheses. The valve murmur is an additional disadvantage of some mechanical valves. Hemodynamic dysfunction also can occur when a small mechanical prosthesis is inserted into a small aortic or mitral annulus. The resulting high gradients worsen during exercise and provide for elevated perioperative mortality rates (Bjork et al., 1974; Dale et al., 1980).

Dissatisfaction with the incidence of valve related morbidity and mortality after cardiac valve replacement with a mechanical prostheses led to introduction of xenograft tissue valves. Work on the xenograft valve started in 1965 (Duran and Gunning, 1965). The valves of the pig and calf seemed to be the best choices because of relative vascularity and similarity of physical properties to those of human valves, and it was found that both were technically satisfactory for clinical usage (O'Brien, 1967). Originally, mercurial salts were used for the treatment of xenograft valves and later formalin for the sterilization and reduction of antigenicity. However, both reagents were found to be

unsuitable for treatment of biological valves because they weakened the chemical cross-linkage bonds in collagen and resulted in collagen degeneration (Binet et al., 1965; O'Brien and Clarebrough, 1960).

Glutaraldehyde, used for tanning leather in the shoe industry, produces stable collagen covalent cross-linkage bonds and actually increases tissue strength. The tissue also loses viability, and its antigenicity is markedly reduced. Carpentier et al. (1969), in Paris, first used glutaraldehyde for tissue valve preservation in 1968 (Gerbode, 1970) and facilitated the use of bioprostheses, both porcine and bovine pericardium, as a satisfactory alternative to mechanical valves.

In 1970, commercially available glutaraldehyde-treated porcine xenograft valves, that were readily available in all sizes, were introduced by Hancock laboratories. Carpentier-Edwards xenograft valves were subsequently offered by Edwards laboratories. The Hancock standard and the Carpentier-Edwards standard are high-pressure glutaraldehyde-fixed bioprostheses. These bioprostheses are presently used only in the United States. The Hancock standard valve is mounted into a Dacron-covered polypropylene stent. There are various sewing ring configurations to facilitate interrupted or continuous suturing techniques. The Carpentier-Edwards bioprosthetic porcine valve uses a

flexible Elgiloy (an alloy of spring steel) stent, which is radiopaque. Tubular knitted porous Teflon, which surrounds a silicone rubber insert, is used as the sewing ring material. A specially buffered and stabilized glutaraldehyde solution is used to fix the valves.

Hancock laboratories introduced a Hancock modified orifice porcine valve since hemodynamic performance of the original Hancock prosthesis was not ideal (Lurie et al., 1976; Stinson et al., 1977). In this modified prosthesis, the right coronary cusp with its muscular shelf has been replaced by a matching noncoronary cusp that does not contain the muscle shelf from another porcine valve. In vitro studies showed that this modified prostheses had better hydraulic function, and intraoperative studies revealed much lower transvalvular gradients (Levine et al., 1977). However, late postoperative catheterization studies have revealed only slightly better hydraulic performance than the standard valve (Rossiter et al., 1980). Edwards laboratories introduced a modified xenograft prosthesis, Carpentier-Edwards supraannular bioprosthesis, with improved hydraulic function by the further thinning of both the cloth and metal components of the frame.

Porcine bioprostheses have a low rate of serious thromboembolism, essential lack of thrombosis, and freedom from anticoagulant-related

hemorrhage (Carpentier et al., 1969; Hannah and Reis, 1976; Lakier et al., 1980; Pipkin et al., 1976), but hydraulic performance is still limited in the smaller sizes (Yoganathan et al., 1986). The main disadvantage of the porcine xenograft is limited durability due to calcification (Gallo et al., 1984). Calcification of biological heart valves results in degeneration and dysfunction.

The Ionescu-Shiley pericardial valve was the first and most widely used model of the bovine pericardial heart valve. In 1966, M. Ionescu and G. Wooler of England introduced a formalin-fixed heterograft supported by a titanium stent. Two years later, Ionescu tried a series of autologous fascia lata mounted on that three-legged stent covered inside and out with Dacron velour, but the results with fascia were unsatisfactory. In 1970, he mounted glutaraldehyde-fixed bovine pericardium on a cloth-covered titanium frame with its posts slightly splayed outward to provide a dull orifice. The first valves were made in small numbers in Ionescu's hospital laboratory, but since 1976 they have been made at Shiley laboratories and commercialized under the trade name Ionescu-Shiley pericardial valve (Ionescu et al., 1977; Ionescu et al., 1982; Ionescu et al., 1985). New pericardial-based bioprostheses, the Mitroflow and Carpentier-Edwards pericardial valve, are being developed and used clinically today (Anderson et al., 1986; Pelletier et al., 1990; Relland et al., 1985).

The pericardium valve's fabrication concept offers important design and manufacturing advantages, since the end product is free of the natural variation which is always a problem in an intact valve removed from an animal. Pericardium has made possible the development of trileaflet stent-mounted bioprostheses with very low transannular gradient (Revuelta et al., 1984). This valve is better than the porcine valve in terms of hydraulic performance due to the absence of a muscle shelf at the base of the valve (Cosgrove et al., 1985). Wheatley's group in Glasgow (Fisher et al., 1986) tested six different types of tissue valves and six mechanical valves in vitro and reported that the porcine valves had higher forward flow pressure gradients than pericardial, tilting disk, or bileaflet mechanical valves. Thromboembolism is not a major problem with the bovine pericardium bioprosthesis (Cooley et al., 1986; Ionescu et al., 1986), and this valve may have lower thromboembolic problems than porcine valves. However, the glutaraldehyde-treated pericardium has a clear tendency toward calcification (Gallo et al., 1985). Experimental clinical studies of the incidence of primary tissue failure between porcine and bovine pericardium valves show that the bovine pericardial valve deteriorates sooner and more frequently than the porcine valve (Gallo et al., 1987; Odell et al., 1986). Generally, tissue failures of pericardial valves occur sooner in the mitral than in the aortic position, and different models of valves fail differently at different times (Gabbay et al., 1984; Gabbay et al., 1988; Walley and Keon, 1987;

Wheatley et al., 1987).

In spite of substantial progress made in the preparation of prostheses (mechanical and bioprosthetic valves), an adequate valve substitute meeting the requirements of an ideal cardiac valve has yet to be identified. The various types of prosthetic valves introduced are only partially of the quality of natural, healthy, human heart valves. In a search for the perfect valve replacement, researchers turned to the human heart valve as the best valve substitute. Numerous clinical problems with both mechanical and xenograft valves are solved by use of a allograft (homograft) valve: low incidence of thromboembolic complications, freedom from long-term anticoagulation therapy, reduced hemodynamic obstruction especially in the small aortic root size of children, and resistance to endocarditis.

Implantation of allograft cardiac valves was preceded by documented success in the preservation and reimplantation of homologous vascular material experimentally and clinically by Robert Gross in 1948 (Gross et al., 1948). In 1952, Lam and his coworkers reported that it was experimentally possible to transplant canine aortic valve homografts into another dog's descending thoracic aorta. In 1953, Robicsek in Hungary completed the first true orthotopic transplant of a valvular homograft, a canine tricuspid valve. Gross's clinical

experiences with preserved arterial grafts and Lamb's experimental success in implantation of a homologous cardiac valve became the basis for Murray and other investigators to develop the techniques for clinical operations. In 1956, Murray and his coworkers in Toronto implanted fresh and fully living aortic valve homografts into the descending thoracic aorta for the relief of aortic insufficiency. Although this operation was only partially successful hemodynamically, the homograft valves had remarkable durability and performance. Kerwin et al. (1962) reported that these valves had satisfactory functions extending to up to 6 years.

Implantation of fresh homograft aortic valves into the subcoronary position was proposed by Duran and Gunning (1962) and was completed with full clinical success in England by Donald Ross in 1962. Sir Brian Barratt-Boyes in New Zealand reported in 1964 that he, too, had initiated subcoronary insertions of aortic valve homografts in 1962, almost at the same time as Donald Ross and independent of each other (Barratt-Boyes, 1964). These initial valves had excellent durability both initially and long-term and were virtually free of thromboembolic complications without anticoagulant therapy. These results gave impetus for early workers to pursue this method of aortic valve replacement.

The first clinical use of aortic valve allografts was reported (Davies et al., 1968), but the use of these valves was soon extended for the repair of ventricular outflow tract defects (Ross and Somerville, 1966), mitral and tricuspid valve diseases (Ross and Somerville, 1972), and finally for the repair of the entire aortic root and valve (Lao et al., 1984). Although the use of mitral and tricuspid allografts was proposed early, the results of mitral valve replacements with allografts were not favorable (Graham et al., 1971; Robicsek, 1953). Eguchi and Asano in 1968 proposed the use of pulmonary allografts for the right side of the heart, and Kay et al. in 1986 presented the results with the first clinical series.

Since the inception of the allograft aortic valve for clinical subcoronary implantation in 1962, changes in methods of sterilization and storage techniques have been implemented. Allograft valves that were sterilized by gamma radiation or chemicals such as β-propiolactone, ethylene oxide, or chlorhexidine had a great propensity for cusp rupture and degeneration (Barratt-Boyes et al., 1977; Heimbecker et al., 1968; Malm et al., 1967; Smith, 1967). Storage techniques such as freeze-drying have resulted in poor performance with diminished durability of transplanted allograft valves (Beech et al., 1973; Parker et al., 1977). The use of harsh methods for valve preparation has been completely abandoned because of increased failure rates.

In the late 1960s, the use of gentle antibiotic sterilization and storage at 4 °C in nutrient media began to be used. Although a fresh wet-stored allograft lacks donor cellular viability (O'Brien et al., 1987), valves prepared with these gentler techniques have been found clinically to be satisfactory and to function for a reasonable period of time due to their excellent hemodynamic performance even in small sizes (Ross and Yacoub, 1969; Thompson et al., 1979; Thompson et al., 1980), reduction of thromboembolism and hemolysis rates without anticoagulant therapy (Matsuki et al., 1988), and enhanced resistance to endocarditis (Kirklin and Barratt-Boyes, 1986). A number of groups demonstrated excellent medium-term (7-10 years) results with the wet-storage technique (Anderson and Hancock, 1976; Barratt-Boyes, 1979; Khanna et al., 1981; Miller and Shumway, 1987; Penta et al., 1984; Teply et al., 1981; Thompson et al., 1980).

Although clinical results with fresh wet-stored antibiotic-sterilized human allografts were good, the difficulties involved in obtaining the valve, the availability of valve size matching for recipients, and lack of certainty concerning preservation and storage techniques prevented the wide-spread use of this graft for valve substitution. Based on these problems, cryopreservation was introduced as the best method for indefinite and convenient storage and frozen valve banks have been developed which allow prolonged storage of

tissues and thus increase availability.

Luyet et al. (1940) in America established the foundations of modern cryobiology. In 1949, Polge et al. from London actualized the immense advantage of cryogenic temperatures in organ banking. They reported that glycerol enabled rooster sperm to survive freezing at -79 °C. This technique subsequently was applied for the preservation of various cell types and even entire embryos (Bunge and Sherman, 1953; Lovelock, 1953; Schaeffer et al., 1972; Smith and Polge, 1950). Smith also performed studies on numerous frozen mammalian cellular systems, including ova, blood, corneas, hearts, and even whole animals (Smith, 1961). The use of glycerol and other cryoprotectants has enabled considerable advances in the long-term preservation of mammalian cells in suspensions and organs.

The technology of cryopreservation has been applied to preserve aortic allografts. Gross, Bill, and Peirce (1949) demonstrated that simple freezing of aortic homografts to -72 °C without a cryoprotectant resulted in a high risk of graft rupture. In the early 1970s, Angell and associates (1987) reported on the maintenance of viability in DMSO cryopreserved aortic allografts stored for prolonged periods in liquid nitrogen and their clinical application. The Brisbane group, under the direction of O'Brien, followed this early clinical

application of the cryopreservation process with a series of valve replacements utilizing allograft valves that had undergone gentle antibiotic sterilization, controlled freezing with dimethylsulfoxide (DMSO) to low temperature, and then storage in liquid nitrogen at -190 °C. His group confirmed the cellular viability of tissue by histologic and biochemical data (O'Brien et al., 1987; O'Brien, Kirklin et al., 1987). In their study, the cryopreserved aortic valves had remarkable results with 100 % freedom from reoperation because of valve degeneration for up to 10 years. The high durability of the viable cryopreserved valve was considered to be related to continuous cellular viability. Explanted valve tissue, up to 10 years after operation, have shown continuous viability of leaflets from the time of implantation (O'Brien, Kirklin., 1987).

Several studies have shown that cryopreserved allograft aortic valves have increased functional integrity in comparison to fresh aortic allografts and xenografts, which is an extremely attractive feature with regard to the pediatric population (Angell et al., 1987; Jonas et al., 1988; O'Brien, Kirklin et al., 1987). The frozen viable allograft has superior durability in addition to the advantages of the nonviable fresh allograft. This superior durability may be due to improved matrix preservation by the limitation or reduction of cell death prior to and during cryopreservation rather than continued fibroblast cellular

function following transplantation. In current world experience, most aortic and pulmonary homografts are used as valved conduits for insertion in the right side of the heart (Bodnar and Ross, 1991).

C. Cryobiology of Heart Valves.

Cryopreservation techniques for heart valves are derived empirically from knowledge developed for cryopreservation of single-cell suspensions and simple tissues (van der Kamp et al., 1981), and finally applied to cardiovascular tissues. Since the initial development of the cryopreservation technology for heart valves (Angell et al., 1976), there have been considerable advances in the characterization of many cryoprotective agents, the development of computer-controlled freezing equipment, and active cryobiology research in universities and organ/tissue processing groups has validated specific aspects of the techniques involved in cryopreservation.

Properly cryopreserved tissues have yielded excellent clinical results for extended periods of time. It now becomes necessary to determine the reason(s) for this good performance. Human heart valve cryopreservation can be divided into five steps: (1) harvesting and transport of the donor heart to the processing facility; (2) tissue preparation and antibiotic disinfection; (3) control-rate

freezing with cryoprotectants; (4) storage in vapor-phase liquid nitrogen; and (5) thawing/diluting for transplantation. It is generally accepted that cryopreservation protocols must ensure integrity of the extracellular matrix and maintenance of cellular viability of the tissues for prolonged clinical durability after implantation. A number of studies (Angell et al., 1976; Hu et al., 1989; Khanna et al., 1981; O'Brien, 1986; Ross et al., 1979; Strickett et al., 1983; van der Kamp et al., 1981) investigating valve preparation procedures reported several general principles which provide for better integrity of tissues and cellular viability: short warm ischemia times (the time period from cessation of donor heart beat to initial cooling of valve tissue with cold storage solution) following death of donor, antibiotic concentrations which are nontoxic to valve cells yet effectively disinfect the allograft (Hu et al., 1989; Khanna et al., 1981), cryopreservation by cooling at 1 °C/min with 10 % dimethyl sulfoxide as a cryoprotectant, and storage and transport of processed valves in the vapor phase temperature of liquid nitrogen (Adam et al., 1990).

Even though fibroblast cells in heart valve cusps are relatively resistant to anoxia, most procurement programs seek to remove the heart within the first hour after cessation of heartbeat. The procurement of donor hearts for allograft heart valve transplantation should include aseptic technique, retention of proper length of aorta and pulmonary conduits, and the avoidance of valve cusp injury.

Cold ischemic time (the time interval after transfer of the heart to cold transport solution until cryopreservation) during transportation and processing of hearts should be less than 24 hours after cardiectomy. St. Louis, et al (1991) reported that protocols designed to harvest valves between 2 and 24 hours after donor death resulted in depleted aerobic metabolic reserves (i.e., lowered high-energy phosphate stores). Such valves, however, continued active anaerobic metabolism and contained numerous morphologically intact fibroblasts. Organ perfusion studies (Belzer et al., 1983; Henry et al., 1988; Southard et al., 1984; Southard et al., 1985) have suggested that transport media containing adenosine and metabolic phosphates preserve cellular viability by stimulating ATP synthesis, Na^+ reabsorption, maintenance of near normal concentrations of tissue K^+, and reversal of tissue edema.

Since it is not generally practical to collect donor valves under sterile conditions, an efficient method of disinfection is required to provide a sterile allograft for transplantation. The method used should have no deleterious effect on the integrity of the valve while still maintaining efficient antibacterial and antifungal activity. Many antibiotic disinfecting mixtures have been investigated, not only to determine the efficiency of disinfection but also to assess effects on the physical properties of the grafts, i.e. cellular viability, host tissue ingrowth, and valve survival (Barratt-Boyes et al., 1977; Gavin et al.,

1973; Hu et al., 1989; Lockey et al., 1972; Strickett et al., 1983; van der Kamp et al., 1981; Wain et al., 1977; Waterworth et al., 1974; Yacoub and Kittle, 1970). Currently, nearly all allograft heart valve programs use low-concentration, broad-spectrum antibiotics in a sterile-filtered nutrient tissue culture medium to disinfect cardiovascular tissues (Ross et al., 1979; Strickett et al., 1983).

Suspended animation (inhibition of cellular activities) through reduced temperature is the only way to achieve organ preservation. Living cells can be preserved for years by storage at temperature of the order of -150 to -200 °C by inhibiting molecular motion. Physical processes in biological systems, such as osmotic pressure and thermal expansion, are completely dependent on the rate of molecular motion. All physiological and biochemical processes of the cell can be arrested with the inhibition of molecular motion, and aging becomes nearly impossible. Cryopreservation generally involves tissue freezing. The consequences of freezing in biological systems arise from three factors: inhibition of chemical and physical processes by the low temperatures, the effects of ice in the biological system, and the physicochemical effects of increased solute concentrations as the volume of liquid water decreases during ice crystal formation.

As the temperature becomes lower, molecular movement decreases. A decrease of molecular motion slows down both physical and chemical processes in biological systems in proportion to the fractional change in absolute temperature.

Damage to biological systems during freezing and thawing results from the conversion of liquid (solvent) water into ice. Rapid cooling (greater than 1 °C/minute) during freezing produces a myriad of small crystals because of insufficient time for the growth of large crystals. Cells rapidly cooled during freezing appear unshrunken and contain intracellular ice. The physical presence of ice crystals inside cells is lethal and can result in a failure of the cryopreservation protocol. Damage to the cells by the intracellular ice primarily occurs as these crystals grow in size during warming or storage at relatively high subzero temperatures through a process described as recrystallization (Mazur, 1977). Slow cooling (less than 1 °C/minute) during freezing results in the formation of a small number of very large crystals. With slow cooling, intracellular ice may not form, but the intracellular environment is exposed to biochemical and physical adversities caused by high solute concentrations, dehydration, pH shifts (van der Berg and Rose, 1959; van der Berg and Soliman, 1969), and cell shrinkage (Meryman, 1970). The viability of the intact cell, tissue, or organ of which the cell is a part is governed by the

resulting influences of these changes on the biochemical properties of intracellular structures. High salt concentrations are the most provable cause of cell damage since they are disruptive to cell membranes and protein structure. Optimal survival of various cell types occurs at a cooling rate somewhere between fast and slow. This optimal rate varies with different cell types; specimen size, volume, and shape; the presence of cryoprotectants, and the concentration(s) of the cryoprotectant(s).

Although post-thaw viability of a frozen system can be optimized by carefully selecting the best combination of cooling and warming rates, the degree of viability is likely to be unsatisfactory when preserving bulky tissues and organs for transplantation. Chemical cryoprotectants offer the most feasible means of protecting cells from damage due to freezing and thawing. Suitable cryoprotectants are characterized by a relatively low toxicity to the cellular materials and very high solubility in water. There are marked differences in the major functional groups and compositions of compounds that have cryoprotective efficacy. The most widely used group of protectants are those with low molecular weight that are significantly permeant to the membrane of cells, such as dimethyl sulfoxide (perhaps the most widely used protectant of all), propane diol and methanol (Grout, 1991). Compounds such as glycerol, sucrose, and a number of the sugars have varying degrees of

permeation depending on cell type and incubation conditions (Shlafer, 1981). The primary role of the cryoprotectants is the protection of cells from freeze desiccation, but they may also provide an element of protection to specific molecular species within the cell by stabilizing macromolecules (Meryman et al., 1978). Since cryoinjury is linked to conversion of liquid water to ice, the colligative role of protectants depends on an effective reduction in the water content of the cell, which in turn reduces the effective ion/solute concentrations that can occur as a result of freeze-dehydration (Grout, 1991).

Generally, cells remain stable for only a few months in the range of -70 to -100 °C. Cells kept for longer periods at -70 to -100 °C undergo appreciable "aging" as a result of enzymatic activity and physical/chemical reactions (Luyet, 1960). All biological material can be stored without significant change at liquid nitrogen temperature (-196 °C) or at that for the vapor phase of a liquid nitrogen (-150 to -190 °C) for at least 10 years since no ordinary, thermally driven reactions occur in aqueous systems at these temperatures (Heacox et al., 1988).

Small ice crystals are thermodynamically unstable because of their high surface energy, and they tend to undergo recrystallization to improve their thermodynamic stability. Recrystallization generally occurs during warming

and may result in cell damage (Wolfinbarger et al., 1989). Rapid thawing is recommended for rewarming cryopreserved heart valves since it suppresses recrystallization. The process is accomplished by immersion of the frozen tissue in a 42 °C water bath directly from the liquid nitrogen storage conditions. After thawing cryopreserved tissues, cryoprotectants may removed by a stepwise-washing procedure to minimize osmotic stress to the cells (Bank and Brockbank, 1987; May and Baust, 1988), although the effectiveness of some stepwise-washing procedures in removing the cryoprotectant may questioned (Hu, 1992).

D. Calcification of Cardiovascular Implants.

Calcific degeneration of cardiovascular implants and diseased cardiovascular tissues is common (Schoen et al., 1988). Calcification of cardiovascular implants often results in clinical device failure due to mechanical dysfunction, vascular obstruction, or embolization of calcific deposits. Primary tissue degeneration due to intrinsic cuspal calcification is the most frequent cause of clinical failure of porcine aortic valve bioprostheses (Ferrans et al., 1980; Gallo et al., 1984; McClung et al., 1983; Schoen et al., 1983; Schoen, 1987; Schoen et al., 1988; Valente et al., 1983; Valente et al., 1985). Bovine pericardial bioprostheses also fail frequently because of calcification (Reul et

al., 1985; Schoen, 1987; Schoen et al., 1988).

Pathological analyses of failed bioprosthetic valves reveal dystrophic calcific deposits within the cusps involving both cellular remnants and collagen fibrils (Reul et al., 1985; Schoen, 1987; Schoen et al., 1988). Calcium salt incorporations are most extensive at regions of greatest hemodynamic stress, such as the commisures (the free edges of the leaflets) and annular attachments. Experimental calcification events in rat subdermal implants of porcine bioprosthetic heart valve tissue have been shown with cells devitalized by glutaraldehyde pretreatment within the cusps followed later by collagen mineralization (Levy et al., 1983; Schoen et al., 1985). Although the infrastructure of porcine aortic valves and bovine pericardium is different, the kinetics and morphologic features of mineralization of these materials are similar. Calcific deposits in pericardial tissue also initially involve cell remnants followed by mineralization of collagen. Nearly all forms of cell-oriented calcification occur by crystal formation on remnants of cell membranes, usually in the form of extracellular vesicles. Late mineralization in collagen could be due to either extension of cell-oriented mineral deposition, or to an independent extracellular matrix mineralization mechanism. Initial calcium phosphate deposits increase in size and number with the process of calcification. Proliferation of nucleation sites, crystal growth, and progressive

confluence of diffusely distributed microcrystals result in the formation of gross nodules which focally obliterate implant architecture leading to ulceration, and eventual deformation of the valve structure (Schoen et al., 1987). Although bioprosthetic tissue calcification causes either stenosis or regurgitation or both, regurgitation due to tearing at calcific deposits is most frequent, and calcific emboli can also occur. Calcification of bioprosthetic tissue does not appear to be completely explained by a single mechanism. Indeed this process most probably occurs as an interaction of host, implant, and mechanical factors. The physical forces to which the valve tissue is exposed (Thubrikar et al., 1983; Wright et al., 1982), recipient mineral metabolism (Carpentier et al., 1984), and host environment at the valve locus (Schoen et al., 1988) may all play a role in facilitating calcification. Exposure to the extracellular ions associated with the host mineral metabolism is a prerequisite for bioprosthetic calcification. Glutaraldehyde fixation is identified as the single most important implant factor leading to calcification of bioprosthetic valves (Golumb et al., 1987; Levy et al., 1983). Mechanical stress plays a role in promotion of calcification, but mechanical deformation is not necessary for mineralization to proceed (Levy et al., 1983; Schoen et al., 1985; Schoen et al., 1988).

Although valved aortic homografts develop calcification much less frequently than xenograft and bioprosthetic valves, calcification is one of the

general causes of homograft failure in the late ultimate results (Maxwell et al., 1989; Miller and Shumway, 1987). Calcification is significantly greater in the conduit wall than in the valve leaflets (Brock, 1968; Gonazalez-Lavin et al., 1988; Jonas et al., 1988; Maxwell et al., 1989; Miller and Shumway, 1987; Saravalli et al., 1980; Webb et al., 1988), and the consequent lack of distensibility limits their long-term durability. In addition to cell-and collagen-oriented calcification of the aortic homograft, aortic wall calcification occurs in close association with elastin as a prominent feature in experimental (subdermal and circulatory models) and clinical species (Gonazalez-Lavin et al., 1988; Jonas et al., 1988; Khatib and Lupinetti, 1990; Saravalli et al., 1980). Urist and Adams (1967) demonstrated that the calcification of transplanted aorta in rats was localized mostly to the elastic structure after degradation or splitting of elastic fibers where an increased rate of calcium uptake was observed. In fresh and cryopreserved aortic homograft conduit, calcification was also present in the elastic component of the media with the elastosis (Gonazalez-Lavin et al., 1988; Jonas et al., 1988).

The pathophysiology of cardiovascular implant calcification is complex and poorly understood, and there are no satisfactory preventive measures or therapies to reverse degenerative calcification. However, calcification occurring normally in skeletal and dental tissues, and pathologically in

cardiovascular implants share important features (Anderson, 1983; Anderson, 1989; Schoen et al., 1988). Some mechanistic factors are shared by the various types of cardiovascular implant calcification.

Chief among the common elements in the various types of cardiovascular implant calcification is the various cell-derived components, such as cellular debris and subcellular vesicle-like organelles, which serve as the initial locus of calcification in direct analogy to the matrix vesicles of endochondral skeletal and dental mineralization (Anderson, 1984). Membranous structure of matrix vesicles in bone mineralization are derived from the surface membrane of chondrocytes, bone cells, and nonskeletal cells (Anderson, 1983) and initiate calcification by concentrating calcium within their already phosphate-rich structure (Anderson, 1984; Valente et al., 1985). Vesicles present a confined microenvironment for mineral initiation in which calcium is attracted by acidic phospholipids concentrated in matrix vesicles (Peress et al., 1974; Wuthier, 1975) and inorganic phosphate is concentrated in the vesicles by phosphatases (e.g., alkaline phosphatase, adenosine triphosphatase, and pyrophosphatase) residing in the matrix vesicle membrane (Cyboron et al., 1981; Matsuzawa and Anderson, 1971). These phosphatases facilitate the initiation of mineralization both by raising the local phosphate concentration and by diminishing the mineral inhibiting effects of pyrophosphate and adenosine triphosphate. Matrix

vesicles serve only for mineral initiation, the mineral proliferation is dependent on the concentration of calcium and phosphate in the extracellular fluid and the presence of mineral inhibitors, pyrophosphate, adenosine triphosphate, and anionic proteins. Matrix vesicle structures may be comparable to the early cell-oriented calcification noted in clinical and experimental cardiovascular implants.

Calcification of cells and cell fragments is also due to a disruption of normal physiology for cellular calcium regulation. Normal living animal cells have low intracellular free calcium concentrations (approximately 10^{-7} M) and high extracellular free calcium concentrations (approximately 10^{-3} M)(Schoen, 1987; Schoen et al., 1988). In healthy cells, despite the entrance of calcium into cells through several types of calcium channels, the 10,000-fold gradient across the plasma membrane is maintained by energy-requiring metabolic process such as plasma membrane-bound Ca^{2+}-ATPase. Ca^{2+}-ATPase uses the energy of ATP hydrolysis to pump Ca^{2+} out of the cell (Schoen, 1987; Schoen et al., 1988). In damaged cells of cardiovascular implants, mechanisms for calcium exclusion are no longer functional (decreased efflux) (Schoen et al., 1986), and the injured membrane is more permeable to calcium (increased calcium influx). A net calcium influx reacts with phosphorus in the membrane of cell and may contribute to the initiating mechanism of calcium phosphate crystallization in cardiovascular implants.

The first mineral that forms in matrix vesicles is amorphous calcium phosphate, which exhibits little or no crystallinity to roentgen ray or electron diffraction. However, it is usually converted to crystalline, insoluble calcium phosphate mineral in the form of mature hydroxyapatite. Hydroxyapatite is a complicated compound with ten calcium atoms, six phosphate groups, and two hydroxyls and must be built from calcium and phosphate ions available in serum or extracellular fluid. The concentration of calcium and phosphate in serum or extracellular fluid is not sufficient to initiate mineral deposition spontaneously, but is sufficient to support crystal proliferation once a few preformed crystals of hydroxyapatite are present. Preformed crystals serve as templates for new crystal formation (Anderson, 1983).

E. Proteoglycans in Aorta.

There are two main classes of extracellular molecules in aorta: (1) heterogeneous proteoglycans (PGs) as ground substance, (2) elastin and collagen fibers which provide structural function. The proteoglycans in the arterial wall are synthesized by smooth muscle cells and endothelial cells in cardiovascular structures (Morita et al., 1990). Extracellular matrix proteoglycans are functionally important components of the arterial wall. The aorta undergoes repetitive, transient pressure changes, and it is likely that the

proteoglycans, in concert with the elastin component of the tissue, buffer these cyclical changes. In addition, these macromolecules contribute to maintain structural integrity of the tissue, influence calcification, and regulate the permeability of the tissue (Berenson et al., 1973; Castellot et al., 1982; Ruoslathi, 1988; Ruoslathi, 1989). Proteoglycans are high molecular weight polyanionic substances consisting of a central core protein to which many different glycosaminoglycan (GAG) chains are covalently bound. Glycosaminoglycans are long heteropolysaccharide molecules, consisting of repeating disaccharides units in which one sugar is a hexosamine (N-acetylglucosamine or N-acetylgalactosamine) and the other is uronic acid. In most cases the amino sugar is sulfated. The nature of glycosaminoglycans is highly charged polyanions because of the carboxyl and sulfate groups in the disaccharide units. There are six classes of glycosaminoglycans distinguished by their sugar residues, the type of linkage between these residues, and the number and location of sulfate groups: (1) hyaluronic acid (2) chondroitin sulfate (3) dermatan sulfate (4) heparan sulfate (5) heparin (6) keratan sulfate. Hyaluronic acid is the largest glycosaminoglycan. Hyaluronate differs from the other glycosaminoglycans in that it does not contain sulfate groups and is not covalently bound to protein. It is nevertheless classified as a glycosaminoglycan because of its structural similarity to these other polymers. It consists of repeating disaccharide units of N-acetylglucosamine and

glucuronic acid. Chondroitin sulfate is found widely distributed throughout various tissues. It is an unbranched hetropolymer consisting of repeating disaccharides of N-acetylgalactosamine and D-glucuronic acid. The disaccharides may be sulfated in either the 4 or 6 position of N-acetylgalactosamine. Dermatan sulfate differs from chondroitin sulfates in that its predominant uronic acid in the repeat disaccharide is found as iduronic acid, although glucuronic acid is also present in variable amounts. Glucosamine and glucuronic acid or iduronic acid form disaccharide repeated units of heparin, as in dermatan sulfate. Heparin is particularly highly sulfated, containing up to three sulfate residues per disaccharide unit. Unlike the other glycosaminoglycans, which are predominantly extracellular components, heparin is an intracellular component of mast cells. Heparan sulfate is very closely related to heparin, in that it contains a similar disaccharide repeat unit. However, it varies from heparin, in that it is less sulfated and contains higher proportions of glucuronic acid than heparin. Heparan sulfate appears to be extracellular in distribution. Keratan sulfate is characterized by molecular heterogeneity and is composed principally of a repeating disaccharide unit of N-acetylglucosamine and galactose. The length and charge distribution within the glycosaminoglycan chains mainly determine physico-chemical properties (polydispersities in terms of molecular size, and charge density) of proteoglycans.

Proteoglycans exist on three levels of organization: (1) individual subunits (2) individual aggregates and (3) the level created by the interaction of subunits of one aggregate with subunits of another aggregate (Castellot et al., 1982). Subunits consist of a protein core filament with multiple covalently bound glycosaminoglycan chains. The proteoglycan aggregate is composed of many proteoglycan subunits in noncovalent associations with a single hyaluronic acid molecule through a terminal hyaluronic acid-binding region on the core protein. This association is stabilized further by the link-protein which has affinity for both hyaluronate and for the hyaluronate binding region of the proteoglycan subunits. A single link-protein is involved in the stabilization of each proteoglycan subunit in the aggregate.

Little information is available describing the characteristics of individual proteoglycans and their precise localization in the aortic tissue. However, arterial proteoglycans have certain physico-chemical properties that are similar to those of hyaline cartilage (Gardell et al., 1980; Oegema et al., 1979). Recent advances in understanding of the cartilage matrix and the process of mineralization indicate that matrix proteoglycans have an inhibitory effect on cartilage mineralization (Hirschman and Dziewiatkowski, 1966; Joseph, 1983; Larsson et al., 1973; Lohmander and Hjerpe, 1975; Mitchell et al., 1982). Lohmander and Hjerpe (1975) reported that the cartilage lost approximately half

its content of proteoglycans with the onset of mineralization. The proteoglycans remaining in the mineralized cartilage differed in size from those of nonmineralized tissue. There are decreased proportions of very high molecular weight proteoglycans in mineralized tissue. Reddi et al. (1978) also found that there was a decline in the synthesis of proteoglycans and a large proportion of the newly synthesized molecules are of lower molecular weight in the cartilage undergoing extensive mineralization. Although the mechanism by which proteoglycans inhibit mineralization is not immediately apparent, proteoglycans might inhibit mineralization by the following mechanisms. First, the polyanionic chains of glycosaminoglycans may help to hold the extended network, repel phosphate anions, and bind calcium. Secondly, proteoglycan aggregates inhibit matrix calcification more effectively than proteoglycan subunits because aggregates physically shield or sequester small mineral clusters within their network of subunits, preventing enlargement of small mineral clusters beyond a critical size that would spread mineralization through the matrix (Cuerro et al., 1973). This inhibition occurs because subunits bound to aggregates can not be easily displaced, and they are organized to provide a large, uniformly dense network of negatively charged glycosaminoglycans chains, and are essentially immobilized in the matrix.

There has been little information describing the effects of

cryopreservation on proteoglycan changes in allograft heart valves. However, cryopreserved allografts have superior durability over cold-stored valves possibly due to improved proteoglycan matrix preservation which may provide for sustained cell activity and reduced potential for calcification following transplantation. Previous studies on cryopreserved allograft heart valves have focused on donor cell viability in the valves (Hu et al., 1989; Hu et al., 1990; Parker et al., 1978; Reichenbach et al., 1971). The present study focuses on the matrix components of the arterial conduit tissue since valved aortic homografts develop calcification (late after transplantation) significantly greater in the aortic wall than in the valve leaflets.

The research to be described was designed to assess the effects of cryopreservation on matrix proteoglycans with the ultimate objective of permiting prediction of the tendency of aorta conduit tissue to calcify following transplantation. The basic premise, or hypothesis, for this study is that proteoglycan changes in the conduit tissues of allograft valves contribute to valve calcifications by mechanisms similar to those mechanisms associated with bone mineralization.

Specifically, the aims of this study are divided into the following: I. Development of method for optimal extraction of proteoglycans from porcine

aorta tissue. II. Quantitative analysis of proteoglycans present in fresh and cryopreserved aorta conduit tissue. III. Study of the size distribution of proteoglycans in fresh and cryopreserved tissue. IV. Assessment of the distribution of proteoglycans in aorta conduit tissue which are present in soluble matrix and covalently linked to collagenous and elastic fibers. V. Evaluation of the morphology of fresh and cryopreserved porcine aorta tissue. VI. Study of the distribution of proteoglycans within the arterial wall using transmission electron microscopy. VII. Analysis of calcium in fresh and cryopreserved porcine aorta conduit tissue. VIII. Determination of phosphorus content of fresh and cryopreserved aorta conduit tissue.

CHAPTER II

EXPERIMENTAL DESIGN AND METHODS

A. Procurement of Tissue.

Porcine aorta conduit tissue was used in this study. The porcine tissues were obtained from a local abattoir, Gwaltney meat-packing company in Smithfield, Virginia. The general age and weight of the pigs were nearly equivalent. The pig hearts were removed within 20 minutes of slaughter with the assistance of Gwaltney's staff. Each heart was immediately washed with 200 ml of cold lactated Ringer's solution to remove residual blood and transferred to cold tissue culture media (RPMI 1640, GIBCO) and packed in ice for transport to the laboratory. The aortic conduits (about 6 cm in length) were quickly dissected from the hearts on arrival at the laboratory.

B. Preparation of Fresh and Cryopreserved Tissues.

Fresh and cryopreserved tissues were used for this study. Fresh aorta conduit tissues are defined as tissues dissected from the hearts immediately on arrival at the laboratory. Cryopreserved tissues are defined as tissues taken through a standard preimplantation processing for cryopreservation as described by Lange and Hopkins (1989). The detailed methods in each step (allowable warm ischemic time, antibiotic treatment regimen, concentration of cryoprotectants, and technique for storage) of cryopreservation protocols differ between allograft heart valve programs in different tissue banks and valve processing companies. This study employed the methods of LifeNet Transplant Services in Virginia Beach, VA (Lange and Hopkins, 1989; Wolfinbarger and Hopkins, 1989) for valve cryopreservation.

For the preparation of cryopreserved tissue which was used in this study, excess adipose tissue of porcine aortic conduit was trimmed after dissection of aorta conduit tissue from hearts. Tissues were rinsed in cold 0.9 % NaCl and disinfected with antibiotic mixtures. The following antibiotics were premixed with tissue culture medium (RPMI 1640) and approximately 125 ml of the antibiotic solution was added to each tissue: cefoxitin (240 μg/ml medium), lincomycin (120 μg/ml medium), polymyxin B sulfate(1 μg/ml medium) and

vancomycin (50 μg/ml medium). The tissue, immersed in the antibiotic medium, was then stored at 4 °C for 24 hours. The conduit tissue was removed from the antibiotic medium container and rinsed three times with 150 ml of fresh medium (RPMI 1640) at 4 °C.

The freezing medium was prepared as follows: RPMI 1640 tissue culture medium supplemented to a final concentration of 10 % (v:v) with fetal calf serum (FCS, GIBCO) were premixed. Dimethylsulfoxide (DMSO), to a final concentration of 10 % (v:v) was added to the cooled (4 °C) premixed medium at 4 °C. The conduit tissue was placed in volumetric container, and the freshly prepared freezing solution was added to produce a total volume of 100 ml. The conduit tissue in freezing solution was placed into a clear 4 by 6 inches of polyester-polyolefin modified bag (Kapak Corporation). All air was removed from the pouch by gently squeezing it and the bag was heat-sealed using a commercially available sealer (Scotchpak brand, Kapak Corporation). This bag was then inserted into a slightly larger 5 by 8 inches bag of trilaminate aluminum polypropylene (Kapak Corporation) and this outer bag was also heat-sealed. Packaging of tissue in freezing media was performed in the cold room (4 °C). The doubly packaged tissue was placed in a specially designed styrofoam box, placed in a freezer (-70 °C) for 5 hours, and then stored at -150 °C (ultralow freezer, Harris Corporation) until used.

Prior to extraction of cryopreserved tissues for proteoglycans, the cryopreserved aortic conduit was removed from the ultralow freezer, the outer pouch was opened and discarded, and the frozen conduit tissue was thawed by immersion of the inner bag in a 40 °C water bath (for approximately 5 minutes) (Wolfinbarger, 1992). When the ice in the bag turned to slush during the thawing step (freezing medium was not allowed to completely thaw), the inner pouch was opened and the aortic conduit in freezing medium was transferred into a large flask (500 ml). Dilution medium consisting of RPMI 1640 medium supplement with FCS, 10 % final concentration, (33 ml) was added to the tissue-containing flask, and it was gently agitated for 1 minute. An additional 66 ml of the dilution medium was added and again gently agitated for 1 minute. More dilution solution (200 ml) was added to the aortic conduit and agitated for an additional one minute. The conduit tissue was then transferred to 100 ml of fresh dilution solution. This "cryopreserved tissues" was then treated in a manner similar to fresh tissues.

C. Proteoglycan Extraction from Conduit Tissue.

Conduit tissues were rinsed in isotonic saline and minced as finely as possible with dissecting scissors. Weighed samples of 1 g of finely minced aorta conduit tissues were placed in Oak Ridge Centrifuge Tubes (Nalgene) and

aliquots of 5 ml of appropriate cold extraction solutions (Gdn-HCl) with protease inhibitors were added. A number of protease inhibitors were included in the extraction solutions: 0.1 M aminocaproic acid (for plasmin and plasmin activators), 0.005 M benzamidine-HCl (for trypsin-like activity), 0.01 M EDTA (for metalloproteases), 0.005 M N-ethylmaleimide (for sulfhydryl-dependent proteases and to prevent nonspecific disulfide exchange, which can occur in denaturing solvents), 0.001 M iodoacetamide (for thiol-dependent proteases), and 0.001 M phenylmethylsulfonyl fluoride (for serine-dependent proteases). Phenylmethylsulfonyl fluoride was dissolved in small volumes of methanol (0.17 g/ml for 1 M) and added as small volumes of concentrated solutions (for final 0.001 M of phenylmethylsulfonyl fluoride depending on total volumes of extraction medium) at several times during extraction. Extraction of tissue was performed using a rocker platform (Bellco Glass, Inc.) at 4 °C. Extracts were centrifuged at 10,000 x g using a JA-20 rotor in a refrigerated centrifuge (Beckman Model J2-21) for 30 minutes, and the supernatants (approximately 4 ml) were transferred into dialysis membrane tubing (Spectra/por, molecular weight cut off 3,500) and dialyzed repeatedly (five times) against 200 ml of ultra-pure water at 0 °C with stirring.

D. CsCl Centrifugation of Proteoglycans.

Once the crude proteoglycans were extracted, CsCl density gradient centrifugation was used for further purification from other macromolecules and for separating proteoglycans from each other. Proteoglycans in the extracts of aorta were adjusted to a density of 1.33 g/ml by the addition of solid CsCl (approximately 0.4g/ml extract), transferred to an ultrabottle with an aluminum closure (Nalgene), and centrifuged at 100,000 x g for 40 hours at 8 °C in an ultracentrifuge (Beckman, Model L8-70) using a Type Ti 60 rotor. Following centrifugation, five equal fractions were collected using a syringe with a long needle starting from the bottom of the tube. The densities of the fractions were determined by weighing 1 ml of each fraction. The bottom three-fifths of the gradient were pooled, and the pooled solution was adjusted to a density of 1.46 g/ml by the addition of solid CsCl (approximately 0.12g/ml extract). These sample were centrifuged again at 100,000 g for 40 hours at 8 °C. Six equal fractions were then collected from each tube using a 10 ml syringe with a long needle, again starting from the bottom of the tube. The densities of each fraction were determined by weighing 1 ml of each fraction. The fractions (20 ml) were transferred to dialysis membrane tubing (Spectra/por, molecular weight cut off 3,500) and exhaustively dialyzed (five times) against 1 liter of ultra-pure water for one day at 0 °C, or until the osmolarity of the dialysate

(measured by Advanced Wide-Range Osmometer 3W2, Advanced Instruments Inc.) approximated that of water. Based on uronic acid and protein content profiles, samples were pooled into three fractions (I, II, and III) and freeze-dried in Freeze Dry/Shell Freeze System (Labconco).

E. Quantitation of Proteoglycan Concentrations.

Glycosaminoglycans were isolated from the extract by alkaline treatment as described by Carlson (1968). Briefly, dialyzed proteoglycans were incubated in 0.05 M sodium hydroxide (NaOH) in 1.0 M sodium borohydride ($NaBH_4$) for 48 hours at 45 °C. Alkali was neutralized by adding 125 μl of 10 M acetic acid/ml of solution, and the solutions were clarified by centrifugation at 6,000 x g using a JA-20 rotor in a refrigerated Beckman Model J2-21 centrifuge for 20 minutes. Trichloroacetic acid was added to the supernatants (10 % , w:v, final concentration) and incubated at 4 °C for 2 hours. The samples were then centrifuged at 3,000 rpm using a Damon/IEC Division centrifuge (IEC Model HN-S) for 30 minutes. The supernatants were transferred into dialysis membrane tubing (Spectra/por, molecular weight cut off 3,500) and dialyzed repeatedly (three times) against ultra-pure water equivalent to 40 times the sample volume at 0 °C. The dialysates were collected and used for the determination of uronic acid concentrations as described by Bitter and Muir

(1962).

Uronic acid content in the proteoglycan fractions was determined by the method of Bitter and Muir (1962) with glucuronolactone (Sigma Chemical Co.) as a standard. Sulfuric acid reagent (0.025 M sodium tetraborate in 36 N sulfuric acid) was prepared and cooled in a freezer (-20 °C) before the assay. The sulfuric acid reagent (3 ml) was added to 0.5 ml of sample or standards containing 0 - 25 μg of uronic acid. The samples or standards and reagent were mixed carefully, heated in a boiling water bath for 10 minutes, and cooled to room temperature. Carbazole (Fluka chemie AG) reagent (0.1 ml of 0.125 % weight/volume solution in absolute ethanol) was then added to the cooled mixture. The samples or standards and carbazole reagent were well mixed, covered to prevent evaporation, heated in a boiling water bath for 15 minutes, and cooled to room temperature. Absorbance was measured at 530 nm in a spectrophotometer (Shimadzu UV 160U).

Protein content in the proteoglycan fractions was measured by the procedure of Lowry (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. To samples or standards of 0 - 500 μg of protein in 0.1 ml water, 4ml of fresh Reagent A (2 % sodium carbonate in 0.1 N NaOH, 2 % potassium tartarate, 1 % cupric sulfate) was added and allowed to stand for 10 minutes at

room temperature. Folin phenol reagent (0.5 ml, 1 N, Sigma Chemical Co.) was then added and mixed well. After 30 minutes, absorbance of the samples and standards were read at 500 nm.

F. Gel Column Chromatography.

Gel chromatography was used for determination of approximate hydrodynamic volumes of purified proteoglycans. Separation of proteoglycans based on size was performed on a column of Sepharose CL-4B (Pharmacia) which is an agarose-based gel permeation matrix having an approximated agarose concentration of 4 %. Dissociative buffer, 4 M Gdn-HCl/0.05 M sodium acetate (pH 5.8) was prepared and filtered through a Whatman, #3 filter. Dissolved gases were removed by placing the buffer in a side-arm vacuum flask and applying a vacuum (precision vacuum pump, GCA corporation, Model S35). The degassing process was stopped when no more small air bubbles were released from the buffer (usually 20 to 30 minutes with 600 ml of buffer).

1. Preparation of Gel Matrix.

The sepharose slurry was washed with dissociative buffer equivalent to

two volumes of the gel volume to equilibrate the gel and remove the Merthiolate present in the gel as a preservative. Briefly, the gel slurry (180 g for 200 ml volume) was poured into a graduated cylinder and suspended in 2 times the gel volume of dissociative buffer (400 ml) and allowed to settle. The supernatant was decanted off carefully to remove fines, and the settling process was repeated. The gel was finally suspended in an equal volume of dissociative buffer.

2. Column Packing.

A long narrow column (1.5 cm in diameter and 100 cm in length) was used for the gel permeation chromatography. The column was filled with the gel slurry mixed with degassed dissociative buffer using a packing reservoir (250 ml) attached to the top of the column. The gel was allowed to briefly settle in the column prior to opening of the column outlet. The flow rate (20 ml/hour) during packing was slightly faster than that to be used during chromatographic separations. After the gel had completely packed, the top of the column was connected to a reservoir containing dissociative buffer, and the column flow rate was reduced to that to be used for separation runs. The column was eluted at a rate of 16 ml/hour. This constant flow rate was maintained by use of a peristaltic pump (Pharmacia, Model P-3). Before use,

one column volume (approximately 160 ml) of buffer was allowed to flow through the column.

3. Collection and Dialysis of Eluent.

A fraction collector (ISCO) was connected to the column and set to collect 3.5 ml (70 drops) of eluent in each fraction. A hollow fiber system (Fleaker hollow fiber concentration system, quantity 88 fibers, Spectrum), for the dialysis of solute molecules against ultra-pure water, was connected between the peristaltic pump and the fraction collector. A reservoir containing ultra-pure water was also connected to the hollow fiber system, and a flow of ultra-pure water through this continual dialysis system was maintained by gravity flow methods (approximately 320 ml/hour).

4. Calibration of the Column.

The void volume (V_o) of the column is the volume in which very large molecules (which are excluded from the gel) would elute. The void volume was calibrated using Escherichia coli by measuring turbidity of the eluent at 600 nm or with blue dextran by measuring absorption of the eluent at 620 nm. An aliquot of Escherichia coli (lyophilized cells of strain B, ATCC 11303,

Sigma Chemical Co.) was weighed (1.5 mg), dissolved in 1 ml of 4 M Gdn HCl/0.05 M sodium acetate, pH 5.8 (dissociative buffer), and chromatographed on the Sepharose CL-4B column. The column was eluted at a flow rate of 16 ml/hour. Fractions of 3.5 ml were collected with fraction collector and measured for absorbance at 620 nm. Blue dextran, (molecular weight 2,000,000, Sigma Chemical Co.) 1.5 mg dissolved in 1 ml of dissociative buffer, was also subjected to gel permeation chromatography on the column (1.5 x 100 cm) of Sepharose CL-4B. The column was eluted at a flow rate of 16 ml/hour, and 3.5 ml fractions were collected. The eluent fractions were monitored for absorbance at 620 nm.

Inclusion volume (V_i) of the column is the volume in which very small molecules, which are completely included in the gel, would elute. The inclusion volume was determined using 5,5'-dithiobis [2-nitrobenzoic acid] (molecular weight 396.3, Sigma Chemical Co.) by measuring for absorbance at 280 nm. 5,5'-dithiobis [2-nitrobenzoic acid] (1.5 mg) was dissolved in 1 ml of dissociative buffer and applied on the Sepharose CL-4B column. Sample was eluted with 4 M Gdn-HCl/0.05M sodium acetate (pH 5.8) at a constant rate of 16 ml/hour. The eluent was measured for absorbance at 280 nm.

Chondroitin sulfate (Sigma Chemical Co., Catolog No. C8529), 0.15 mg

in 1 ml of gel filtration buffer, was chromatographed on the Sepharose CL-4B column. Fractions of 3.5 ml were collected and monitored for absorbance at 530 nm using the carbazole assay method described earlier (Bitter and Muir, 1962).

5. Gel Permeation Chromatography of Proteoglycans.

Lyophilized proteoglycans extracted from tissues (0.5 mg of uronic acid) were dissolved in degassed 4 M Gdn-HCl/0.05 M sodium acetate buffer, pH 5.8, and chromatographed on the Sepharose CL-4B column (1.5x100 cm) previously equilibrated with the same buffer. The column was eluted at a flow rate of 16 ml/hour and 3.5 ml was collected in each fraction with the fraction collector. Aliquots of the fractions were analyzed for uronic acid by carbazole reaction (Bitter and Muir, 1962) and for protein by measuring for absorbance at 280 nm. Each sample for gel filtration was chromatographed more than three times to insure statistical accuracy. K_{av} values for proteoglycans eluting from the column were used to refer to the relative sizes (hydrodynamic volumes) of proteoglycans. The K_{av} values were calculated from the mean elution volume (V_e) of the proteoglycans using the formula: $K_{av} = V_e - V_o/V_i - V_o$, where V_o = void volume and V_i = inclusion volume of the column, and V_e = elution volume of the proteoglycans.

G. Enzymatic Extraction/Digestion of Conduit Tissue.

1. Collagenase-Digestion of Conduit Tissue.

Gdn-HCl extracted conduit tissue (5 g) described earlier was washed three times with ultra-pure water and hydrolysed with collagenase from Clostridium histolytium (clostridiopeptidase A; EC 3.4.24.3, Sigma Chemical Co., 1 mg of enzyme/g of wet tissue) in 10 ml of 10 mM $CaCl_2$/50 mM Tris-HCl buffer, pH 7.6. for 48 hours at 37 °C. All protease inhibitors, as previously described, were added to the sample to inhibit non-specific proteolysis. The hydrolysate was centrifuged at 6,000 x g using a JA-20 rotor in a Beckman J2-21 centrifuge for 30 minutes, and supernatant (collagenase digest) was removed.

2. Elastase-Digestion of Conduit Tissue.

The residue tissue, not digestible by collagenase, was washed three times with ultra-pure water and hydrolysed with elastase (Type I: from porcine pancreas, EC 3.4.21.36. Sigma Chemical Co., 250 units/g of wet tissue) for 48 hours at 37 °C in 0.2 M Tri-HCl buffer, pH 8.8, containing protease inhibitors. The supernatant (elastase digest) was removed after centrifugation

of the digestion mixture at 6,000 x g in a Beckman J2-21 centrifuge for 30 minutes.

3. Isolation of Glycosaminoglycan from Residual Tissue.

The pellet remaining after the digestion with elastase was washed with ultra-pure water and treated with 2.0 % NaOH overnight at room temperature and further digested with papain (from papaya latex, EC 3.4.22.2, Sigma Chemical Co.,1 mg papain/g of wet tissue), after adjusting the pH to 6.4 with phosphoric acid. Digestion was performed at 65 °C for 24 hours in the presence of 0.01 M EDTA and 0.01 M cysteine hydrochloride.

H. Light Microscopy and Transmission Electron Microscopy.

In order to localize proteoglycans in histology sections of porcine aorta, the cationic phthalocyanine-like dye Cuprolinic Blue (CB, Polysciences, Inc) was used as a specific stain (Van Kuppevelt et al., 1985; Volker et al., 1986).

Small pieces of aorta, both fresh and cryopreserved, (1 mm x 1 mm x 1 mm) were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, overnight at 4 °C. Half of the fixed samples were stained for 12 hours with

1.0 % Cuprolinic Blue in 0.025 M veronal acetate buffer, pH 5.6, containing 2.5 % glutaraldehyde. The rest of the fixed tissues were stained with 1.0 % Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. After staining, the specimens were washed three times (10 minutes for each wash) with staining solution lacking Cuprolinic Blue and 0.3 M $MgCl_2$. The tissues were dehydrated in a graded ethanol series (30 %, 50 %, 70 %, 95 %, twice with 100 %, twice with acetone) for 10 minutes each. The 30 % and 50 % ethanol solution contained 0.5 % sodium tungstate (Sigma Chemical Co.). The aorta blocks were embedded in low viscosity epoxy resin. Tissue sections were prepared using a RMC ultramicrotome (Model MT2C). For histology study and selection for electron microscopy examination, semi-thin sections were stained with Richardson stain preparation (2 part Azure II, 1 part methylene blue, and 1 part sodium borate in deionized water) and studied by light microscopy (Nikon Biophot V series). Ultra-thin sections, stained with Cuprolinic Blue only were stained with uranyl acetate and lead citrate. Briefly, droplets (one for each grid) of 2 % uranyl acetate were formed on the petri dish, and the grids were immersed. After 20 minutes, grids were rinsed three times with 100 ml of deionized water and dried on the petri dish. Droplets (one for each grid) of 0.4 % lead citrate were formed. The grids were placed in the stain droplet for 20 minutes. Grids were rinsed three times with 100 ml of deionized water and dried. Staining of thin sections with uranyl acetate and lead citrate was omitted

in samples stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. Sections were examined using transmission electron microscopy (JEOL 100 CX II).

I. Calcium and Phosphorus Assays.

Fresh and cryopreserved porcine aorta conduit tissues were processed for total calcium and phosphorus determination. Aortic conduit tissue to be used for mineral analyses were rinsed three times with 100 ml of 0.9 % NaCl. Tissue (1 g) was placed in crucible (Fisher), and ashed in muffle furnace (Sybron, Thermolyne 2000) at 550 °C overnight. The ashed tissues were solubilized in 5 ml of 1N HCl overnight and analyzed for calcium and phosphorus.

The arsenazo III method (Bauer, 1981; Janssen and Helbing, 1991; Michaylova and Ilkova, 1971; Rowatt and Williams, 1989) was used for determination of total calcium content per gram of tissue. $CaCl_2$ solution (0.01 mM in 1N HCl) was prepared as standard stock. Samples or standards (0.1 ml) containing 0 - 4 μg calcium in 1N HCl were mixed with 4 ml Arsenazo III solution, 0.05 % Arsenazo III (Sigma Chemical Co.) in 0.5 M Tris-HCl, pH 7.4. The absorbance of purple colored complex was read at 650 nm in a

spectrophotometer.

The ascorbic acid method (Chen et al., 1956) was used for the determination of tissue phosphorus in fresh and cryopreserved pig aorta conduit tissues. This method was based on the color formed by the reduction of a phosphomolybdate complex. Ascorbic acid was used for the reduction of a phosphomolybdate. Samples or standards (4 ml) containing up to 8 μg of phosphorus were mixed with 4 ml of reagent C (1 volume of 6 N sulfuric acid, 2 volumes of distilled water, 1 volume of 2.5 % ammonium molybdate, 1 volume of 10 % ascorbic acid). The mixed samples or standards with reagent C were capped with Parafilm, placed in a 37 °C water bath for 1.5 to 2 hours, and cooled to room temperature. The absorbance was read at 820 nm in a spectrophotometer.

J. Statistical Evaluation of Data.

Experiments for the isolation of proteoglycans and sequential extraction of tissue were repeated more than three separate times. Data for the content of uronic acid and protein in proteoglycans were recorded as microgram (μg) per gram (g) wet tissue weight and represented mean $\pm$ standard error of a minimum of 3 replicate assays. Studies for mineral analyses of tissues were

performed in triplicate and repeated at least three separate times. Elemental concentrations were expressed as microgram per gram wet tissue weight. The data were used for calculation of the means and standard error of the means. Linear regression analyses (Energraphic 3.0) were used for standard curves of the uronic acid assay, the Lowry protein assay, calcium analysis with Arsenazo III, and the phosphorus assay. Statistical evaluations of significance were compared by Student's t test and analysis of variance (ANOVA). Means of more than two groups were compared by multiple comparison tests (TUKEY, REGWF. REGWQ) (University SAS program). Significance level was set at 0.05.

CHAPTER III

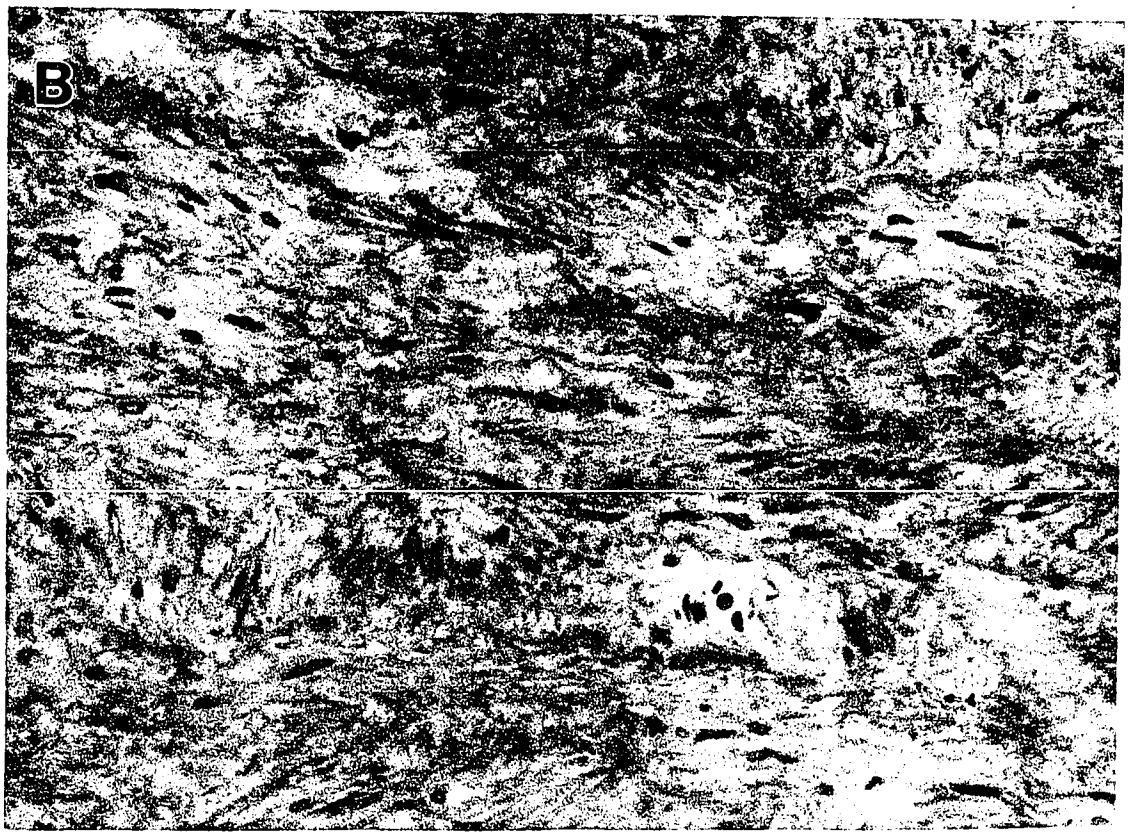
RESULTS

A. Basic Morphology of Fresh and Cryopreserved Porcine Aortic Conduit Tissue.

Light microscopic studies revealed that the tunica media of porcine aortic conduit tissue contains an abundance of smooth muscle cells (represented by the nuclei); however, the most distinct feature of the tunica media is its large amount of elastin material. The elastin material is not present in the form of fibers, but rather as fenestrated membranes (Figure 1A). Careful examination of Figure 1A revealed what appears to be interruptions of some of the laminae. These interruptions are actually the fenestrations or openings in the elastin membrane. The smooth muscle cells of the media are arranged in a closely wound spiral between the elastic membranes, however, this arrangement is difficult to recognize in sectioned material.

A comparative histologic examination of tunica media from fresh and

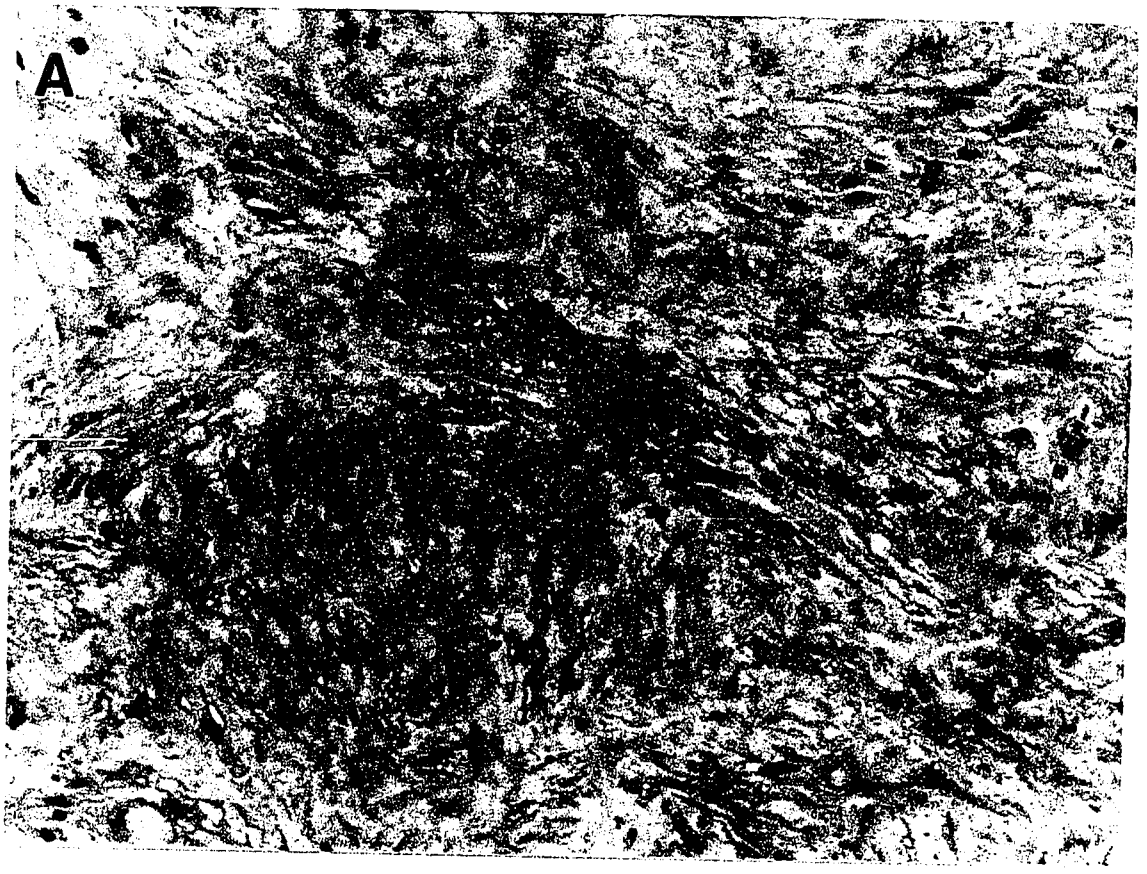
Figure 1. Histologic sections of the tunica media of fresh (A) and cryopreserved porcine aortic conduit (B), showing normal elastic architecture (waves) and distribution of smooth muscle cell, nuclei, (Richardson stain; original magnification x400).

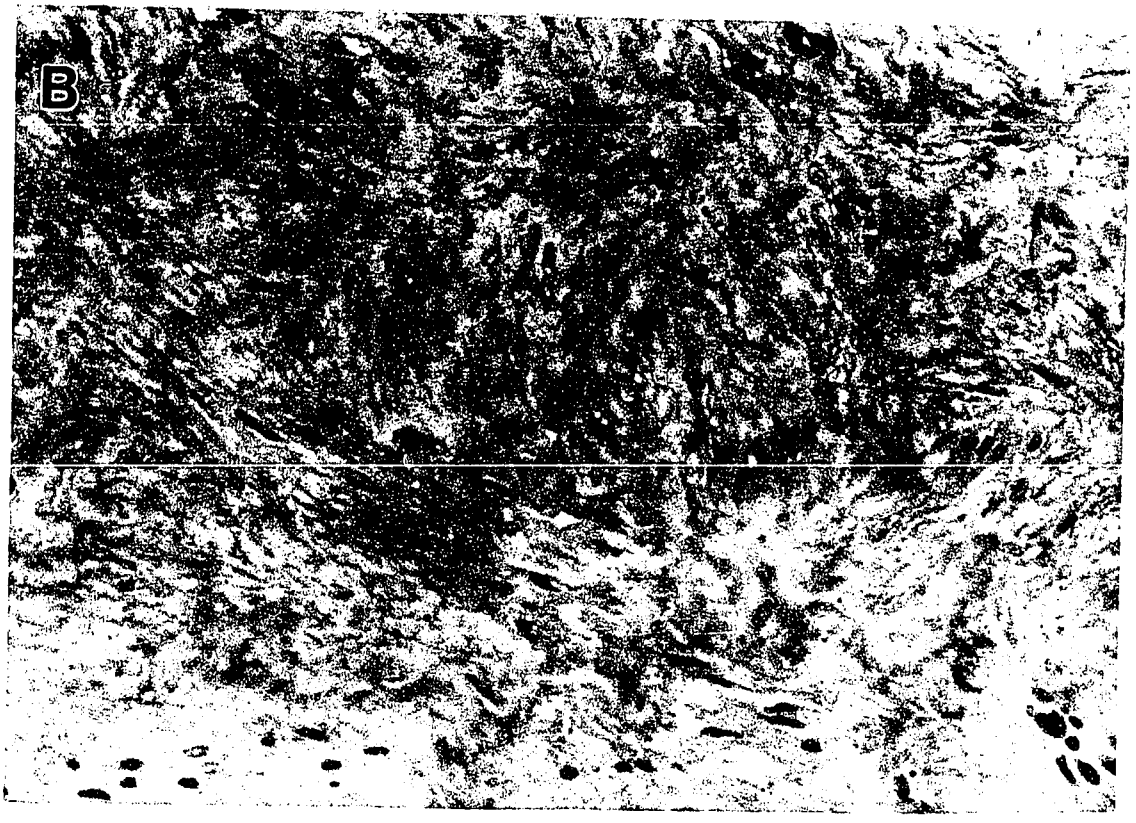


cryopreserved porcine aorta conduit specimens revealed a normal pattern of elastic tissue and distribution of the smooth muscle cells in the medial layer of aorta (Figure 1). The pattern of elastin distribution in fresh aortic tissue consisted of long, uniform, parallel laminae in regular arrangement. The smooth muscle cells had prominent round nuclei, and were distributed between the elastic laminae as single cells (Figure 1A). The normal structure of the media was not affected during tissue preimplantation processing since neither change of the elastic tissue nor evidence of altered distribution of smooth muscle cells was detected in cryopreserved aorta conduit specimen (Figure 1B).

The outermost layer of the porcine aorta, the tunica adventitia, is shown in Figure 2. The tunica adventitia consists mostly of collagenous fibers that course in longitudinal spirals. Their course, like the smooth muscle cells, however, is unrecognizable in individual tissue histology sections. The cells of the adventitia, represented by the nuclei seen in the adventitia in Figure 2, are fibroblasts. There are no elastic laminae in the adventitia; but elastic fibers are present, though relatively few in number. The presence of elastic fibers in histology sections used in this study was unrecognizable since the elastic fibers were not stained with Richardson stain preparation. A comparative histologic examination of tunica adventitia from fresh (Figure 2A) and cryopreserved (Figure 2B) porcine aortic conduit revealed no change in the structure of

Figure 2. Histologic sections of tunica adventitia of fresh (A) and cryopreserved porcine aortic conduit (B), showing the normal collagen structure and fibroblast distribution (Richardson stain; original magnification x400).





collagen and distribution of fibroblasts in tissue that has undergone preimplantation processing and cryopreservation (Figure 2B).

B. Isolation of Proteoglycans.

1. Development of Method for Optimal Proteoglycan Extraction.

Procedures for proteoglycan extraction for subsequent studies must provide for a maximum recovery and occur under conditions that prevent degradation of the macromolecules by chemical or enzymatic processes. It has been reported that the types of electrolytes, the concentrations of electrolytes, and the extraction times appear to be critical to the efficiency of dissociative extraction (Sajdera and Hascall, 1969). Guanidine (Gdn)-hydrochloride (HCl) was earlier reported to be the most effective solvent for the extraction of proteoglycans in cartilagenous tissue (Sajdera and Hascall, 1969).

In this study, fully hydrated tissue was used because dry-defatted tissue (frequently used in earlier proteoglycan studies) would not be suitable for the planned proteoglycan studies. Using smaller tissue pieces was also important in obtaining efficient extractions. Small pieces of tissue can expose more surface area to the solvent, and the diffusion distance for the solutes and solvents is

shorter than with larger (whole) pieces of tissue. Therefore, high concentrations of the dissociative solvents are quickly obtained in the tissue, and the protease inhibitors can rapidly reach their sites of action to prevent degradation.

Figure 3 shows the amounts of proteoglycans (measured as uronic acid content after glycosaminoglycans isolation) extracted from porcine aorta conduit as a function of concentration of Gdn-HCl and various extraction times. A Gdn-HCl concentration of 4 M proved to be the most optimal concentration for extraction (Figures 3 and 4), and 48 hours was an appropriate extraction time (Figure 3). The yields of proteoglycans after 48 hours extraction with different concentrations (0.5, 2, 4, 6, and 8 M) of Gdn-HCl were significantly different from one another statistically ($P < 0.05$)(Figure 4). Based on these results, proteoglycan fractions used in subsequent portions of this study were isolated using 48 hours extraction in 4 M Gdn-HCl.

2. Extraction and Purification of Proteoglycans.

The scheme for the isolation of proteoglycans from conduit tissue is illustrated in Figure 5. Fresh and cryopreserved porcine aorta tissues were extracted once for 48 hours in 4 M Gdn-HCl yielding 460 μg uronate/g wet

Figure 3. Extraction of proteoglycans from porcine aorta conduit tissue with different molarities of Gdn-HCl (0.5, 2, 4, 6, and 8 M) at various extraction times (1, 2, 4, 6, 18, 24, 30, 42, 48, and 54 hours). Data shown are mean values with error bars indicating the standard error of the mean where an error bar is not shown, it was obscured by the symbol used to indicate the data point (n = 3).

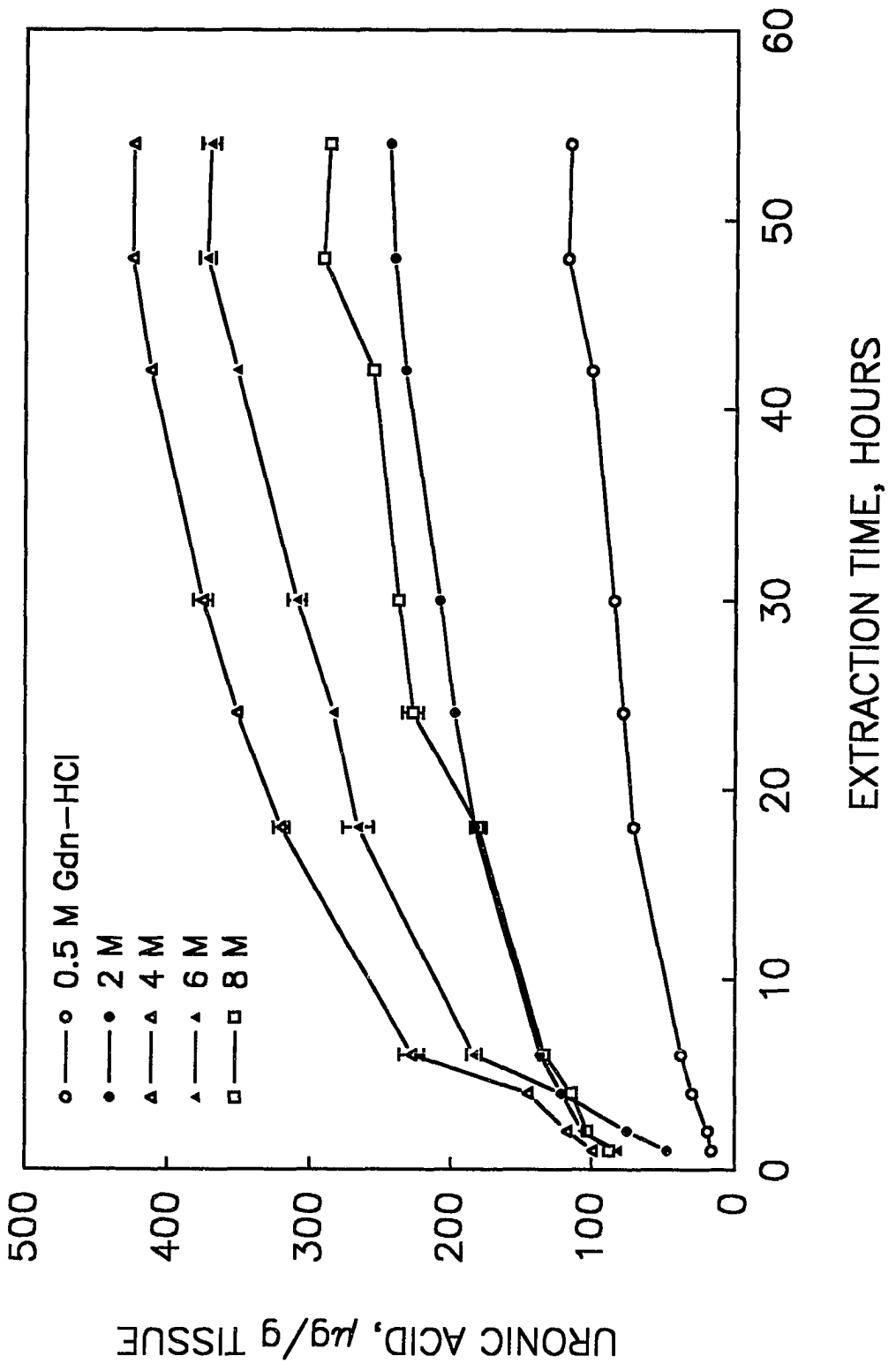


Figure 4. Extraction of proteoglycans from porcine aorta conduit for 48 hours in increasing concentrations of Gdn-HCl.

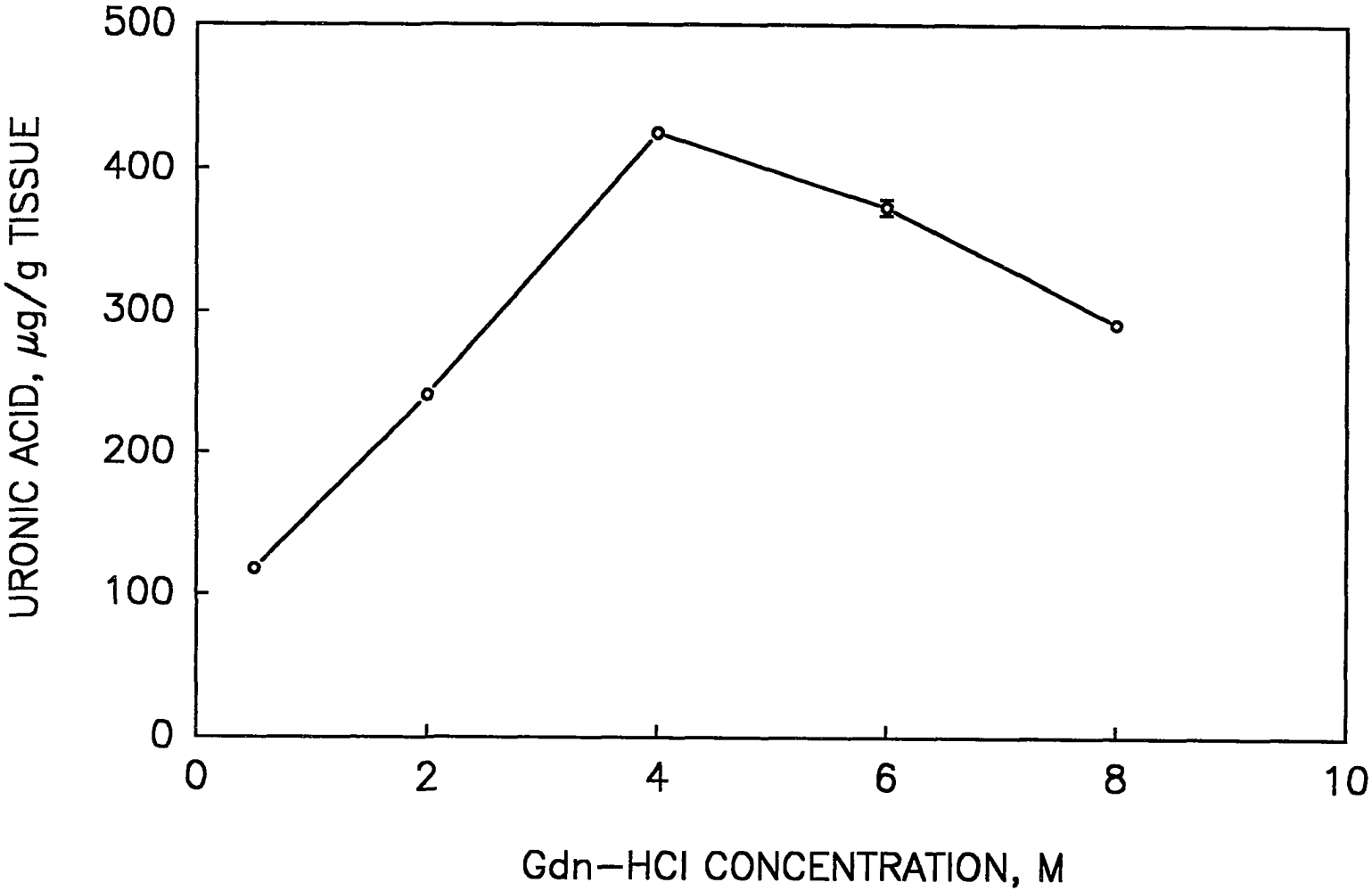
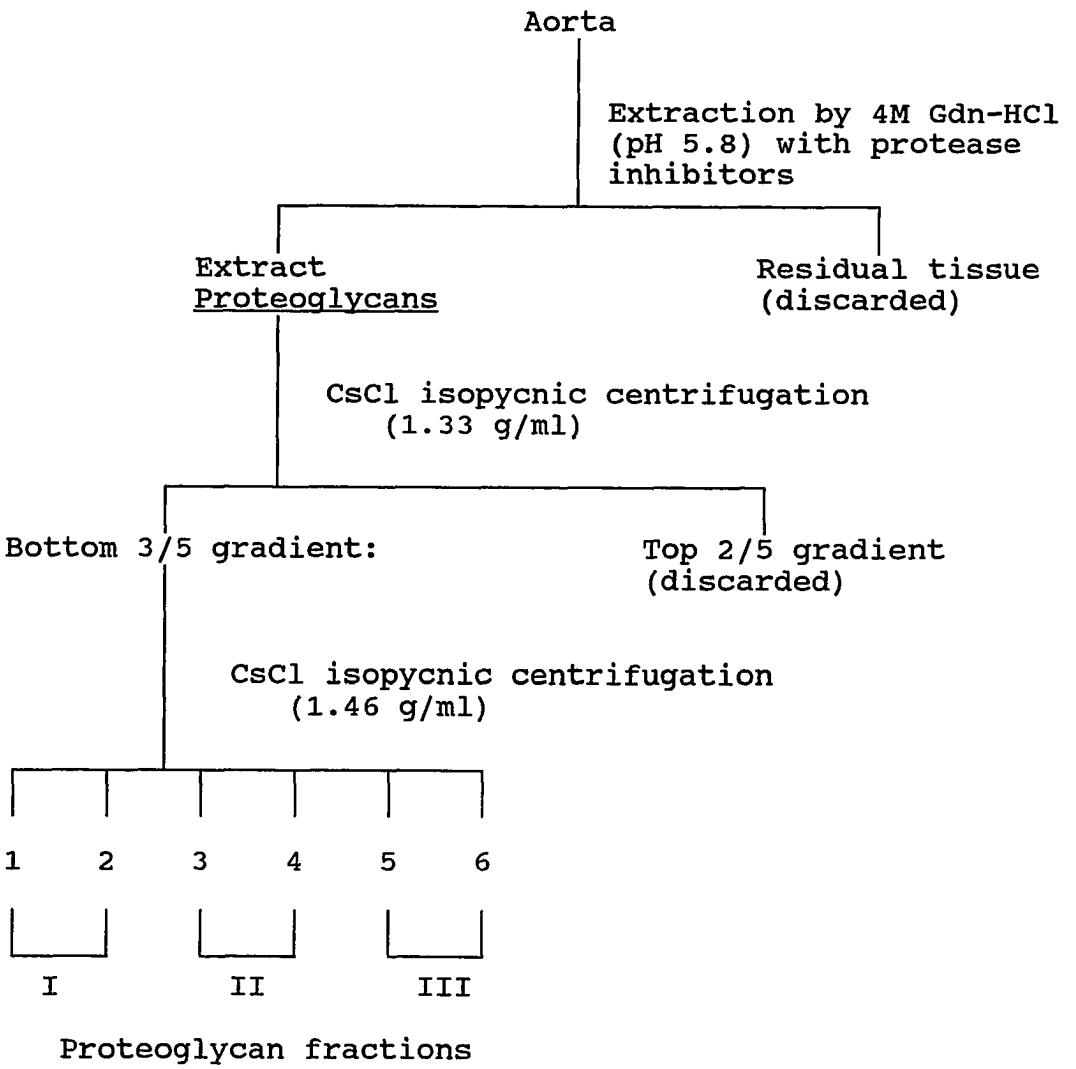


Figure 5. Experimental scheme of the extraction and isolation of proteoglycans from fresh and cryopreserved porcine aorta tissue under dissociative conditions.



tissue (representing about 47 % of total uronate of the tissue) in fresh tissue and 470 μg uronate/g wet tissue (representing about 45 % of total uronate of the tissue) in cryopreserved tissue (Table 1). More uronate could be extracted by a second 48 hours extraction of the tissue, but the extraction was normally not repeated in order to minimize the presence of degraded proteoglycans in the extract.

Once the proteoglycans were in solution, CsCl density gradient centrifugation was used to purify proteoglycans from other macromolecules in the extract and to begin to separate them from each other. The advantage of this method is that CsCl can be added directly to the clarified tissue extract (direct dissociative gradient). It is, therefore, easy to process large volumes of the extract, and the proteoglycans remain in dissociative conditions that should prevent hydrolytic degradation by associated enzymes. Since proteoglycan contents in aorta are small, it is necessary to keep the proteoglycans in dissociative conditions until some partial purification is achieved. Dialysis of extracts of aorta into associative solvents may lead to formation of intractable coacervates.

Proteins usually have partial specific volumes greater than 0.75 ml/g and thus should have buoyant densities less than 1.35 g/ml (Eyring and Yang, 1968;

Table 1. Uronate recovered in the bottom three-fifths of the gradient after the initial ultracentrifugation

	Total UA[a] (μg/g wet tissue)	UA in bottom three fractions (% of total UA)
Fresh	460 $\pm$ 5.7	92.6 $\pm$ 1.7
Cryopreserved	470 $\pm$ 34.4	93.5 $\pm$ 0.6

[a] UA, uronic acid.

Values represent the mean $\pm$ standard error, n = 3.

Luscombe and Phelps, 1967). Glycosaminoglycans, which have partial specific volumes in the range of 0.5 - 0.65 g/ml (Cox and Schumaker, 1961; Ifft and Vinograd, 1962), have buoyant densities of 1.6 - 2.0 g/ml depending on the nature of the supporting solvent. Proteoglycans (proteinpolysaccharide), with partial specific volumes between those of pure protein and glycosaminoglycan, should have buoyant densities in the range of 1.35 - 1.6 g/ml. Because of these differences in density, isopycnic density gradient sedimentation should allow efficient separations of proteins, proteoglycans, and glycosaminoglycans from each other. Degradation of proteoglycan molecules is minimized with this method since degradative enzymes, which are often present in tissue extracts, readily sediment away from the more dense carbohydrate-containing substances.

The proteoglycans from aorta often have lower average buoyant densities than the majority of the population of cartilage proteoglycans, and thus lower initial densities in the dissociative density gradient (1.33 g/ml) were used in the initial centrifugation of extracted proteoglycans. The purification step of CsCl isopycnic centrifugation at a density of 1.33 g/ml in the isolation procedure (Figure 5) removed most of the proteinaceous material from the extract by distributing it to the top of the gradient, which otherwise would have interfered in later fractionation procedures. Most of the uronate containing material centrifuged (> 90 %) was recovered in the bottom three-fifths of the gradients

with fresh and cryopreserved tissue preparations (Table 1). The density range of the gradient formed after the first ultracentrifugation is not, however, sufficient for the optimal separation of proteoglycans from aorta. A further saparation of proteoglycan was therefore performed with the pooled bottom three fractions by a second dissociative isopycnic centrifugation at a beginning density of 1.46 g/ml with density adjusted by adding solid CsCl. There were density variations (between 1.36 g/ml to 1.58 g/ml) in the gradients after the second CsCl isopycnic ultracentrifugation with fresh and cryopreserved tissues (Figures 6 and 7).

C. Characterization of Extracted Proteoglycans.

1. Quantitative Analysis of Proteoglycans.

Ultracentrifugation profiles from the second centrifugation of proteoglycans in Gdn-HCl extracts from fresh tissue is illustrated in Figure 6 and from cryopreserved tissue in Figure 7. The amounts of uronic acid in proteoglycans from fresh and cryopreserved aorta tissues were calculated using a standard curve from the carbazole reaction assay (glucuronolactone as a standard) where the slope was derived from a linear regression analysis of the data (Figure 8). The protein contents in proteoglycans from the fresh and

Figure 6. Ultracentrifugation profiles of proteoglycans from fresh porcine aorta conduit tissue. Ultracentrifugation was carried out under dissociative conditions (4 M Gdn-HCl) in CsCl at an initial density of 1.46 g/ml. Six fractions were collected starting from the bottom of the centrifuge tube and analyzed for uronic acid and protein. Based on analyses, proteoglycans were pooled into three fractions I, II, and III.

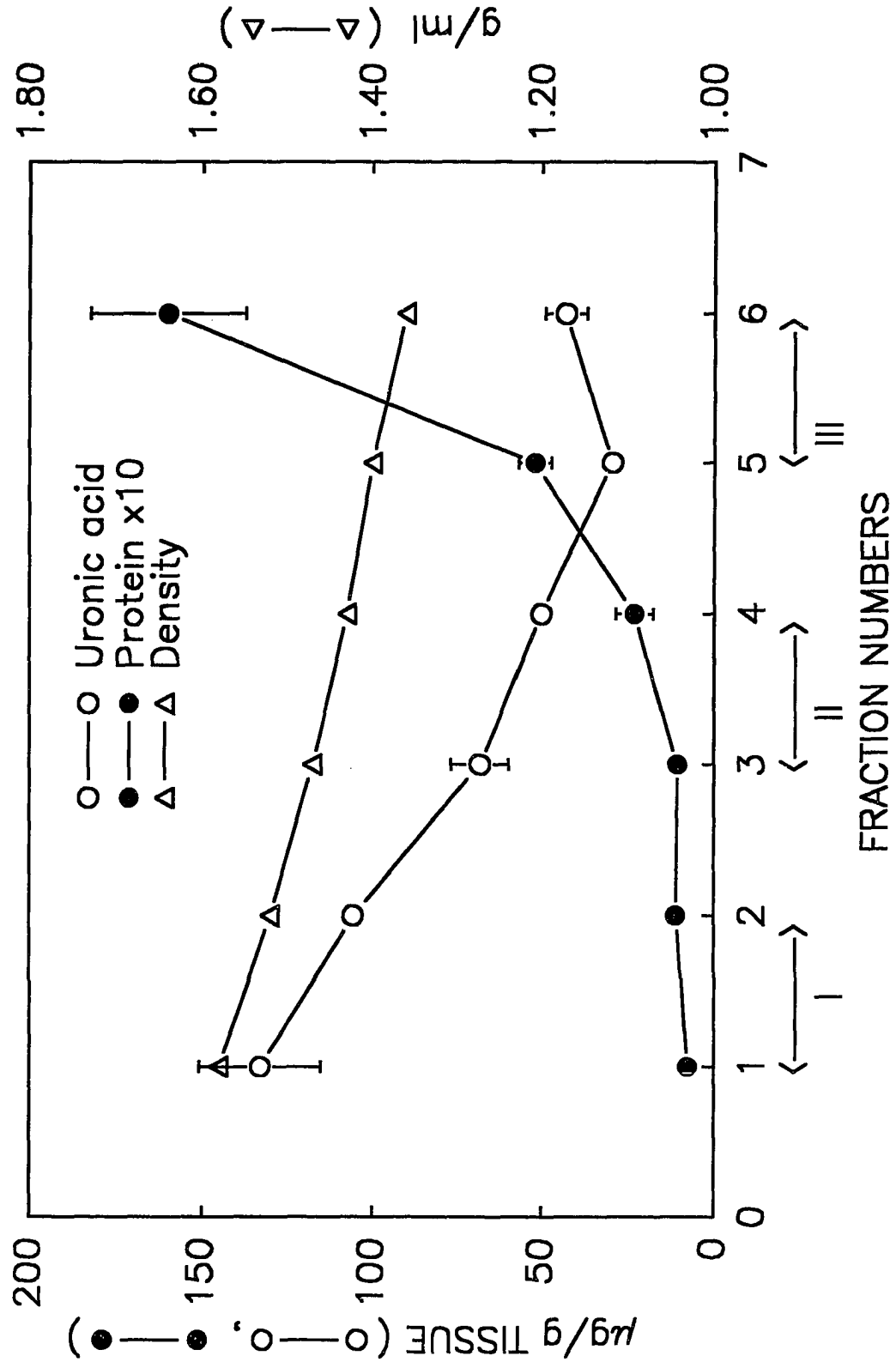


Figure 7. Ultracentrifugation profiles of proteoglycans from cryopreserved porcine aorta conduit tissue. Ultracentrifugation was carried out under dissociative conditions (4 M Gdn-HCl) in CsCl at an initial density of 1.46 g/ml. Six fractions were collected starting from the bottom of the centrifuge tube and analyzed for uronic acid and protein. Based on analyses, proteoglycans were pooled into three fractions I, II, and III.

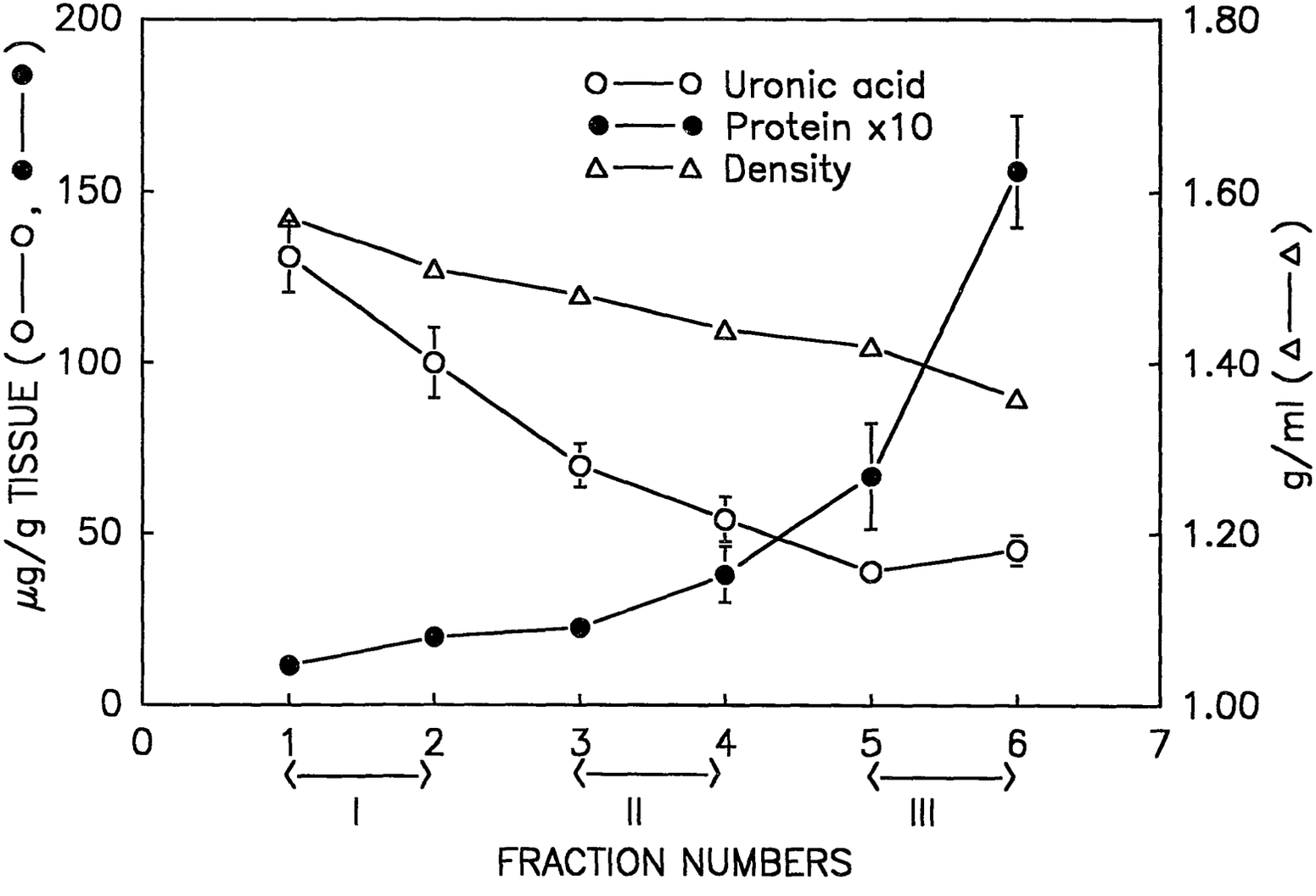
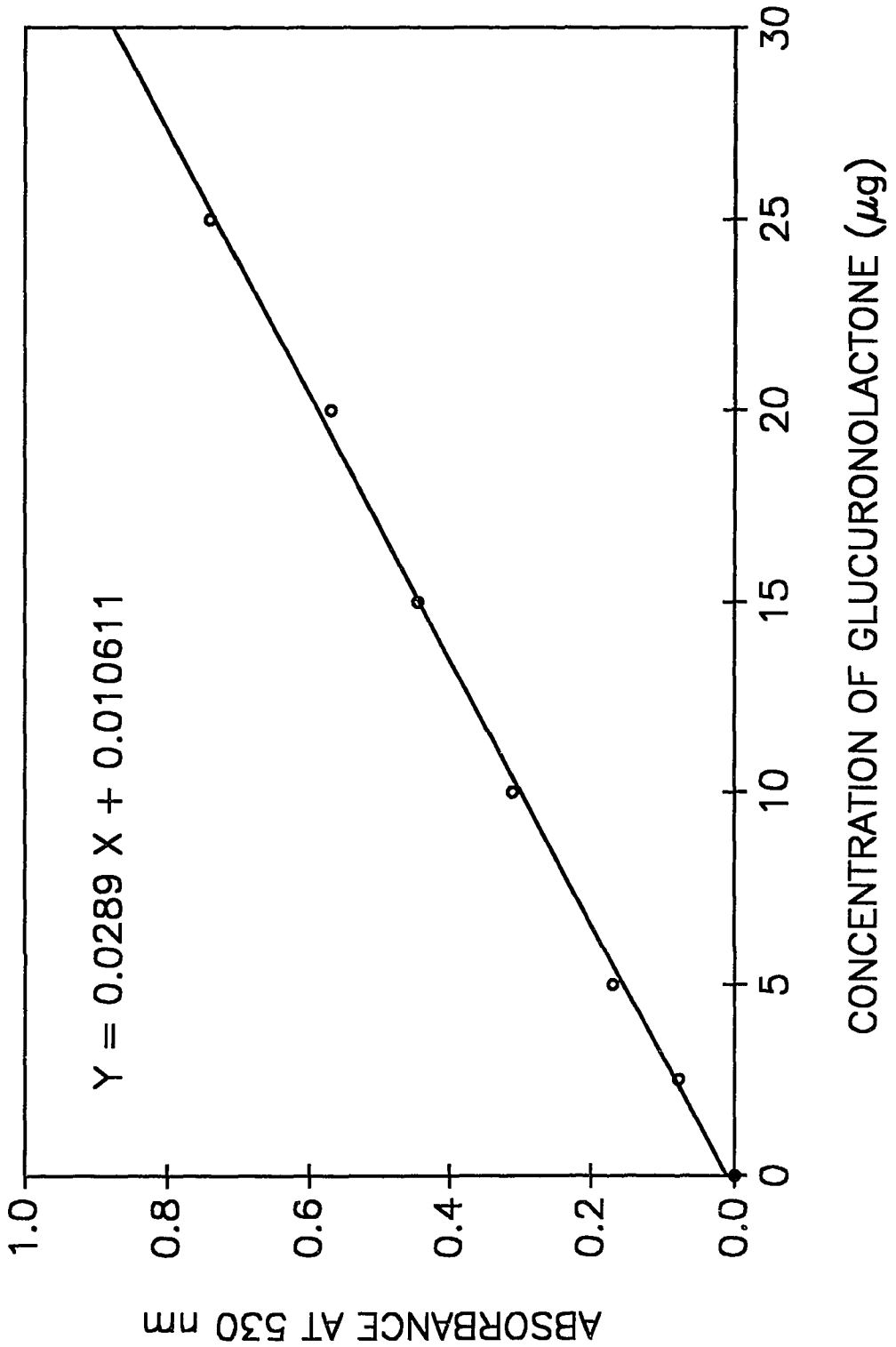


Figure 8. Linear regression analysis for the standard curve of the uronic acid assay with glucuronolactone as a standard. Glucuronolactone in sulfuric acid reagent (0.025 M sodium tetraborate in sulfuric acid) was reacted with carbazole reagent (0.125 % carbazole in absolute ethanol) in boiling water and the absorbance at 530 nm was determined.



cryopreserved tissues were quantitated using a standard curve from the Lowry protein assay with bovine serum albumin as a standard (Figure 9). There was no difference in the dissociative CsCl isopycnic centrifugation profiles of proteoglycans between fresh and cryopreserved porcine aorta tissue (Figures 6 and 7).

Based on the uronic acid and protein profiles, the ultracentrifugation fractions were pooled into three proteoglycan fractions, I, II, and III, as shown in Figures 6 and 7. In extracts from fresh and cryopreserved tissues, fraction I had the highest uronate concentrations (238.46 $\pm$ 20.68 μg uronate/g tissue with fresh tissue and 231.28 $\pm$ 20.78 μg uronate/g tissue with cryopreserved tissue) and fraction III had the lowest (72.38 $\pm$ 9.98 μg uronate/g tissue with fresh tissue and 84.18 $\pm$ 7.76 μg uronate/g tissue with cryopreserved tissue). Fraction III had the highest protein concentration, consistant with the buoyant density characteristic, of the CsCl gradient. Most of the proteoglycan material (83 % of total uronic acid in fresh tissue and 81 % of total uronic acid in cryopreserved tissue) sedimented in the bottom I and II fractions (density $>$ 1.43 g/ml in fresh tissue and density $>$ 1.44 g/ml in cryopreserved tissue). The total uronic acid ($P > 0.05$) and protein contents of proteoglycans ($P > 0.05$) extracted from fresh and cryopreserved porcine aorta tissues were not statistically significantly different (Figure 10).

Figure 9. Linear regression analysis for the standard curve of the Lowry protein assay with bovine serum albumin (BSA) as a standard. Bovine serum albumin in reagent A (2 % sodium carbonate, 2 % potassium tartarate, 1 % cupric sulfate) was reacted with Folin phenol reagent and the absorbance at 500 nm was determined.

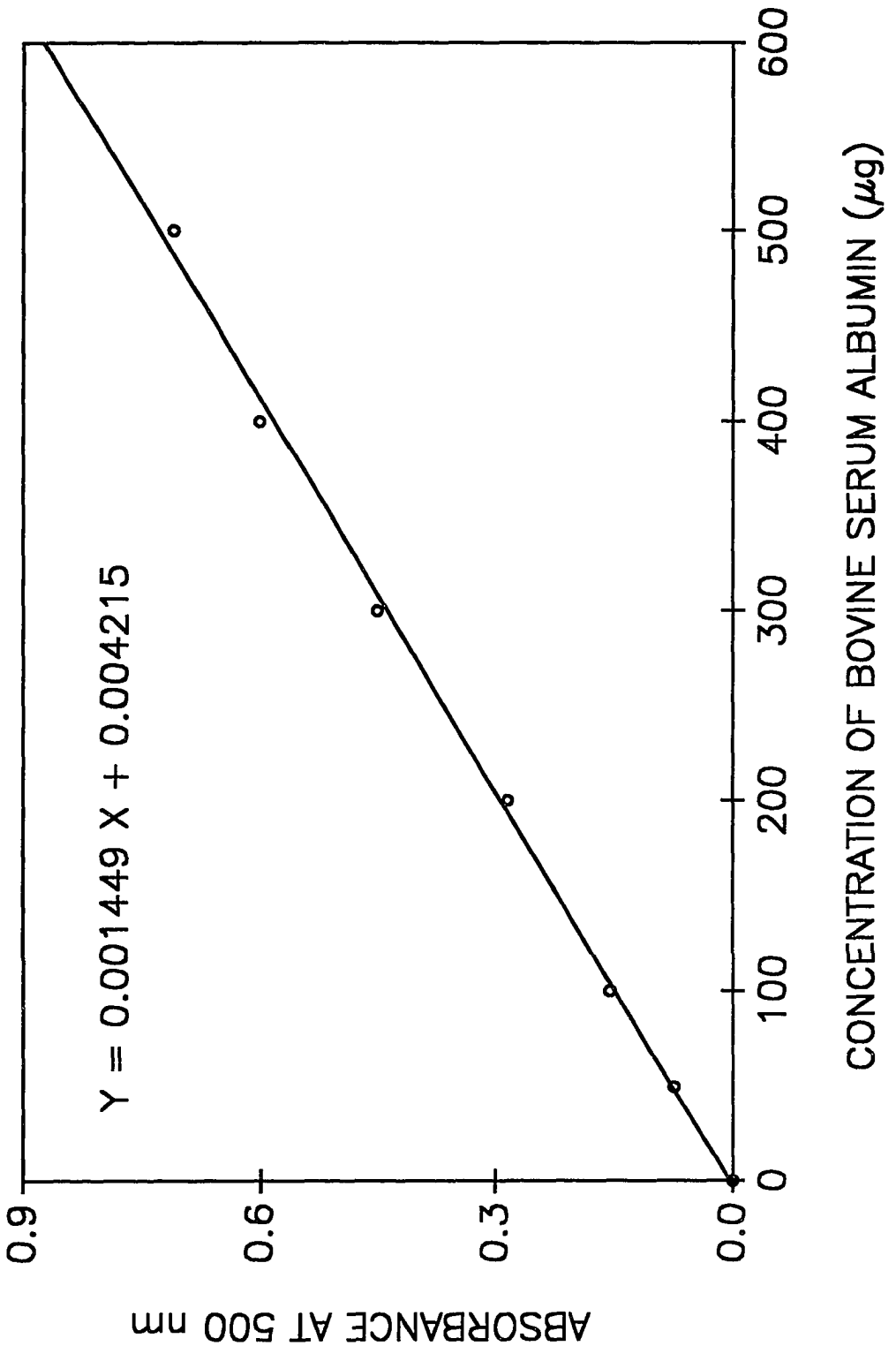
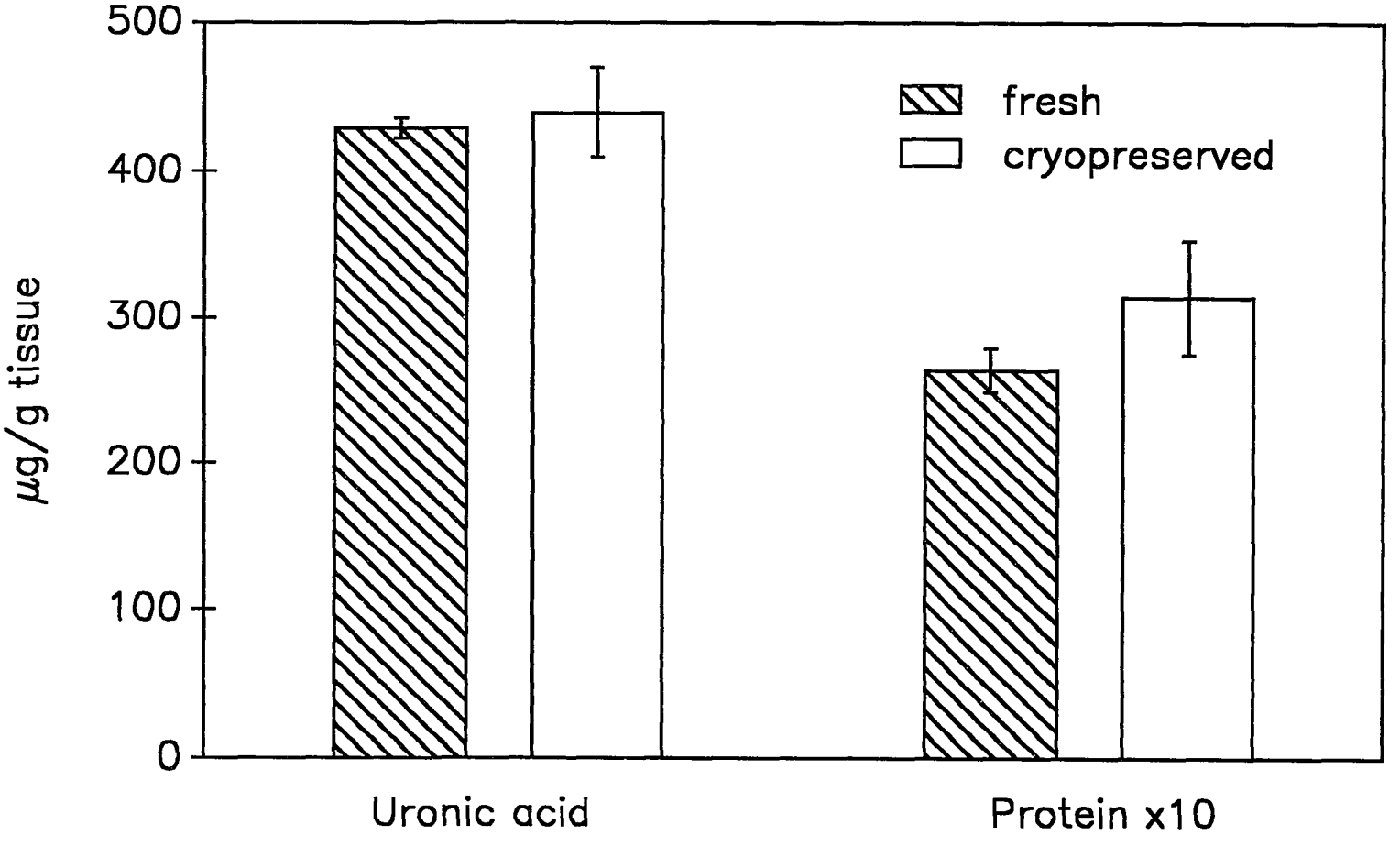


Figure 10. Total concentrations of uronic acid and protein contents from proteoglycans, after the second ultracentrifugation, in fresh versus cryopreserved aorta conduit tissue. Data shown are mean values with error bars indicating the standard error of the mean (n = 3).



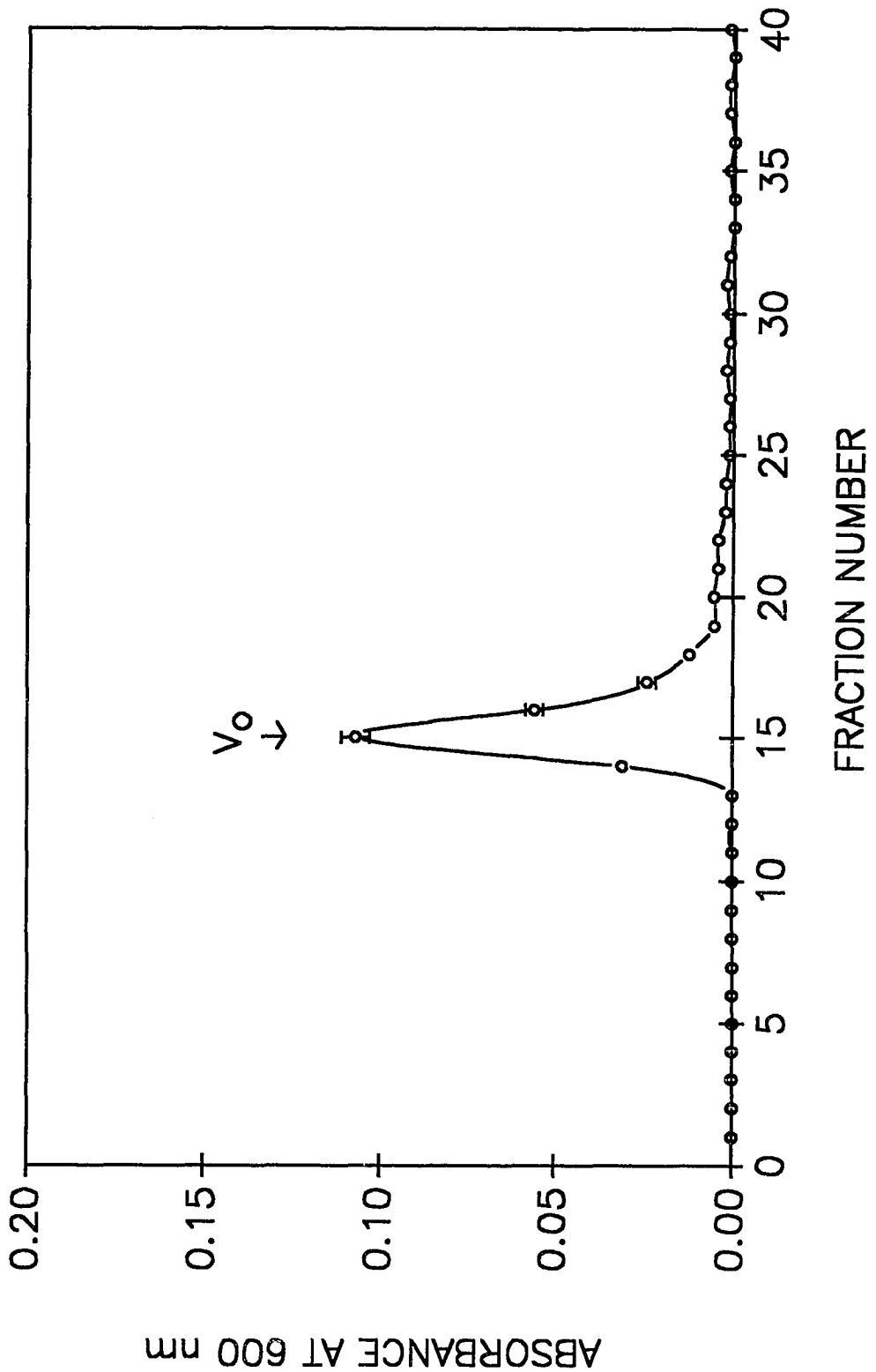
2. Gel Permeation Chromatography Studies.

Gel permeation chromatography is one of the most widely applied analytical procedures for identifying and characterizing proteoglycans. This technique separates groups of proteoglycans containing multiple components and is suitable for obtaining information on the size (hydrodynamic volume) distribution of proteoglycans. The general considerations for choice of an appropriate procedure are the hydrodynamic range and resolving capacity of the support, the capacity of the support, time for elution, recovery of solute from column, and the kinds of analytical procedures to be performed on the eluent solutions.

(a) Determination of Void Volume (V_o) and Inclusion Volume (V_i).

Void volume (V_o) is the total volume surrounding the gel particles in a packed column. The value is determined by measuring the volume of solvent required to elute a solute that is completely excluded from the gel matrix. Most columns can be calibrated for void volume using blue dextran, which has an average molecular weight of 2,000,000. The void volume of the Sepharose CL-4B column (1.5 x 100 cm) used in the present study was determined to be 52.5 ml using Escherichia coli (measuring for turbidity at 600 nm) (Figure 11)

Figure 11. Gel permeation chromatography of Escherichia coli on a Sepharose CL-4B to calibrate the void volume (V_o) of the column. Samples were eluted with 4 M Gdn-HCl/0.05 M sodium acetate buffer, pH 5.8. Fractions were collected and monitored for absorbance at 600 nm.



or using blue dextran by measuring for absorbance at 620 nm (Figure 12). Blue dextran was less suitable for use in determination of void volume than Escherichia coli because it appeared to be partially retained in later fraction of column eluent (Figure 12).

The inclusion volume (V_i) of a column denotes the inner volume of the gel bed and represents that volume accessible in the bed particles to very small molecular weight solutes. The inclusion volume of the Sepharose CL-4B column (1.5 x 100cm) was determined to be 157.5 ml (Figure 13) with 5, 5' - dithiobis [2-nitrobenzoic acid] (DTNB) by measuring absorption of eluent materials at 280 nm.

Samples (Escherichia coli, blue dextran, and DTNB) for calibration of the column were eluted with 4 M Gdn-HCl / 0.05 M sodium acetate (pH 5.8) at a constant flow rate of 16 ml/hour.

(b) Gel Permeation Chromatography of Proteoglycans.

Figure 14 illustrates the procedure used for characterization of proteoglycans by gel permeation chromatography after purification by CsCl isopycnic ultracentrifugation and concentration by freeze-drying. The cross-

Figure 12. Gel permeation chromatography of blue dextran on a Sepharose CL-4B column for the determination of void volume (V_o). The eluent fractions were measured for absorbance at 620 nm.

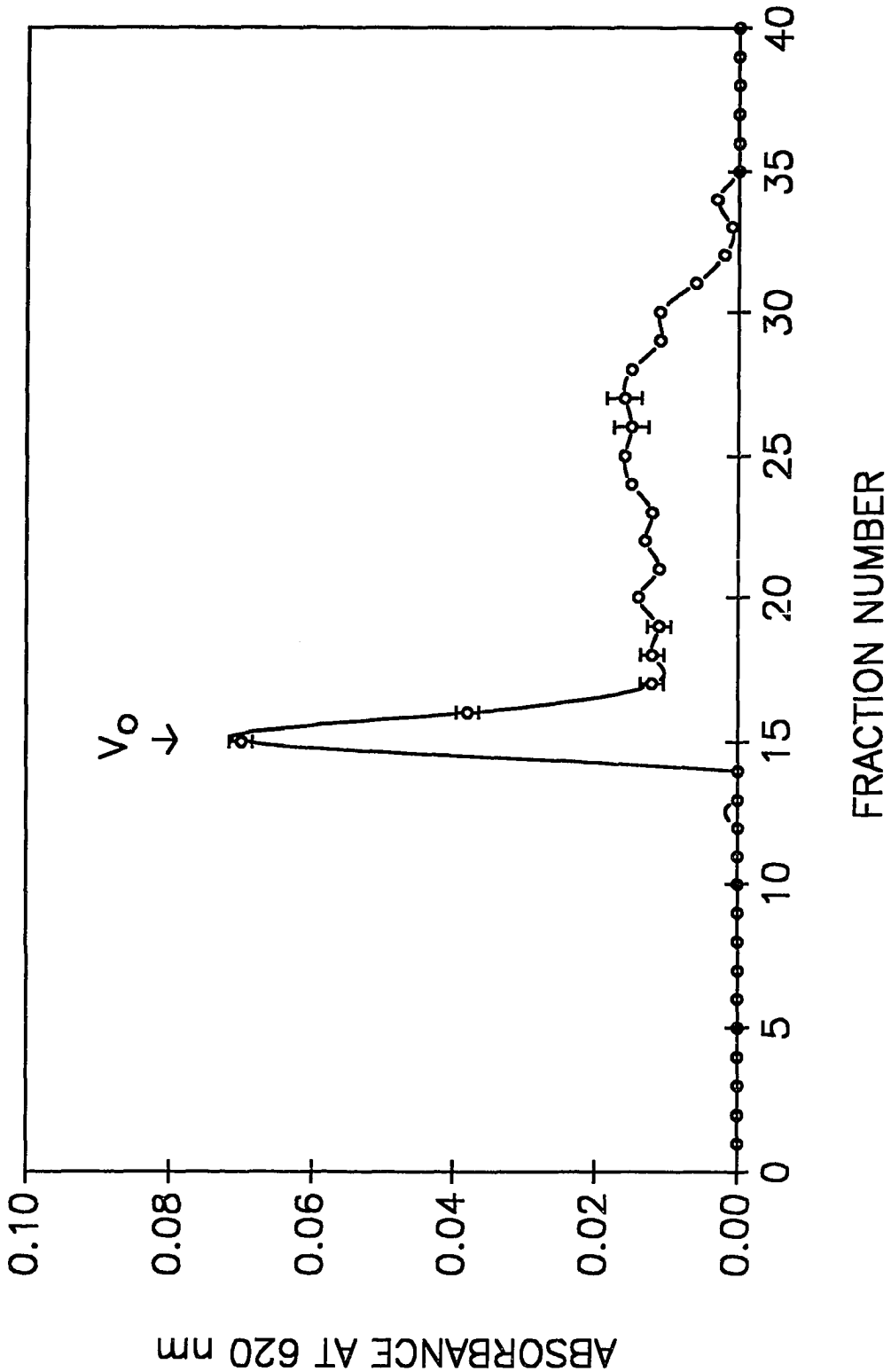


Figure 13. Gel permeation chromatography of 5,5'-dithiobis [2-nitrobenzoic acid] on a Sepharose CL-4B to determine the inclusion volume (V_i) of the column. The eluent was measured for absorbance at 280 nm.

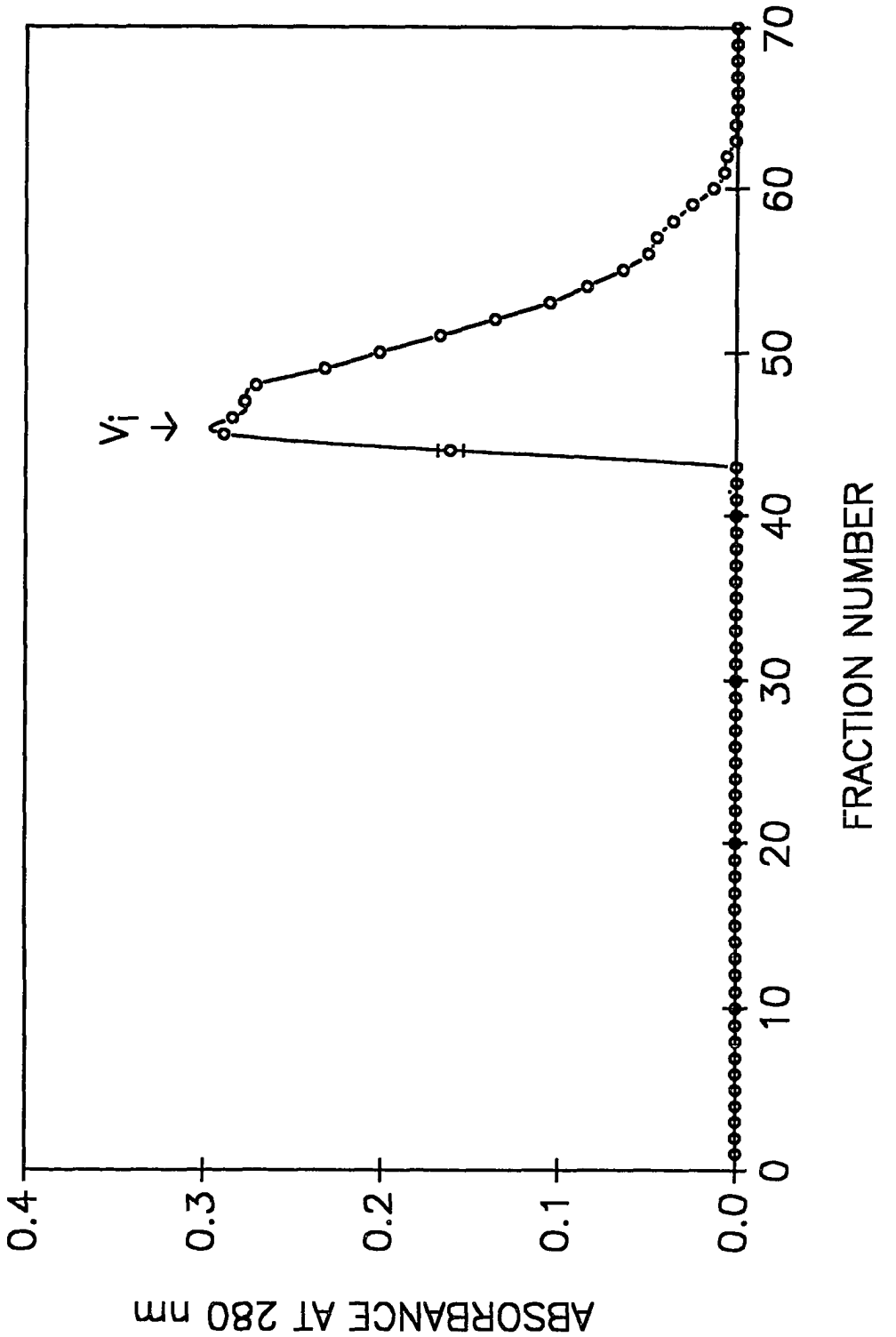
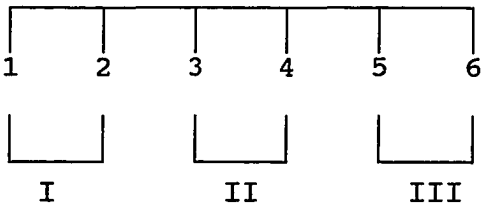


Figure 14. Scheme used for the characterization of proteoglycans from fresh and cryopreserved porcine aorta conduit tissue.



Proteoglycan fractions

Dialysis vs ultra pure water

Dialysate

Freeze-Dry

Concentrated proteoglycans

in 4M Gdn-HCl/0.05 M sodium acetate, pH 5.8

Sepharose CL-4B

chromatography

PG I: K_{av} = 0.13, 0.50 (fresh)
K_{av} = 0.13, 0.47 (cryopreserved)

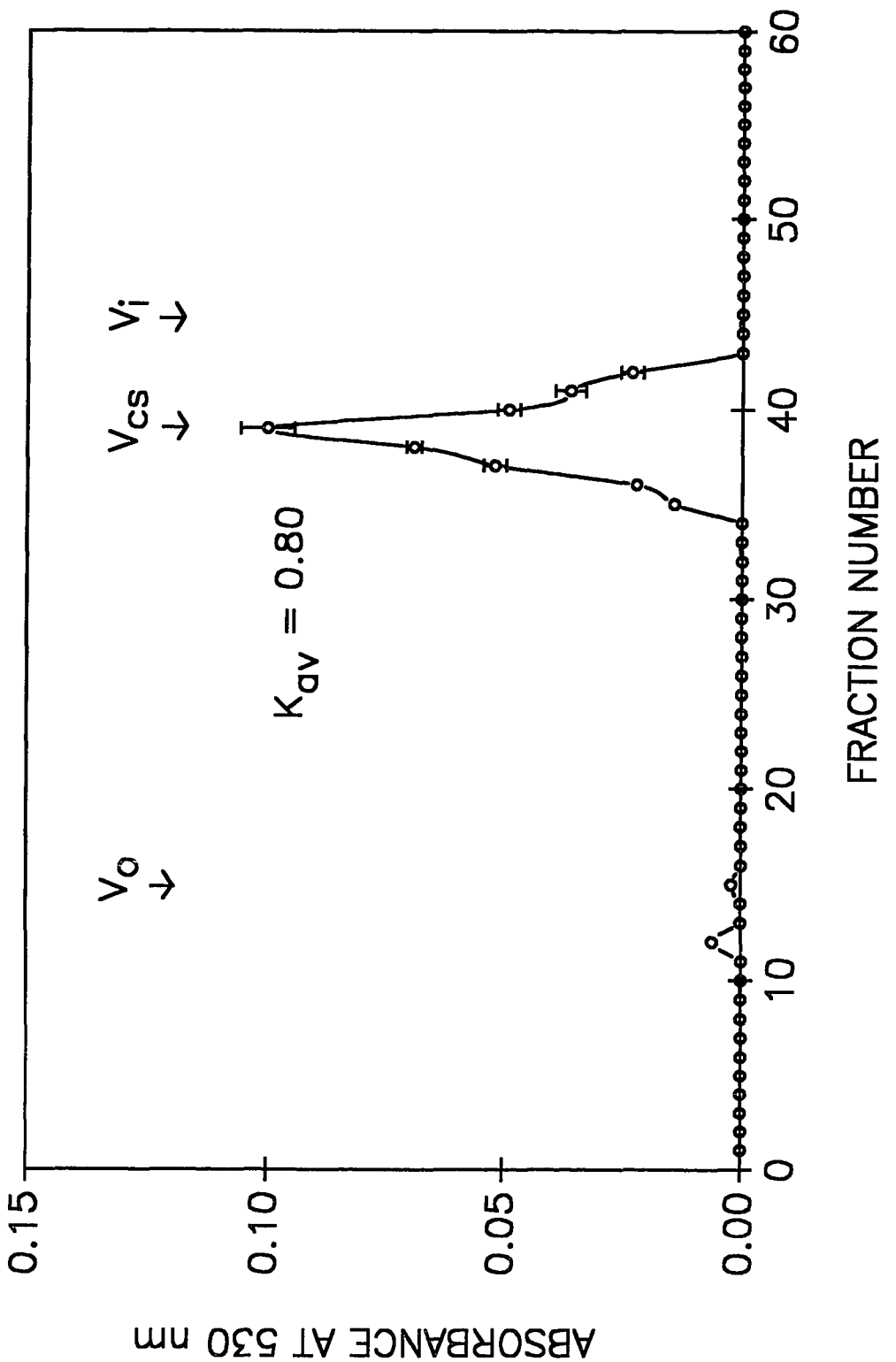
PG II: K_{av} = 0.23 (fresh)
K_{av} = 0.20 (cryopreserved)

PG III: K_{av} = 0.40 (fresh)
K_{av} = 0.43 (cryopreserved)

linked Sepharose 4B support was used since Sepharose CL-4B improves separation of small proteoglycans. To avoid nonspecific aggregation (unwanted interactions), elution was performed with 4 M Gdn-HCl buffered with 0.05 M sodium acetate, pH 5.8. Column eluent was monitored for absorbance at 280 nm for protein content and analyzed for distribution of uronic acid content of the fractions using the carbazole assay procedure. Since no appropriate proteoglycans with known molecular weights were available, it was not possible to determine the absolute molecular weights of these proteoglycans. Chondroitin sulfate, used as a standard glycosaminoglycan, eluted at fraction number 38 (133 ml) for a K_{av} value of 0.80 (Figure 15) and was used to permit evaluation of degradation of proteoglycans into free proteins and glycosaminoglycans.

K_{av} is a definition of the volumetric distribution coefficient for uronic acid-positive and proteinaceous materials. The volumetric distribution coefficient is a physical constant which depends on the molecular size of the solutes (their hydrodynamic volumes) and the inner gel structure (pore size). Its value may vary between 0 and 1. The K_{av} values were calculated from the mean elution volume (V_e) of the proteoglycans using the formula: $K_{av} = V_e - V_o/V_i - V_o$, where V_o = void volume and V_i = inclusion volume of the column, and V_e = elution volume of the proteoglycans. The elution volume

Figure 15. Gel permeation chromatography of chondroitin sulphate on a Sepharose CL-4B. Sample (0.15 mg) was eluted with 4 M Gdn-HCl/0.05 M sodium acetate (pH 5.8) at a constant rate of 16 ml/hour. The eluent was analyzed for uronic acid.



(V_e) is the volume of eluting buffer necessary to remove a particular solute from the packed column. This value depends on the volumetric distribution of the gel bed, the quality of the gel, and the properties of the separated substance. V_e - V_o denotes the net elution volume or the retention volume. This value represents the eluent volume occupied by the solute in the gel phase. If the gel particles do not retain the solute molecules then the retention volume is equal to zero and the substance will elute in the void volume (V_o) of the column.

The elution profiles of proteoglycans in fraction I from the fresh porcine aorta tissue is shown in Figure 16 for uronic acid-positive and proteinaceous materials. The proteoglycans, as the uronic acid-positive material, resolved into two peaks. One hexuronic acid-positive peak occurred near the void volume for a K_{av} of 0.13 and the other peak occurred at a K_{av} of 0.50 (Figure 16). The materials absorbing at 280 nm eluted with a K_{av} of 0.93. All hexuronic acid positive materials were eluted from the column as two peaks without detectable absorption at 280 nm (Figure 16).

The proteoglycans in fraction I from the cryopreserved tissue also resolved into two peaks of uronic acid-positive materials and one peak of proteinaceous materials following gel filtration (Figure 17). One uronic acid-positive peak occurred near the void volume with a K_{av} of 0.13 and smaller

Figure 16. Gel permeation chromatography of proteoglycans in fraction I from fresh porcine aorta conduit tissue. Gel permeation chromatography was performed on Sepharose CL-4B eluting the column with 4 M Gdn-HCl/ 0.05 M sodium acetate buffer, pH 5.8. The column was eluted at a rate of 16 ml/hour, and 3.5 ml fractions were collected. Fractions were analyzed for uronic acid (530 nm) and protein (absorbance 280 nm). The K_{av} values were calculated using the formula: $K_{av} = (V_e - V_o) / (V_i - V_o)$, where V_o = void volume and V_i = inclusion volume of the column, and V_e = elution volume of the peak.

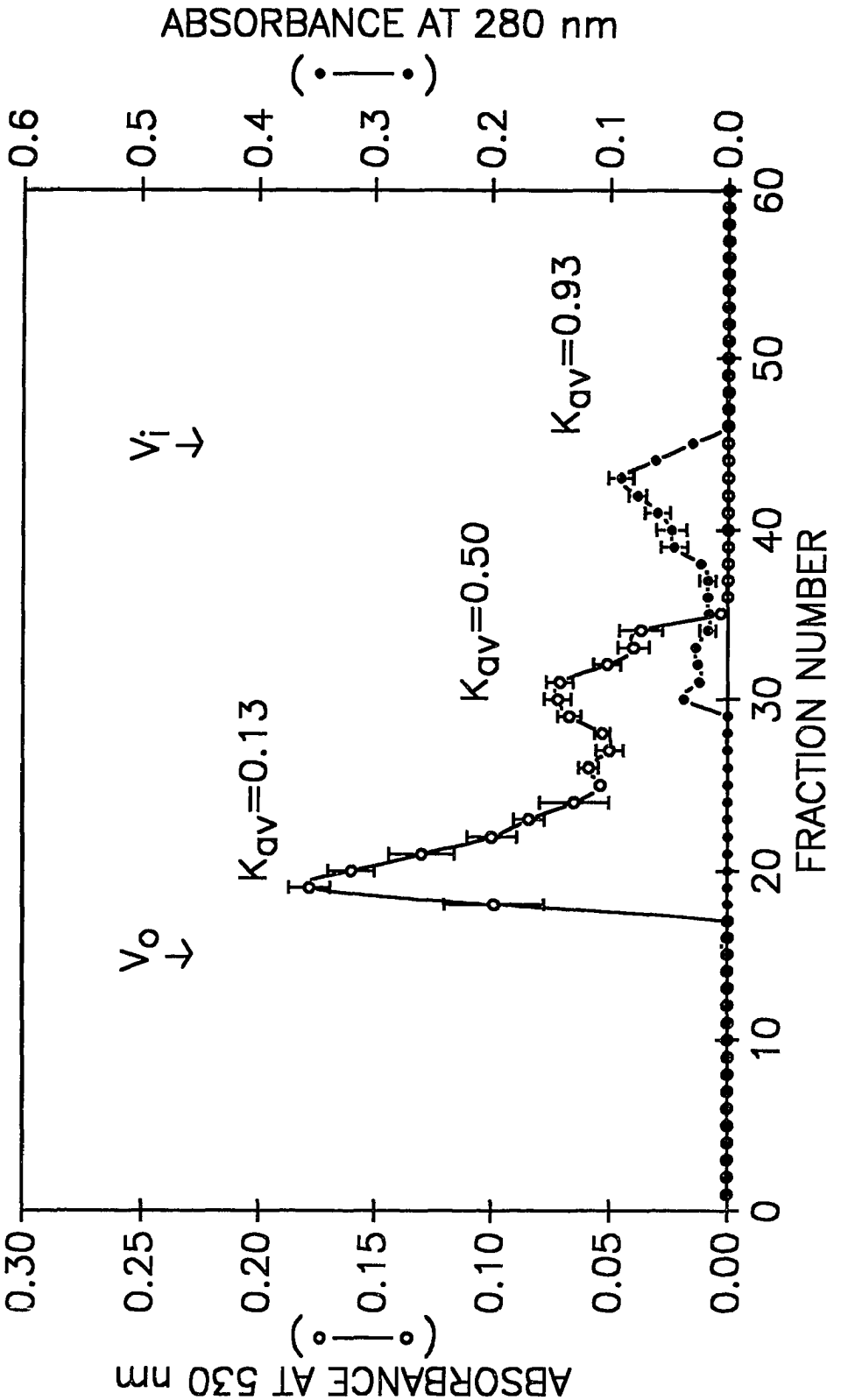
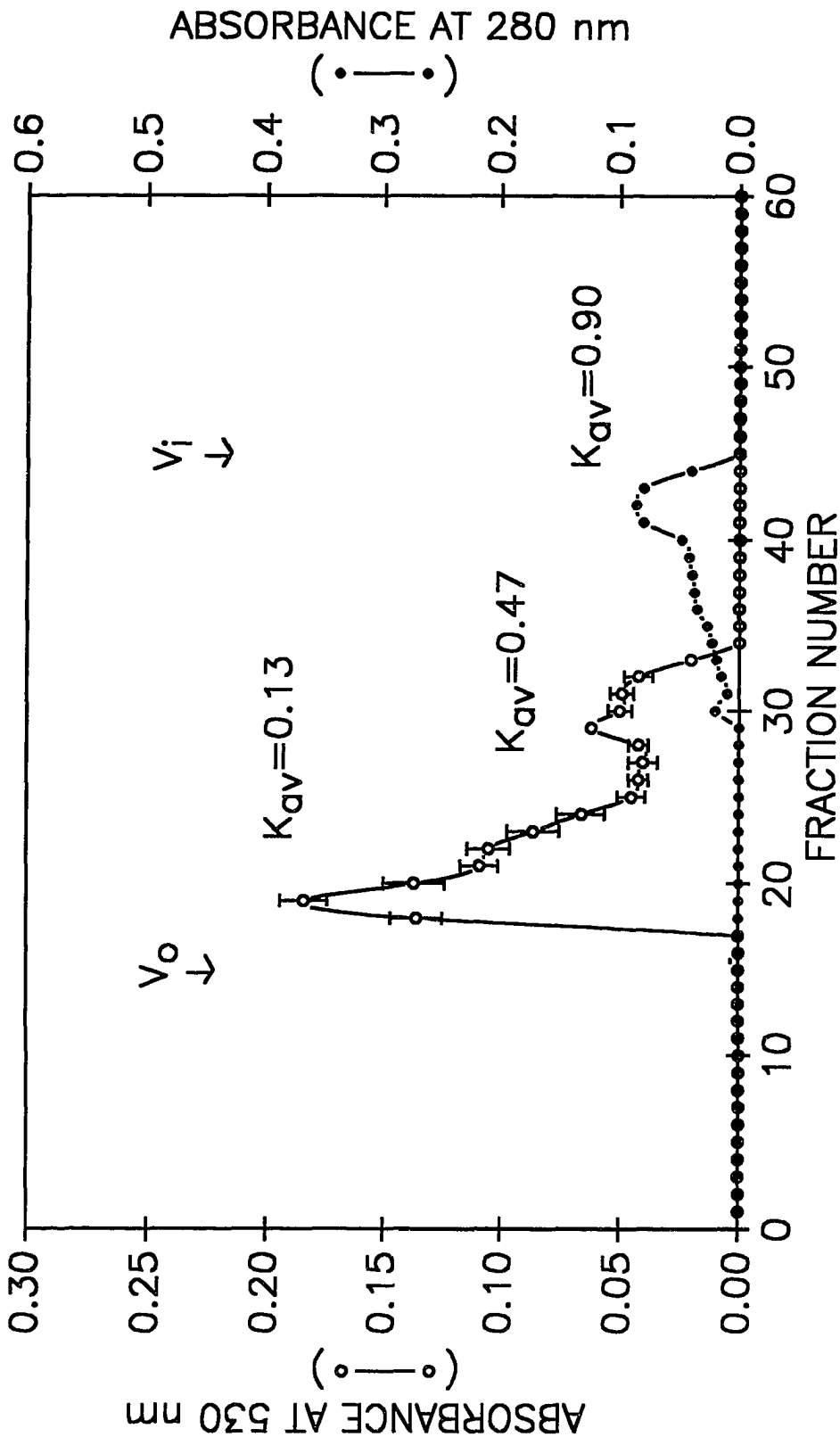


Figure 17. Gel permeation chromatography of proteoglycans in fraction I from cryopreserved porcine aorta conduit tissue. Gel permeation chromatography was performed on Sepharose CL-4B eluting the column with 4 M Gdn-HCl/ 0.05 M sodium acetate buffer, pH 5.8. The column was eluted at a rate of 16 ml/hour, and 3.5 ml fractions were collected. Fractions were analyzed for uronic acid (530 nm) and protein (absorbance 280 nm).



peak occurred at a K_{av} of 0.47. Proteoglycans in fraction I from the cryopreserved tissue was resolved into a single peak of proteinaceous materials with K_{av} of 0.90 (Figure 17). Hexuronic acid positive materials from the proteoglycan fraction I of cryopreserved tissue eluted from the column without detectable absorption at 280 nm and was similar to the elution profile of the proteoglycans in fraction I from fresh tissue.

The chromatographic elution profiles of proteoglycans in fraction II from the fresh porcine aorta tissue are shown in Figure 18 for uronic acid-positive and proteinaceous materials. Hexuronic acid-positive materials present in fraction II from fresh tissue eluted from the column providing for a K_{av} value of 0.23 and was broader than the equivalent peak derived for proteoglycans in fraction I from fresh tissue (Figure 16). Proteoglycans in fraction II from fresh tissue was resolved into two proteinaceous materials peaks with K_{av} of 0.13 and K_{av} of 0.93, but only the larger peak (K_{av} of 0.13) of proteinaceous materials paralleled the uronic acid positive materials present in fraction II (Figure 18).

The proteoglycans in fraction II from cryopreserved aorta tissue was resolved into one peak of uronic acid positive materials and two peaks of proteinaceous materials by gel filtration through Sepharose CL-4B (Figure 19). The K_{av} value of hexuronic acid-positive materials from the cryopreserved

Figure 18. Gel permeation chromatography of proteoglycans in fraction II from fresh porcine aorta conduit tissue. Gel permeation chromatography was performed on Sepharose CL-4B eluting the column with 4 M Gdn-HCl/ 0.05 M sodium acetate buffer, pH 5.8. The column was eluted at a rate of 16 ml/hour, and 3.5 ml fractions were collected. Fractions were analyzed for uronic acid (530 nm) and protein (absorbance 280 nm).

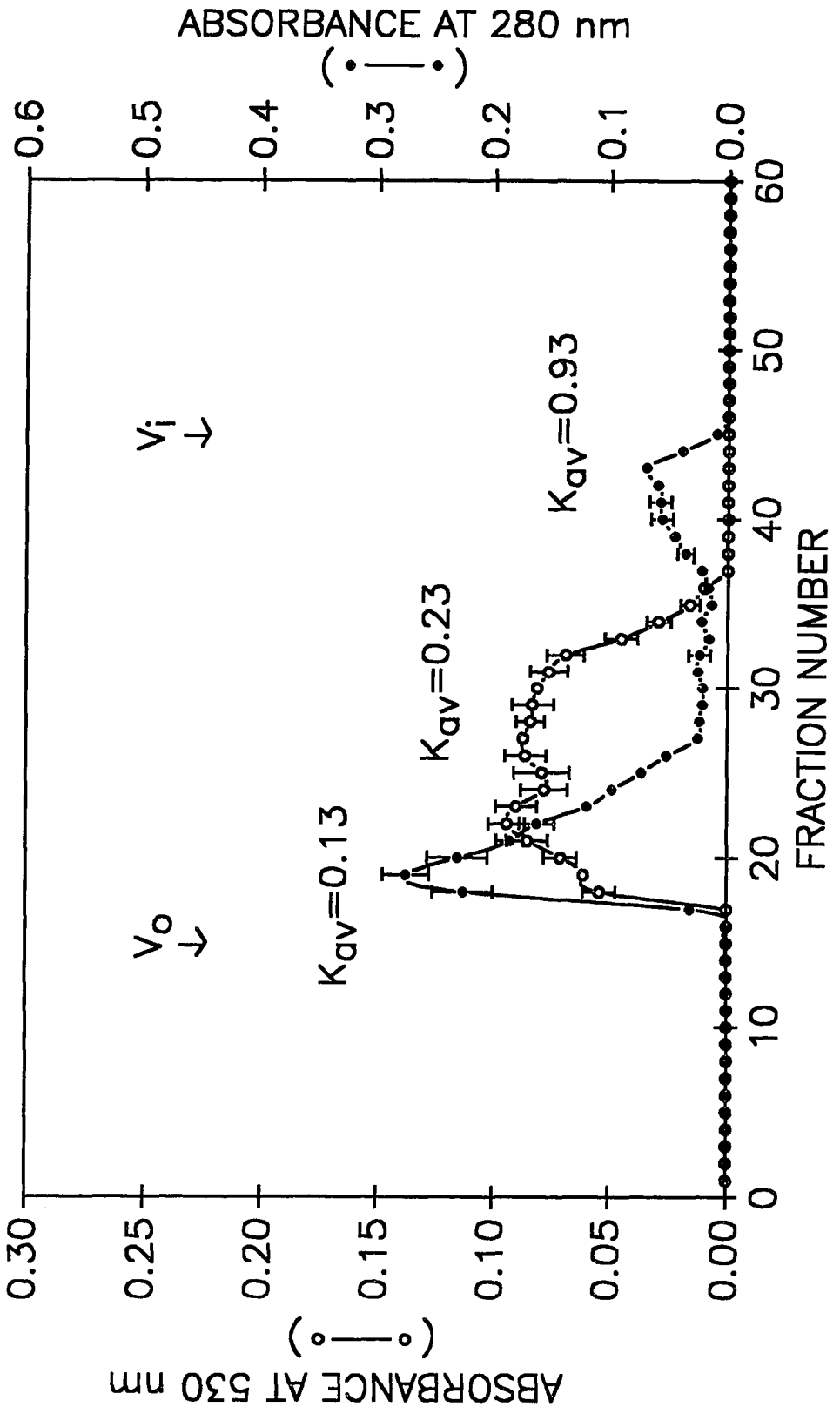
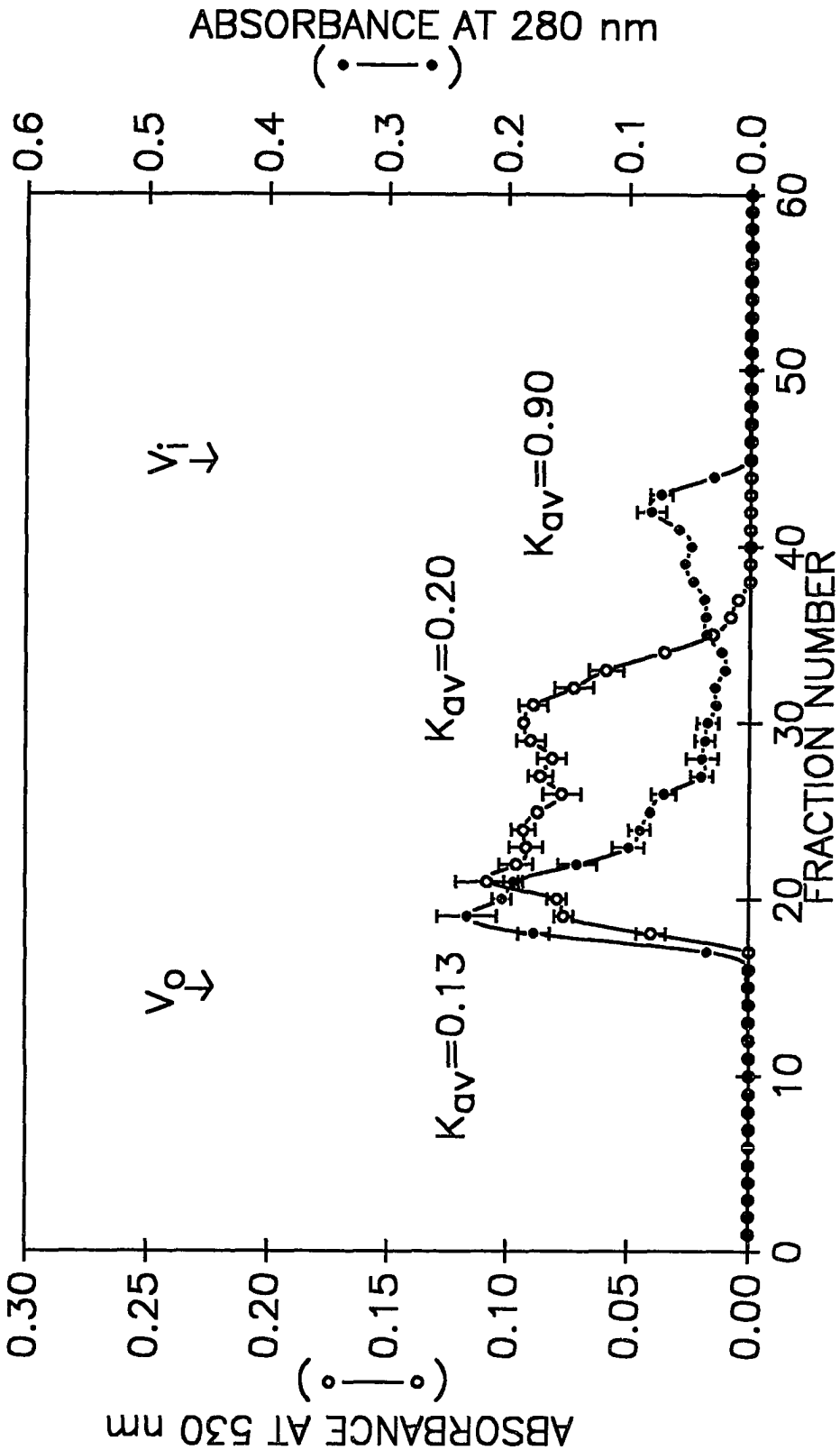


Figure 19. Gel permeation chromatography of proteoglycans in fraction II from cryopreserved porcine aorta conduit tissue. Gel permeation chromatography was performed on Sepharose CL-4B eluting the column with 4 M Gdn-HCl/ 0.05 M sodium acetate buffer, pH 5.8. The column was eluted at a rate of 16 ml/hour, and 3.5 ml fractions were collected. Fractions were analyzed for uronic acid (530 nm) and protein (absorbance 280 nm).



tissue was 0.20. The first peak of proteinaceous materials occurred near the void volume with a K_{av} of 0.13 and the second peak of proteinaceous materials occurred near the inclusion volume with a K_{av} of 0.90 (Figure 19).

Proteoglycans in fraction III from fresh tissue showed the most heterogeneity among the fractions in gel filtration profiles of hexuronic acid-positive materials (Figure 20). Hexuronic acid-positive materials in this fraction provided for a K_{av} value of 0.40 and was broader than similar peaks obtained for fractions I and II from fresh tissues. Materials in fraction III from fresh tissue resolved into two groups of proteinaceous materials (Figure 20). One peak of proteinaceous material eluted from the column near the void volume with a K_{av} of 0.10 and the other eluted near the inclusion volume with a K_{av} of 0.83.

Fraction III from cryopreserved tissue was also chromatographed on the Sepharose CL-4B under dissociative conditions. The uronic acid-positive materials eluted with a K_{av} value of 0.43 (Figure 21) and was also very broad indicating heterogeneity of eluting proteoglycans. The materials absorbing at 280 nm eluted near the void volume as a small peak with K_{av} of 0.10 and at K_{av} 0.80 as a larger peak (Figure 21).

Figure 20. Gel permeation chromatography of proteoglycans in fraction III from fresh porcine aorta conduit tissue. Gel permeation chromatography was performed on Sepharose CL-4B eluting the column with 4 M Gdn-HCl/ 0.05 M sodium acetate buffer, pH 5.8. The column was eluted at a rate of 16 ml/hour, and 3.5 ml fractions were collected. Fractions were analyzed for uronic acid (530 nm) and protein (absorbance 280 nm).

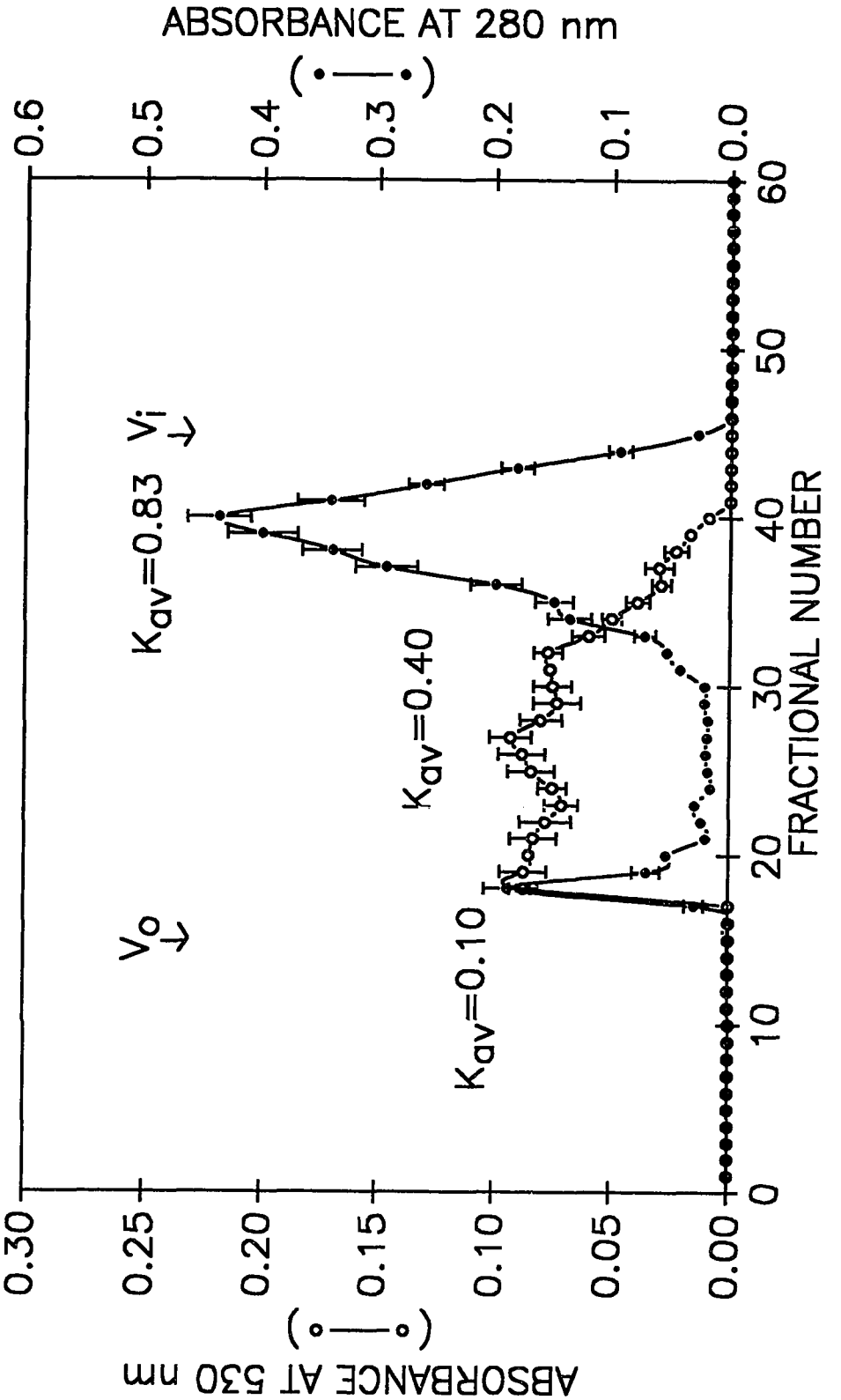
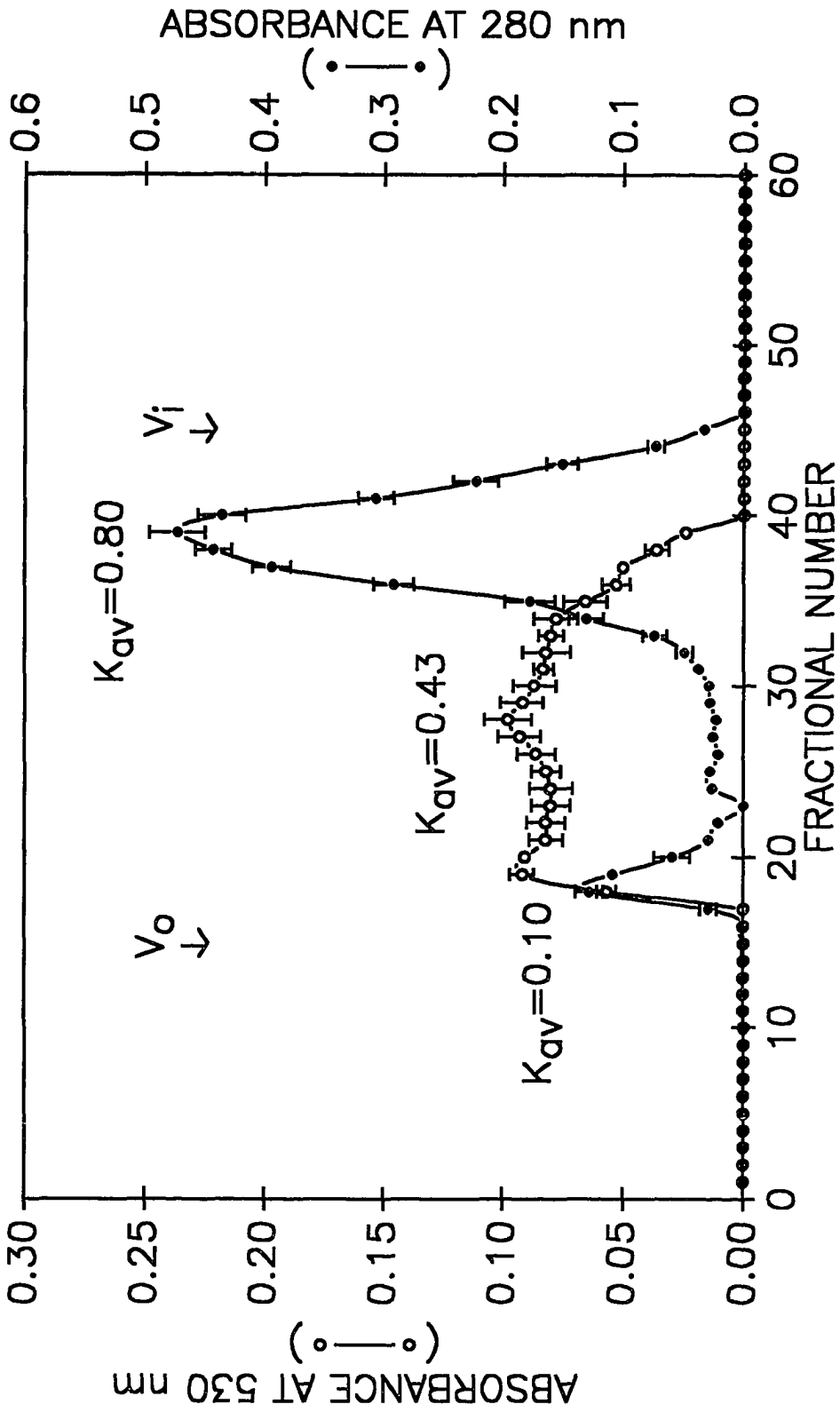


Figure 21. Gel permeation chromatography of proteoglycans in fraction III from cryopreserved porcine aorta conduit tissue. Gel permeation chromatography was performed on Sepharose CL-4B eluting the column with 4 M Gdn-HCl/ 0.05 M sodium acetate buffer, pH 5.8. The column was eluted at a rate of 16 ml/hour, and 3.5 ml fractions were collected. Fractions were analyzed for uronic acid (530 nm) and protein (absorbance 280 nm).



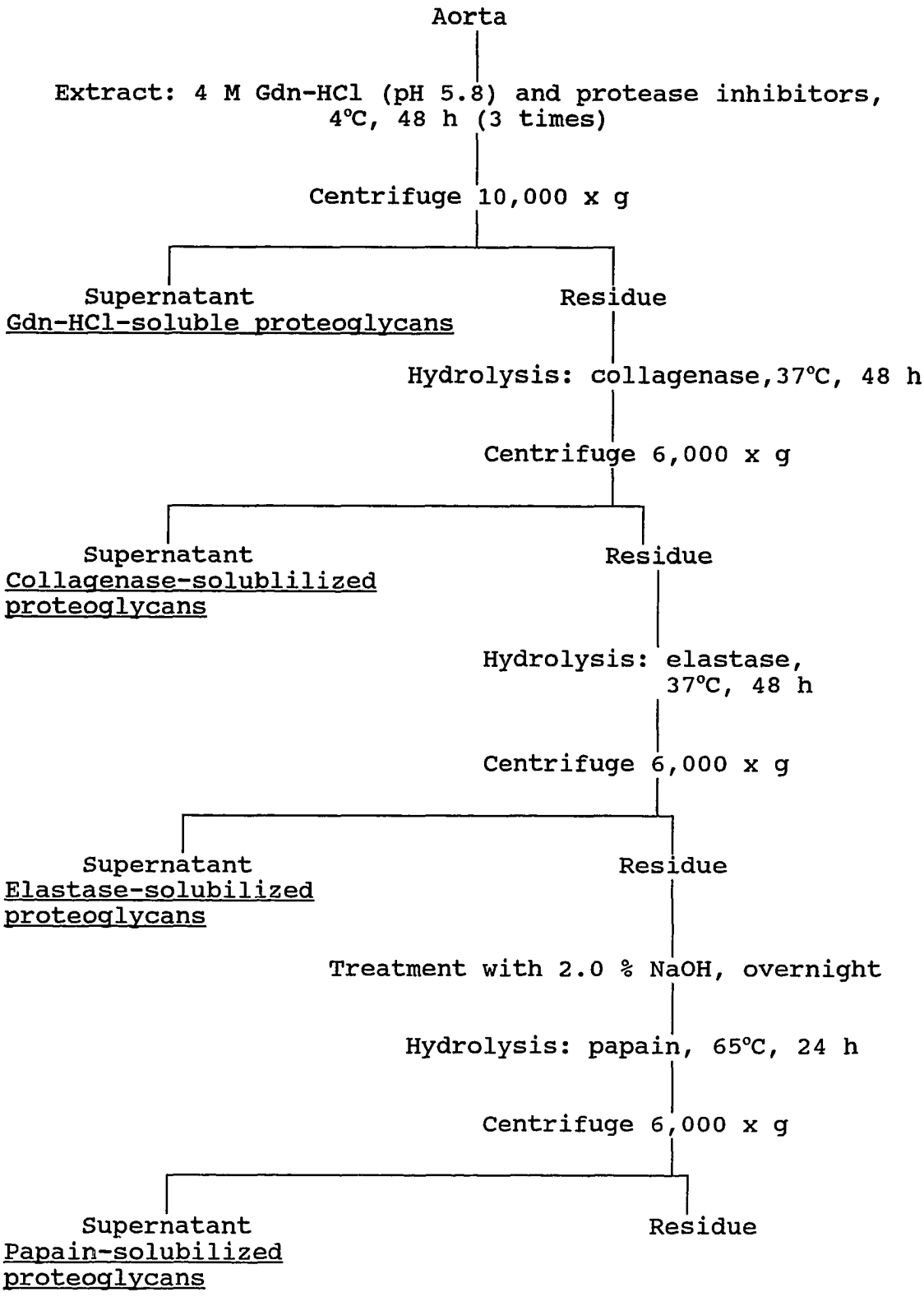
3. Distribution of Proteoglycans in Porcine Aorta.

Some proteoglycans in tissue are present in the soluble matrix while others are associated with other components, i.e. collagen and elastin. Proteoglycans in the soluble matrix could be extracted by use of dissociative solvent (Gdn-HCl) by dissociating most noncovalent interactions between macromolecules. However, the proteoglycans bound to collagen and elastin require solubilization of fibrous components of the tissue for extraction. The quantitative distribution of proteoglycans in porcine aorta was thus studied using sequential extraction of tissue.

The procedure used for extraction of aorta with 4 M Gdn-HCl (three times) and hydrolysis of residual tissue by collagenase, elastase, and papain is shown in Figure 22. Porcine aorta conduit tissue was repeatedly (three times) extracted until no more appreciable quantities of hexuronic acid-positive material was obtained in the extraction solution. The tissue was then digested using enzymes (collagenase, elastase, and papain) to study how much proteoglycans could be released from the residual tissue by these enzymes.

Analysis of initial Gdn-HCl extracts of the fresh and cryopreserved tissues showed that 491 μg and 500 μg of hexuronate /g of tissue were

Figure 22. Procedure for isolation of proteoglycans by sequential extraction of fresh and cryopreserved porcine aorta tissue with Gdn-HCl, collagenase, elastase, and papain.



extracted from fresh and cryopreserved tissues, respectively. Two additional extractions solubilized 73 μg (second extraction) and 40 μg of uronate /g of tissue (third extraction) from fresh tissue and 110 μg (second extraction) and 33 μg of uronate /g of tissue (third extraction) from cryopreserved tissue. Hydrolysis of the Gdn-HCl extracted tissue with collagenase for 48 hours released 125 μg uronate/g tissue from fresh tissue and 211 μg uronate/g tissue from cryopreserved tissue. Elastase digestion of the centrifuged pellet of the collagenase hydrolysis released 154 μg and 110 μg uronate/g tissue from fresh and cryopreserved tissues, respectively. After the digestion of the tissue with elastase a small amount of tissue remained, which was hydrolyzed with 2 % NaOH and papain. The yield of proteoglycan solubilized by papain was 88 μg uronate/g tissue from fresh and 86 μg uronate/g tissue from cryopreserved tissue (Figure 23).

Total uronic acid (as μg of the wet weight of tissue) and uronic acid distribution (as the % of total uronic acid) in the fresh and cryopreserved tissues obtained by sequential extractions of the tissues are reported in Table 2. There was no significant difference in total uronate between fresh and cryopreserved tissues. Based on analysis of the total uronate, after 48 hours of extraction with 4 M Gdn-HCl, 50.6 % (in fresh tissue) and 47.7 % (in cryopreserved tissue) of total tissue hexuronic acid content was solubilized.

Figure 23. Uronic acid analysis of proteoglycans from sequential extractions of fresh and cryopreserved porcine aorta conduit tissue. The tissue was sequentially extracted by 4 M Gdn-HCl (3X) and then digested with collagenase, elastase, and papain. Bars represent standard error of mean (n = 3). (a) Gdn-HCl, 1st, (b) Gdn-HCl, 2nd, (c) Gdn-HCl, 3rd, (d) collagenase solubilized, (e) elastase solubilized, (f) papain solubilized.

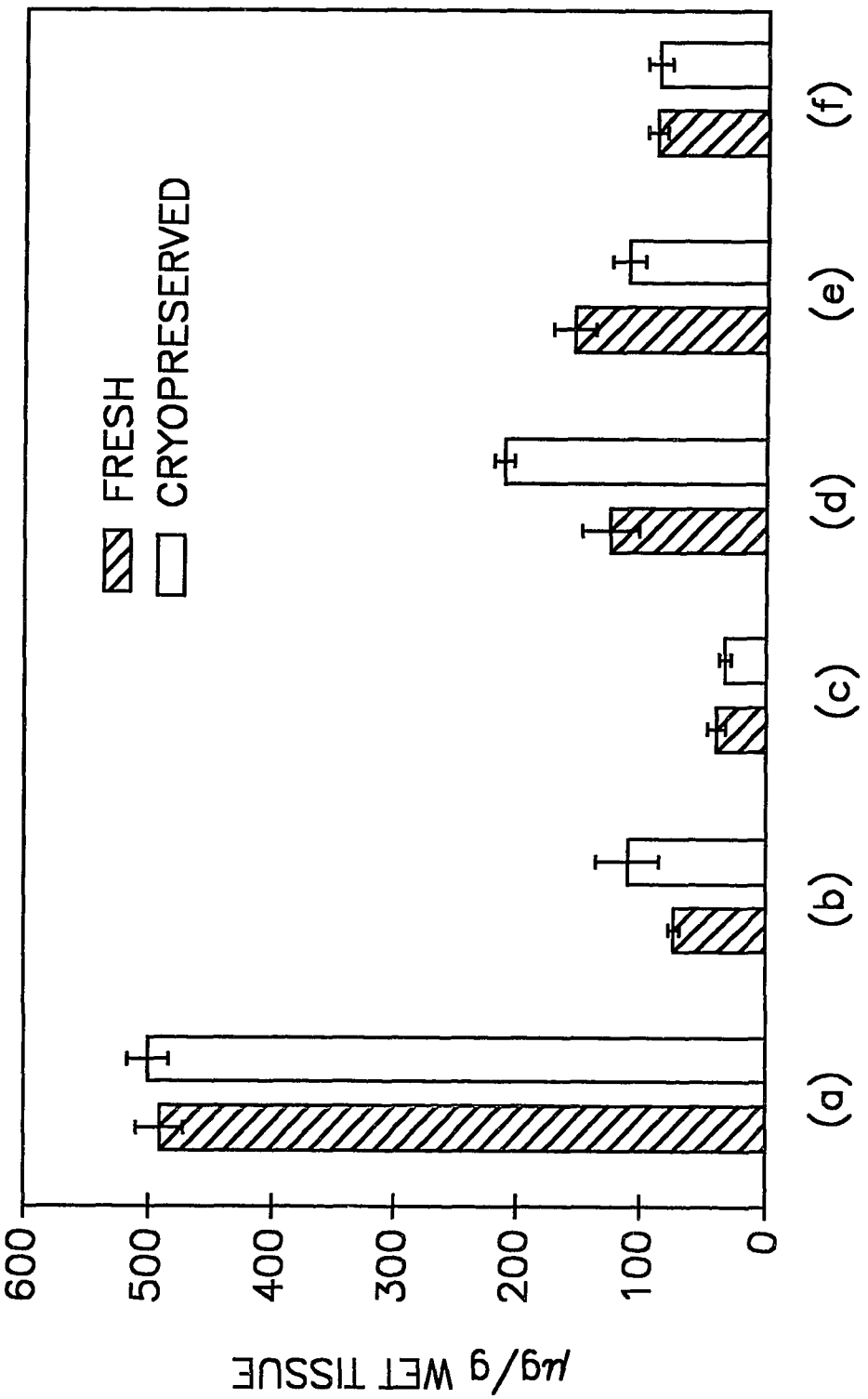


Table 2. Uronate distribution in sequential extracts of proteoglycans from fresh and cryopreserved porcine aorta conduit tissues. The aorta was sequentially extracted by 4 M Gdn-HCl (3X) and digested by collagenase, elastase, and papain.

			Fresh	Cryopreserved
Total UA[a] (μg/g wet tissue)			970 ± 44	1051 ± 25
UA distribution in different extraction steps (% of total UA)	Gdn-HCl extract	1st	50.6 ± 0.5	47.7 ± 2.6
		2nd	7.5 ± 0.2	10.4 ± 2.1
		3rd	4.1 ± 0.6	3.1 ± 0.4
	Collagenase solubilized		12.8 ± 2.1	20.1 ± 1.0
	Elastase solubilized		16.0 ± 1.8	10.5 ± 1.1
	Papain solubilized		9.1 ± 0.4	8.2 ± 0.9

[a] UA, uronic acid.

Values represent the mean ± standard error of replicate assays, n = 3.

Two additional extractions solubilized 7.5 % and 4.1 % of the total hexuronic acid content in fresh tissue and 10.4 % and 3.1 % of the total hexuronic acid content in cryopreserved tissue, respectively. Hydrolysis of the extraction residue with collagenase released 12.8 % of the total uronate in fresh tissue and 20.1 % of the total uronate in cryopreserved tissue. Subsequent elastase hydrolysis of the fresh and cryopreserved tissues released 16.0 % and 10.5 % of the total hexuronic acid content, respectively. The remaining uronate positive materials (9.0 % from fresh and 8.2 % from cryopreserved tissue) were obtained through papain hydrolysis. There was essentially no difference between fresh and cryopreserved tissues in the relative distribution of uronate positive materials in the various tissue extracts.

4. Ultrastructural Studies.

Cuprolinic Blue (CB) was used for the ultrastructural staining for proteoglycans in the tunica media from porcine aortic conduit since it has been shown (Scott, 1972; van Kuppevelt et al., 1984) that Cuprolinic Blue staining gives a better representation of the proteoglycans than, for instance, Alcian Blue or Ruthenium Red.

Ultrastructural morphology and distribution of proteoglycans in the

tunica media of porcine aortic conduit was compared between fresh and cryopreserved tissues. Cell types in the medial layer of both fresh and cryopreserved porcine aorta conduit were primarily smooth muscle cells, and the intercellular spaces contained abundant elastin and collagen fibers (Figures 24 and 25). Viable nuclei were observed, and nuclear morphology was not altered in cryopreserved tissue, which may be indicative that no significant cellular injury was induced by the cryopreservation processing (Figure 25) (partially substantiating earlier work by Dr. Jianfei Hu in this laboratory: Hu, 1992).

Staining of porcine arterial tissue with Cuprolinic Blue resulted in the formation of stick - or egg - shaped precipitates (Figures 24 and 25). The precipitates were detectable in the extracellular matrix as a complex perifibrillar and interfibrillar array of proteoglycans. The precipitates were closely associated with other matrix components, collagen and elastin (Figures 24 and 25). The Cuprolinic Blue - induced precipitates in this study represent polyanionic matrix components which are predominantly sulfated proteoglycans since there is no dissociation of the tetracationic Cuprolinic Blue from the polyanionic matrix components at a critical electrolyte concentration of 0.3 M $MgCl_2$ (Figures 26 and 27) and the stick - shaped precipitates, suggested to represent proteoglycan monomers, retained their characteristic appearance.

Figure 24. Fresh porcine aortic conduit stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Proteoglycan-Cuprolinic Blue precipitates are found in the extracellular matrix surrounding smooth muscle cells (SMC). These precipitates are present in the soluble matrix (SM), and are associated with collagen (C) and elastin (E) fibers. Original magnification x16,240.

Figure 25. Cryopreserved porcine aortic conduit stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Proteoglycan-Cuprolinic Blue precipitates are found in the extracellular matrix surrounding smooth muscle cells (SMC). These precipitates are present in the soluble matrix (SM), and are associated with collagen (C) and elastin (E) fibers. Original magnification x13,440.

Figure 26. Tunica media of fresh porcine aortic conduit stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. Post-staining with uranyl acetate and lead citrate was omitted. Proteoglycan-Cuprolinic Blue precipitates are present in the extracellular matrix. These precipitates are found within the soluble matrix (SM), and are associated with collagen bundles (C) and elastin (E). Original magnification x28,000.

Figure 27. Tunica media of cryopreserved porcine aortic conduit stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. Post-staining with uranyl acetate and lead citrate was omitted. Proteoglycan-Cuprolinic Blue precipitates are present in the extracellular matrix surrounding smooth muscle cells (SMC). These precipitates are found within the soluble matrix (SM), and are associated with collagen bundles (C) and elastin (E). Original magnification x13,440.

Three categories of Cuprolinic Blue precipitates (proteoglycan - dye complex) were identified in fresh tissue, and these three types of precipitates were also found in cryopreserved tissue. Each type of precipitated dye showed preferred localization in the extracellular matrix.

A large-heavy staining type of Cuprolinic Blue precipitate was designated as type I (Figures 28 and 29). Type I precipitates in fresh tissue were found accumulated in areas that at low magnification appear to be amorphous and devoid of fibrillar component (Figures 24 and 26). Type I precipitates are therefore suggested to represent a major component of the tissue known as the "soluble matrix". To a lesser extent, type I precipitates are also observed at solitary collagen fibers (Figures 30 and 31) and at the external surface of elastin fibers (Figures 32 and 33). In cryopreserved tissue, the type I precipitates were also found in tissue areas corresponding to soluble matrix (Figures 25, 27, 34, and 35), and some of these precipitates were found at the boundary of bundles of collagen fibers (Figure 31) and at the external surface of elastin (Figures 33, 36). The distribution of type I precipitates were essentially equivalent in histology sections from fresh and cryopreserved tissues.

A weakly stained proteoglycan - Cuprolinic Blue precipitate in the arterial wall was denoted as type II. This type of precipitate was usually found

Figure 28. Proteoglycan-Cuprolinic Blue precipitates (small arrows) in the soluble matrix (SM) of fresh porcine aortic conduit. The medial layer of tissue was stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Cross-sectioned precipitates reveal circular profiles (large arrows). Original magnification x53,200.

Figure 29. Electron micrograph demonstrating large proteoglycan-Cuprolinic Blue precipitates (small arrows) in the soluble matrix (SM) from fresh porcine aortic conduit. Tissue was stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. Post-staining with uranyl acetate and lead citrate was omitted. Original magnification x53,200.

Figure 30. Collagen (C) associated proteoglycan-Cuprolinic Blue precipitates (small arrows) from fresh porcine aortic conduit. The specimen was stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. When post-staining with uranyl acetate and lead citrate is omitted, the collagen fibrils appear as electron-lucent lines. Most of the collagen associated precipitates (small arrows) lie perpendicular to the fibril axis, whereas some can be detected lying in a parallel fashion (large arrows). The larger type I precipitates (single triangles) are found at the boundaries of bundles of collagen fibers adjoining soluble matrix areas. Original magnification x53,200.

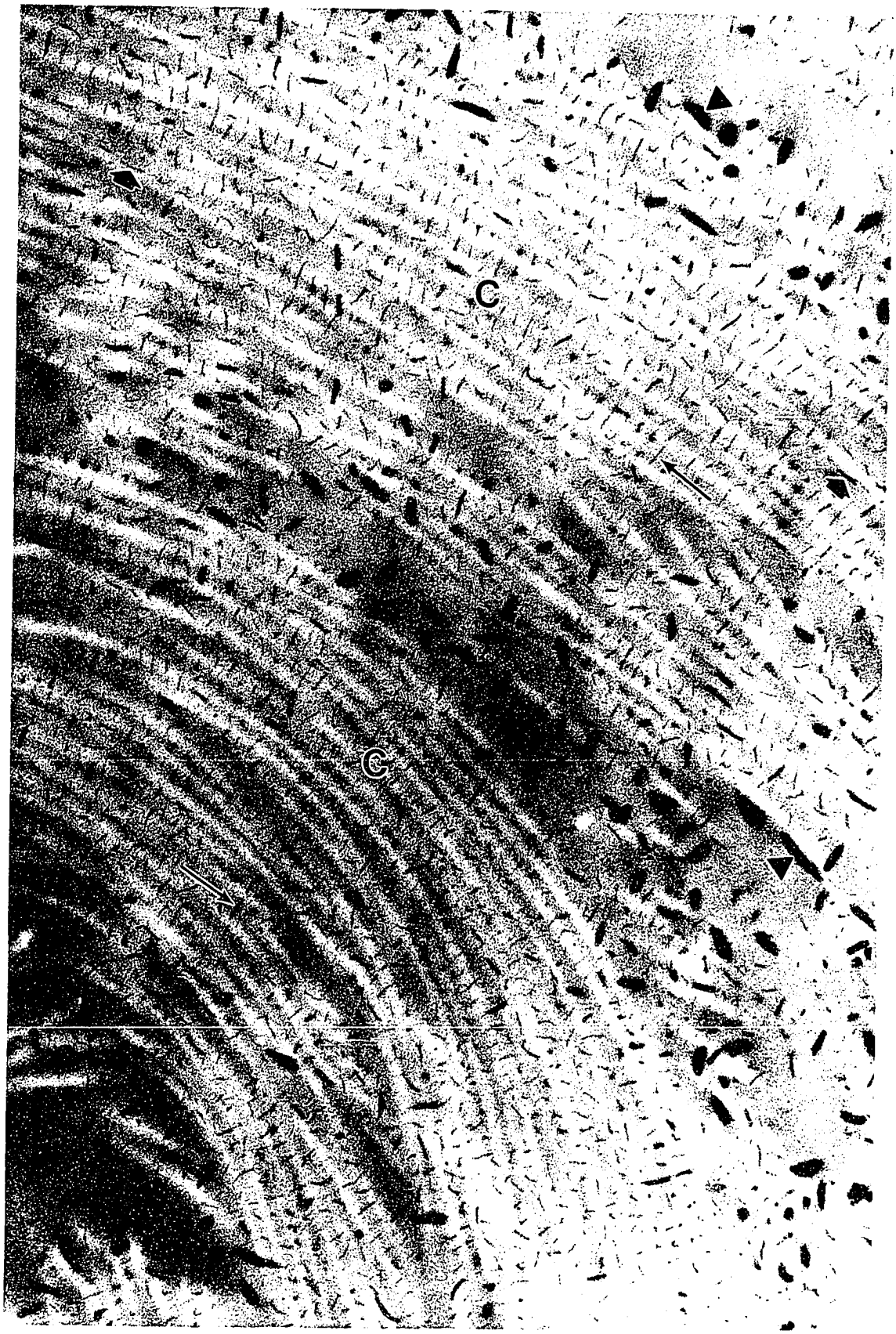


Figure 31. Collagen (C) associated proteoglycan-Cuprolinic Blue precipitates (small arrows) from cryopreserved porcine aortic conduit. The specimen was stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. When post-staining with uranyl acetate and lead citrate is omitted, the collagen fibrils appear as electron-lucent lines. Most of the collagen associated precipitates (small arrows) lie perpendicular to the fibril axis, whereas some can be detected lying in a parallel fashion (large arrows). The larger type I precipitates (single triangles) are found at the boundaries of bundles of collagen fibers adjoining soluble matrix areas. Original magnification x53,200.

Figure 32. Proteoglycan-Cuprolinic Blue precipitates (small arrows) associated with elastin (E) of fresh porcine aortic conduit. The medial layer of aortic tissue was stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Cross-sectional precipitates (large arrows) reveal circular profiles. Type I precipitates (single triangles) are observed at the external surface of elastin adjoining soluble matrix areas. In some cases, proteoglycan-Cuprolinic Blue precipitates (double triangles) appear to serve as a link between collagen (C) and elastin. Original magnification x53,200.

Figure 33. Proteoglycan-Cuprolinic Blue precipitates (small arrows) associated with elastin (E) of cryopreserved porcine aortic conduit. The medial layer of aorta tissue was stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Cross-sectional precipitates (small arrows) reveal circular profiles. Type I precipitates (large arrows) are observed at the external surface of elastin adjoining soluble matrix areas. Original magnification x53,200.

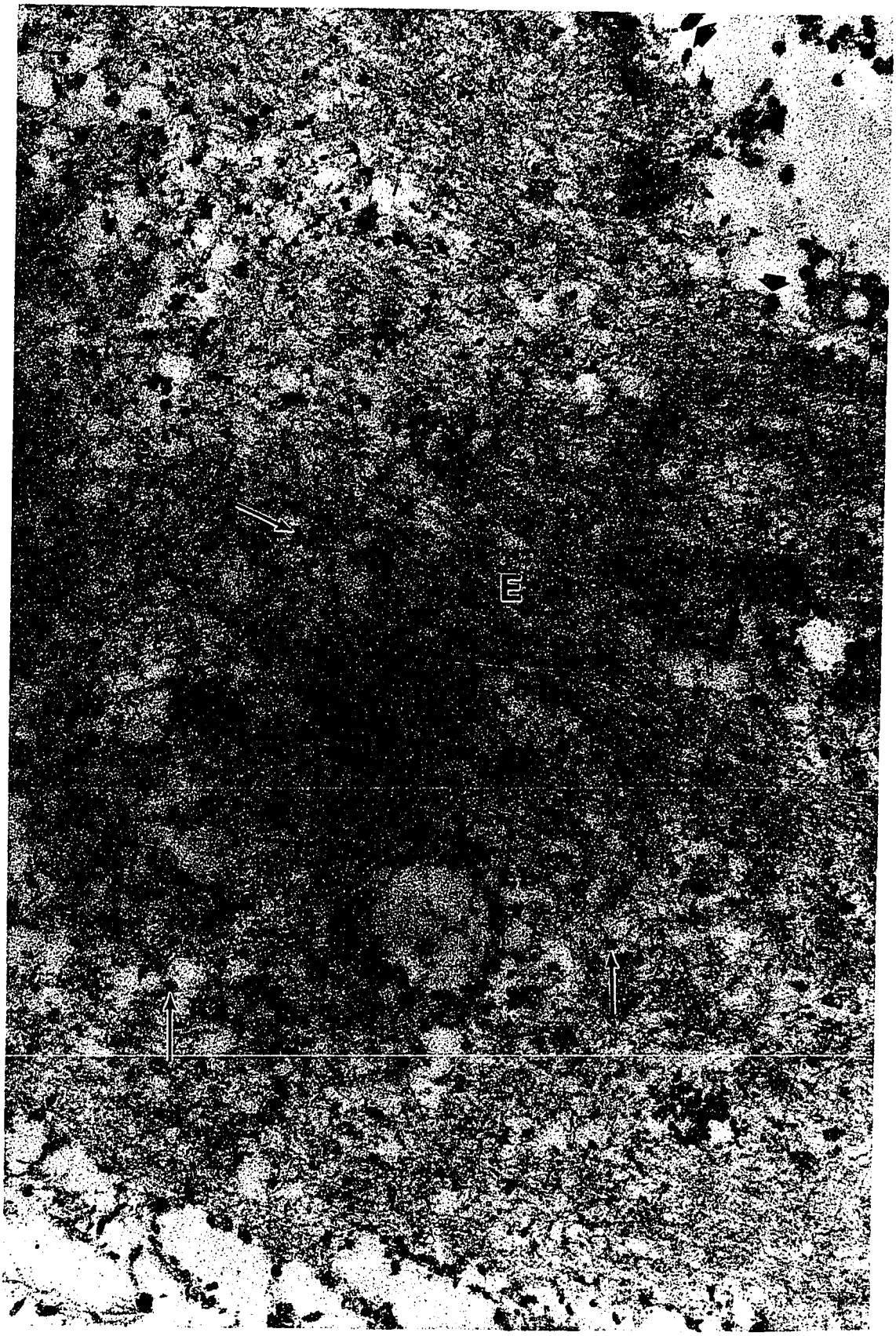
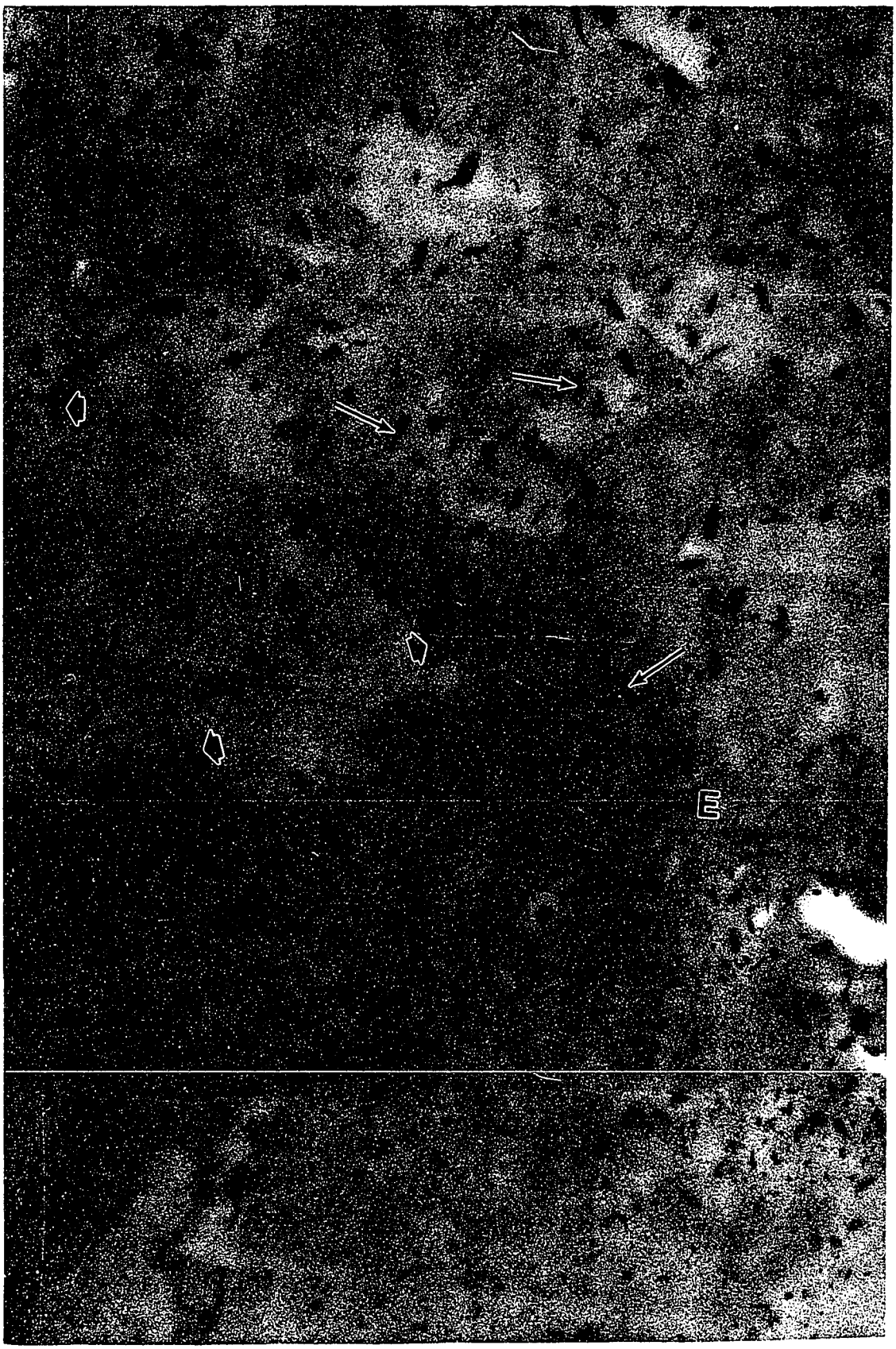


Figure 34. Proteoglycan-Cuprolinic Blue precipitates (small arrows) in the soluble matrix (SM) of cryopreserved porcine aortic conduit. The medial layer of tissue was stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Cross-sectioned precipitates reveal circular profiles (large arrows). Original magnification x39,200.

Figure 35. Electron micrograph demonstrating large proteoglycan-Cuprolinic Blue precipitates (small arrows) in the soluble matrix (SM) from cryopreserved porcine aortic conduit. Tissue was stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. Post-staining with uranyl acetate and lead citrate was omitted. Cross-sectional precipitates (large arrows) reveal circular profiles. Original magnification x53,200.

Figure 36. Proteoglycan-Cuprolinic Blue precipitates (small arrows) associated with elastin (E) in cryopreserved porcine aortic conduit. The specimen was stained with Cuprolinic Blue in the presence of 0.3 M $MgCl_2$. The larger type I precipitates (large arrows) are found at the external surface of elastin adjoining soluble matrix areas. Original magnification x53,200.

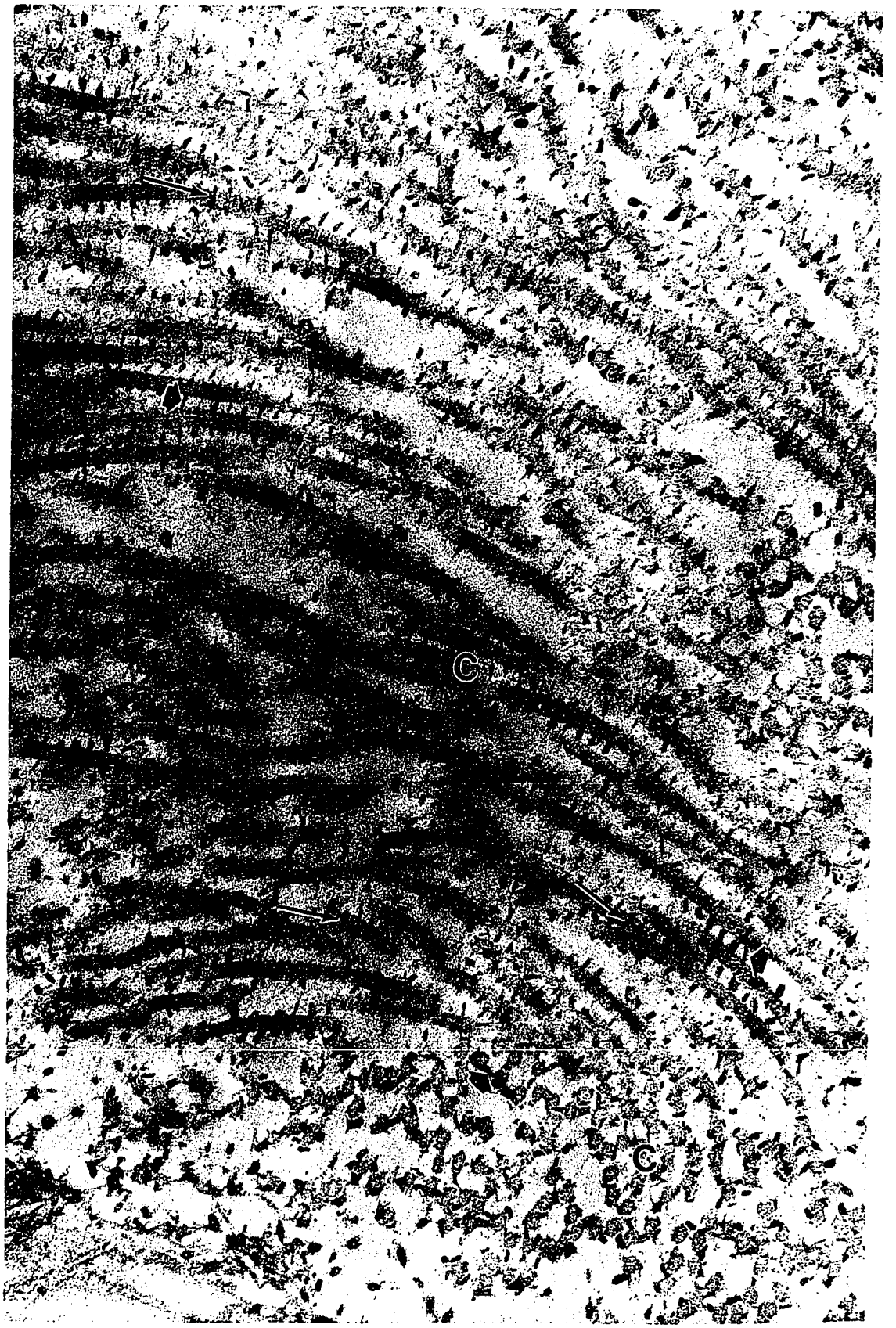


associated within the interior of bundles of collagen fibers (Figures 24, 26, 30, and 37) in fresh tissue. They lie, highly ordered, perpendicular with respect to the fibril long axis and are separated from each other according to the main banding period of the collagen fibrils (Figures 30 and 37). When compared to the type I precipitates, they are clearly less electron - dense (Figures 24 and 30). Although most precipitates lie perpendicular to the fibril axis, some of the dye precipitates lie parallel to the fibril axis (Figure 30). In some cases, type II precipitates appear to connect adjacent collagen fibrils with each other (Figure 37). When poststaining with uranyl acetate and lead citrate is omitted, the collagen fibrils can no longer be detected; the collagen - associated filaments (type II precipitated material), however, remain clearly visible (Figures 26 and 30). From the pattern of the filaments, it is possible to deduce the position of the collagen fibrils.

The ultrastructural morphology of single collagen fibers from cryopreserved tissue was similar to that from fresh tissue. However, the arrangement of collagen fibers in cryopreserved tissue appeared to be more dispersed than that in fresh tissue (Figures 37 and 38). The pattern of the proteoglycan associations to the bundles of collagen fibers in cryopreserved tissue was similar to that in fresh tissue (Figures 25, 27, 31, and 38) and most of the type II precipitates were lying, highly ordered and perpendicular to the

Figure 37. Proteoglycan-Cuprolinic Blue precipitates (small arrows) associated with collagen fibers (C) of fresh porcine aortic conduit. The specimen was stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Some precipitates (large arrows) connect the collagen fibrils with each other. Original magnification x53,200.

Figure 38. Proteoglycan-Cuprolinic Blue precipitates (small arrows) associated with collagen fibers (C) of cryopreserved porcine aortic conduit. The specimen was stained with Cuprolinic Blue and post-stained with uranyl acetate and lead citrate. Some precipitates (large arrows) connect the collagen fibrils with each other. Original magnification x53,200.

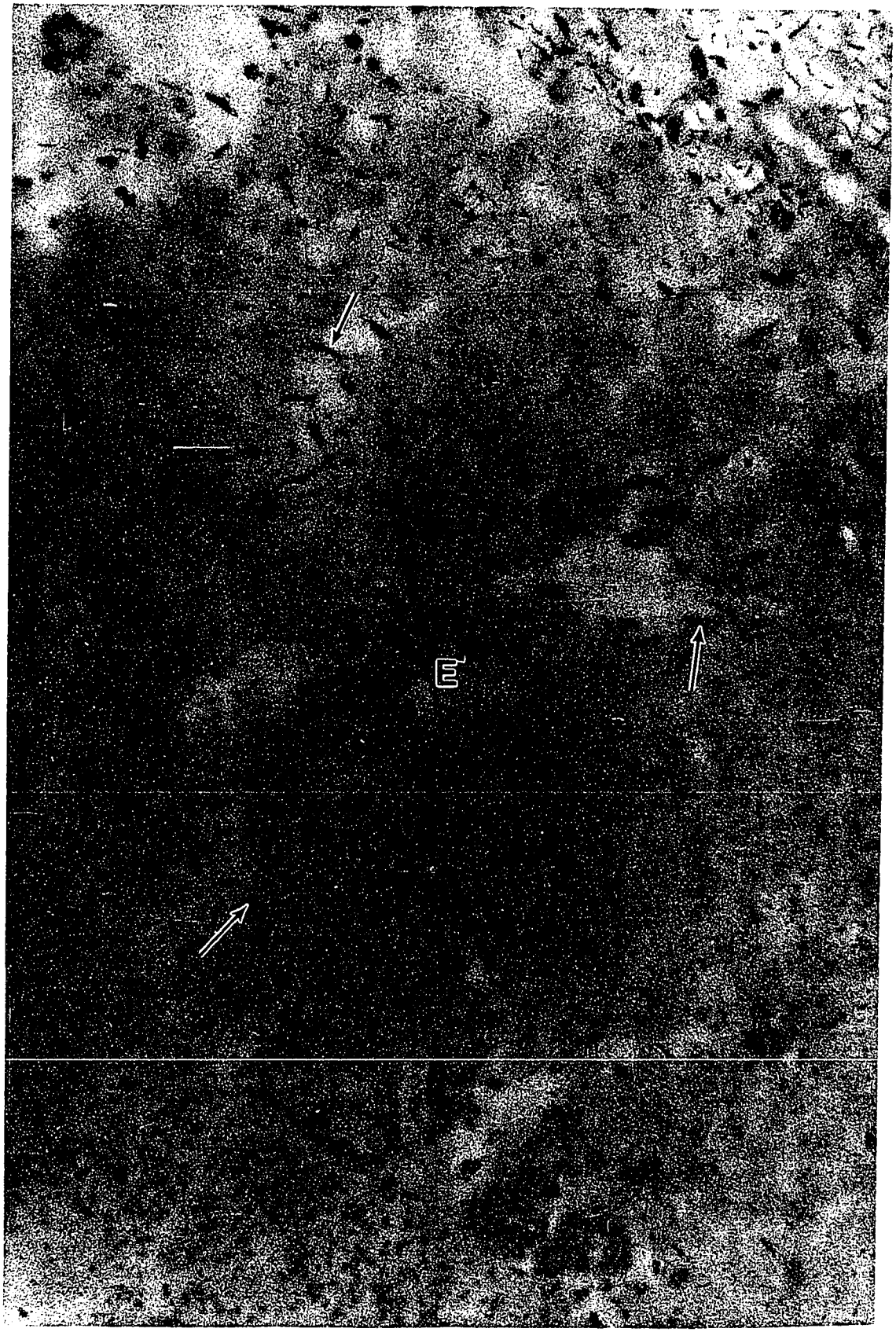


fibril axis. Precipitates lying parallel to the fibril axis can also be seen (Figure 31) as well as precipitates connecting two fibrils (Figure 38).

The intermediately stained proteoglycan-Cuprolinic Blue precipitate was denoted as type III precipitate. Type III precipitates differ from the type II precipitates in width, and they are associated mainly with the elastin proteins (Figures 24, 26, 32, and 39). Matrix precipitate connections between collagen and elastic proteins were also observed (Figure 32). These relationships are particularly well demonstrated in regions containing cross sections of collagen fibrils as seen in Figure 32. The elastin structure of aortic conduit also remained unaffected by cryopreservation procedure because the delicate morphologic feature of elastin tissue was well preserved in cryopreserved porcine aortic conduit tissue (Figures 25, 27, 33, and 36) and was not visibly different from tissue sections obtained from fresh conduit tissue.

Electron microscopic comparisons demonstrated a retention of proteoglycans in the wall of the aortic conduit after cryopreservation, and the relative morphological distribution of proteoglycan content in cryopreserved tissue was similar to that in fresh tissue.

Figure 39. Proteoglycan-Cuprolinic Blue precipitates (small arrows) associated with elastin (E) in fresh porcine aortic conduit. The specimen was stained with Cuprolinic Blue in the presence of 0.3M $MgCl_2$. Original magnification x53,200.



5. Mineral Analyses of Porcine Aorta Tissue.

The procedure for the determination of tissue calcium was based on the interaction of calcium with a metallochromic indicator, Arsenazo III (2,2'-[1,8-Dihydroxy-3,6-disulfo-2, 7-maphthalene-bis(azo)]-dibenzenearsonic acid). Arsenazo III has a specific set of charges and an absorption spectrum which depends on its ionization state and conformation. The spectral properties of arsenazo III were strongly pH dependent and, therefore, the arsenazo III was used in well buffered solutions (0.5 M Tris - HCl, pH 7.4). Arsenazo III specifically complexed with calcium in acidic environment to form a purple colored complex which has an absorbance maximum at 650 nm. The intensity of the purple color was directly proportional to the calcium concentration (Figure 40).

The quantitative calcium levels for fresh and cryopreserved porcine aorta tissue is shown in Table 3. The average amount of chemically detectable calcium was 105.0 $\pm$ 3.9 and 164.8 $\pm$ 3.2 μg calcium/g of wet tissue in the fresh and cryopreserved porcine aorta tissues, respectively. By means of a t test, the value of t was found to be significant at the 0.05 level, which indicates that the calcium content in fresh and cryopreserved tissue was statistically different. The total calcium level increased in tissue cryopreserved by the

Figure 40. Linear regression analysis for standard curve of calcium analysis using the Arsenazo III assay method. Micro amounts of calcium in 1N HCl form a complex with Arsenazo III. This purple colored complex had an absorbance maximum at 650 nm. The intensity of the color measured at 650 nm was directly proportional to the calcium concentration.

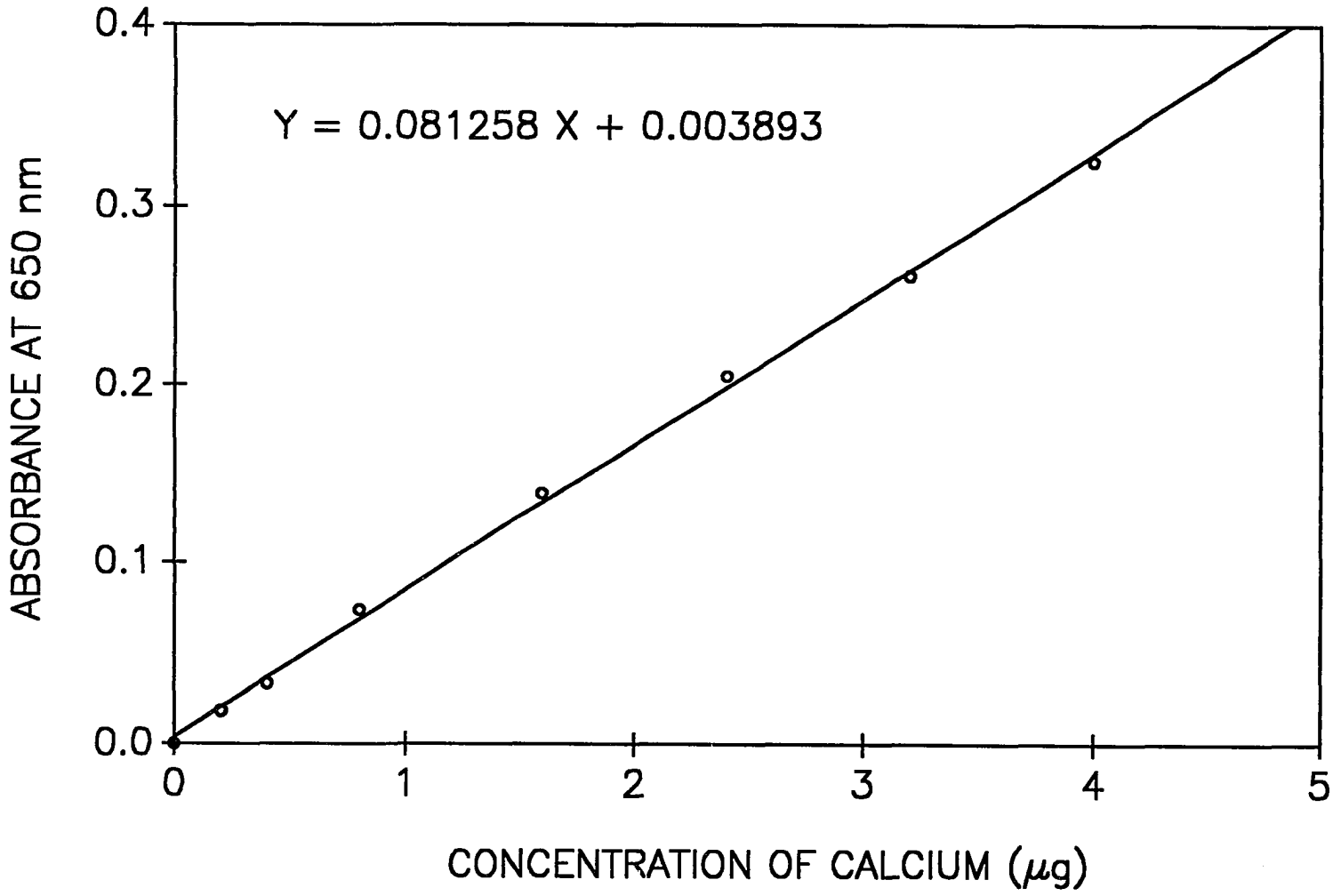


Table 3. The quantitative calcium and phosphorous contents of fresh and cryopreserved porcine aorta conduit tissues. Tissue for calcium and phosphorous analysis was rinsed with 0.9 % NaCl and ashed overnight. The ashed sample was solubilized in 1N HCl and analyzed for calcium using the Arsenazo III assay method and for phosphorous using ascorbic acid assay method.

	Calcium (μg/g wet tissue)	phosphorous (μg/g wet tissue)
Fresh	105.0 $\pm$ 3.9	719.8 $\pm$ 28.5
Cryopreserved	164.8 $\pm$ 3.2	703.1 $\pm$ 39.2

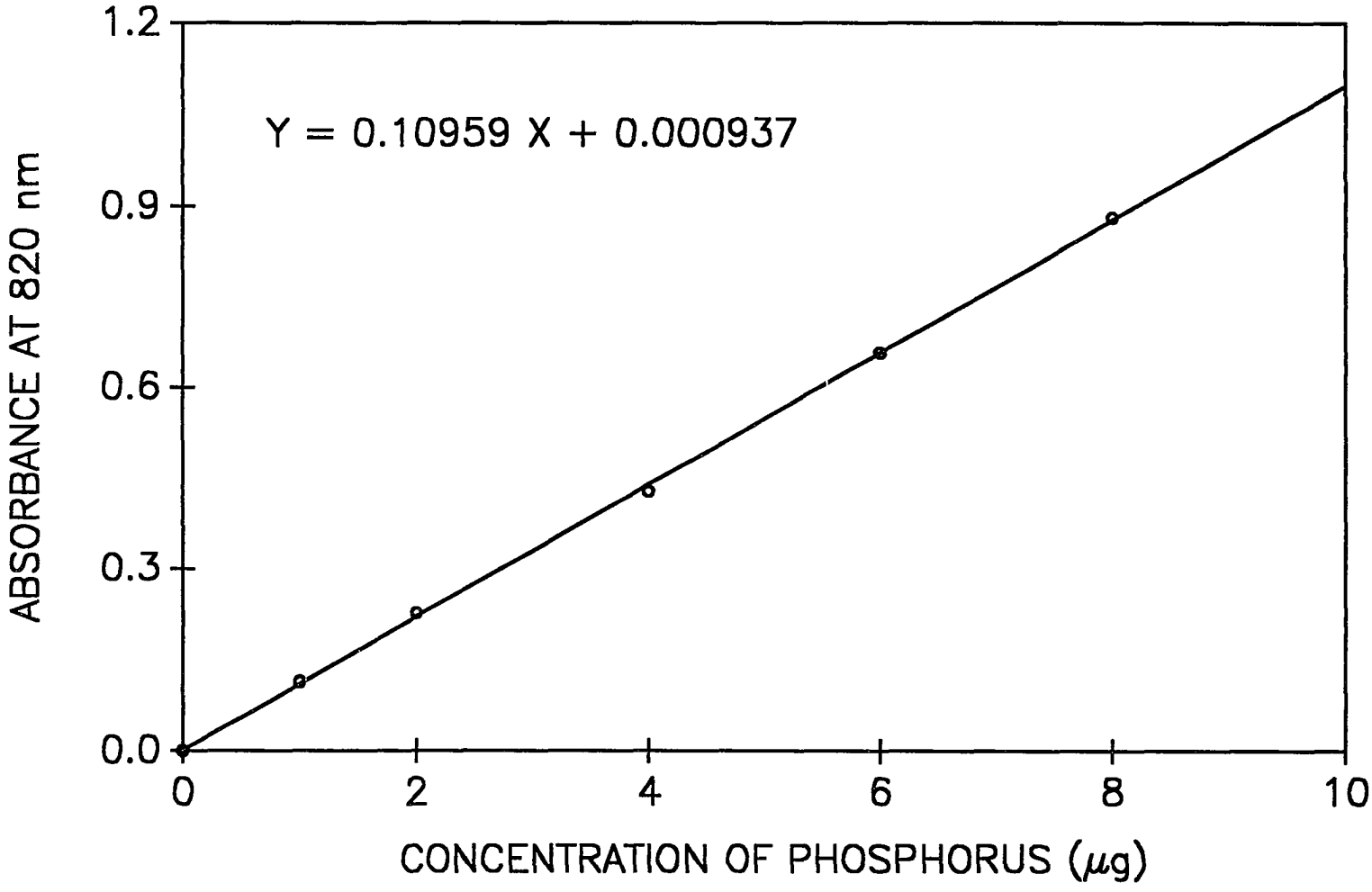
Values represent the mean $\pm$ standard error, n = 9.

cryopreservation process which may be due to the higher calcium concentration of RPMI 1640 tissue culture medium (used for cryopreservation process) compared to calcium content of porcine aorta tissue.

The concentration of phosphorus from the tissues was determined by the ascorbic acid method which was based on the color formed by the reduction of a phosphomolybdate complex. Reagent C (mixtures of 1 volume of 6 N sulfuric acid, 2 volumes of distilled water, 1 volume of 2.5 % ammonium molybdate, and 1 volume of 10 % ascorbic acid) was prepared fresh each day since it was unstable and would lose its ability to form a color with phosphorus.

The stability, constancy, and linearity of this procedure were good. It was not necessary to read solutions immediately or at a certain time after starting a color reaction since the stability of the color developed was very good. Phosphorus standard 8 μg always reads 0.870 to 0.880 in the spectrophotometer with different batches of ascorbic acid and ammonium molybdate. Figure 41 shows standard curves of phosphorus using the ascorbic acid method. The color development was proportional to phosphorus concentration. Standard curves were always linear as ascertained by regression analysis.

Figure 41. Linear regression analysis for the standard curve of the assay for phosphorus using the ascorbic acid assay method. Microdetermination of phosphorus was performed using ascorbic acid solution for the reduction of phosphomolybdate with heating at 37 °C.



Phosphorus content of fresh and cryopreserved porcine aorta conduit tissue is shown in Table 3. The tissues which were ashed and then hydrolyzed in 1 N HCl contained 719.8 $\pm$ 28.5 μg phosphorus/g wet tissue in fresh and 703.1 $\pm$ 39.2 μg phosphorus/g wet tissue in cryopreserved tissue, respectively. There was no difference in phosphorus level between fresh and cryopreserved tissues.

CHAPTER IV

DISCUSSION

The optimal surgical treatment of cardiac valvular disease would involve the restoration of the native valve to a satisfactory functional state through conservative reparative procedures (Deloche et al., 1990). Unfortunately, the valvular pathology most often encountered at operation is irreparable due to advanced rheumatic, degenerative, ischemic, or calcific destruction necessitating valve replacement.

Aortic valve allografts have been used clinically for more than 25 years for the repair of complex heart malformations (Fontan et al., 1984; Moore et al., 1976; Ross and Somerville, 1966). The performance of fresh antibiotic-sterilized valvular homografts has been superior to that of mechanical or xenograft valves. However, because of severe limitations in the supply of aortic homograft valves, a method was sought that would increase the viable lifetime of the valve in storage. Cryopreservation of the viable allograft was thus explored as a possible solution for accumulation of the larger number of

valves necessary for the selection of a proper match in terms of orifice diameter and HLA-defined antigens.

Cryogenic techniques for heart valves developed in the early 1970s and confirmed cellular viability (based on histologic and biochemical data) in tissues stored for prolonged periods in vapor phase temperatures of liquid nitrogen, led to the clinical application of dimethylsulfoxide (DMSO)-cryopreserved aortic allografts in the early of 1970s (Angell et al., 1987). Cryopreserved aortic valve allografts have enhanced the surgeon's ability to correct extremely complex congenital cardiac anomalies.

The clinical data suggest that properly cryopreserved allograft valves have remarkable durability in addition to the other advantages (optimal hemodynamic performance, reduced thromboembolism and hemolysis rates without anticoagulation therapy, and resistance to endocarditis) of the nonviable fresh homograft. However calcification is one of the general causes of allograft failure in late results (Maxwell et al., 1989; Miller and Shumway, 1987) especially in reconstructive surgery in children with congenital cardiac defects. Calcification is significantly greater in the conduit wall than in the leaflets, and the consequent lack of distensibility limits their long-term effectiveness.

The pathophysiology of cardiovascular implant calcification is complex and poorly understood. However, calcification processes occurring normally in skeletal and dental tissues, and pathologically in implanted arterial grafts, share important features (Anderson, 1983; Anderson, 1989; Schoen et al., 1988). The study of cartilage matrix and the process of mineralization indicates that matrix proteoglycans inhibit cartilage mineralization.

Proteoglycans are inherently heterogeneous and polydisperse in nature in terms of molecular size, charge density, constituent protein, and glycosaminoglycan moieties. Some of the aorta proteoglycans have features similar to those attributed to hyaline cartilage. For example, large proteoglycans identified in aorta, at least in part, are capable of forming high molecular weight link-stabilized aggregates with hyaluronic acid which indicates some similarity to the proteoglycans present in cartilage (Gardell et al., 1980; Heinegard et al., 1985; Oegema et al., 1979). However, Heinegard et al. (1985) demonstrated that the large proteoglycans had different peptide maps and showed only partial homology when compared to the aggregating proteoglycans in cartilage suggesting differences in primary structure in the core proteins of these two proteoglycans. In studies with rotatory shadowing, these molecules contain the pair of globules typical for the hyaluronate binding region. A single link protein has also been identified in aortic tissue (Vijayagopal et al., 1985),

indicating that this tissue, like cartilage, possesses accessory proteins which function to stabilize the proteoglycan aggregate. The proteoglycan matrix granules in aorta are very prominent and have a close association with both the collagen and elastic fibers in the extracellular matrix. Often, the proteoglycans can be seen condensed on the collagen fibrils at constant intervals similar to the patterns observed in cartilage. This observation suggests that there are ordered interactions between the proteoglycan molecules and the collagen fibrils in the aorta. Aorta, like cartilage, undergoes repetitive, transient pressure changes, and it is likely that the proteoglycans, in concert with the elastin component of the tissue, buffer these cyclical changes.

Most previous studies on cellular viability and histology of valve tissues were undertaken to assess the metabolic state of aortic leaflets before and after processing for transplantation (Hu et al., 1989; Hu et al., 1990). Although allograft aortic conduits have been in use for almost as long as valves, little study has been done on them. This study was focused on the arterial conduit wall since a major concern in using allograft tissue in cardiac reconstruction is late wall calcification and possible conduit obstruction.

The first objective in studying proteoglycans is to extract them from the tissue as quantitatively as possible and under conditions that reduce the chances

for degradation. Proteoglycans constitute a minor component of aorta (approximately 1- 5 % by weight) as compared to cartilage (approximately 50 % by weight). Proteoglycans isolated from cartilage have been most widely studied, primarily because cartilage contains large amounts of proteoglycans. A number of methods, initially applied to cartilage, have been adapted for soft tissues such as aorta. However, there are great variabilities in the proteoglycans depending on the tissue source, location of sample, and extraction procedure. It is necessary to devise an extraction procedure for porcine aorta conduit tissue. The extraction procedure must satisfy the criteria mentioned above (quantitative yields and inhibition of degradation of proteoglycans).

In early studies of proteoglycans, exhaustive high-speed homogenization (disruptive extraction) in water or low concentrations of salt were used as the primary methods for extraction of proteoglycans (Pal et al., 1966). However, these disruptive isolation procedures can introduce shear artifacts. A new extraction technique, using high ionic strength solution, was introduced for solubilizing proteoglycans from tissue without requiring homogenization and has been designated as dissociative extraction (Sajdera and Hascall, 1969). The ease and efficiency of proteoglycan extraction from the tissues are dependent on the ability of the extracting medium to dissociate proteoglycan aggregates in the

tissue.

The study to find optimal concentrations of Gdn-HCl for proteoglycan extraction from porcine aorta tissue has indicated that Gdn-HCl (denaturing solvent) at a concentration of 4 M is the most effective. The yield of proteoglycans from Gdn-HCl concentrations lower or higher than 4 M Gdn-HCl was significantly less than that obtained with 4 M Gdn-HCl. Antonopoulos et al. (Antonopoulos et al., 1974) also found that the concentration of 4M of Gdn-HCl was the most effective for the extraction of proteoglycans from bovine sclera, and cornea. The amount of proteoglycans extracted from sclera and cornea with 5 M Gdn-HCl was similar to that with 4 M Gdn-HCl. However, the yield of proteoglycans increased slightly with increases in the concentration of Gdn-HCl (from 1 to 5 M) from these tissues in contrast with porcine aorta in the present study. Sajdera and Hascall (1969) suggested that the rate of proteoglycans extraction from bovine nasal cartilage was greater for the optimal Gdn-HCl concentration than for optimal inorganic electrolyte (calcium chloride, magnesium chloride) solution. Gdn-HCl in 3 M concentration was the most effective, and the range of optimal concentrations (between 3 and 8 M) for extraction of cartilage was broad for Gdn-HCl but rather narrow for the inorganic salts. In the present study, the sharp optima for extraction of porcine aorta conduit tissue exhibited by different concentrations of Gdn-HCl indicated

that a reasonable amount of care must be exercised in utilizing Gdn-HCl for the purpose of dissociative extraction.

At a 4 M concentration of Gdn-HCl, most intermolecular noncovalent bonds appear to be broken in aortic conduit tissue. At lower concentrations, the denaturing effect of the Gdn-HCl is less pronounced and extraction is less efficient, and extremely long periods of time may be needed to extract high yields of the soluble proteoglycans. Also, at higher concentrations extraction yields becomes lower, possibly an effect of alterations in the network of fibrillar protein as a result of denaturation.

Another general problem in isolation of proteoglycans is that the core protein is very sensitive to protease activity (Heinegard and Sommarin, 1987). It is therefore important to protect the core protein during extraction from the activities of proteases present in the tissue. One efficient way of inhibiting protease activity is the use of denaturing conditions for extraction. Extraction of tissues at low temperature (4 °C) is another way for inhibiting enzyme activity. Additionally, protease activity may be inhibited by selecting an appropriate pH for the extracting solvent. A pH of 5.8 for the extraction solution was used in this study since it is above the optimum for acid pH proteases and below the optimum for neutral and alkaline pH proteases. A

number of protease inhibitors with a broad range of specificities were also included in the extraction solutions. The choice of conditions utilized in the present study appear to be appropriate since minimal degradation of isolated proteoglycans was observed.

A maximum value of tissue proteoglycans was isolated after 48 hours extraction. More uronate could be extract by a second 48 hours extraction of the tissue, but the extraction was not repeated in order to minimize potential degradation of these macromolecules, facilitating further assessment of aggregate size of extracted proteoglycans.

There were density variations after the second CsCl density gradient centrifugation. Oegema et al. (1979) suggested that these variations in the gradients are the result of changes in the number of glycosaminoglycan chains attached to the protein core(s) rather than changes in the chain length or the presence of different populations of core protein. The patterns of the dissociative CsCl isopycnic centrifugation profiles of proteoglycans with porcine aorta tissue in this present study were similar to those observed in bovine aorta (Radhakrishnamurthy et al., 1986), bovine lung (Radhakrishnamurthy et al., 1980), and human aorta (Dalferes et al., 1987). The results obtained in the CsCl isopycnic centrifugation study indicated that there was no change in the

levels of total proteoglycans after cryopreservation of porcine aorta conduit tissue. Manley (Manley, 1965) observed no significant effect on aorta glycosaminoglycan composition with post mortem intervals up to 90 hours.

Because proteoglycans were extracted in dissociative solvent and fractionated by dissociative isopycnic centrifugation, it was expected that the top fraction would contain the hyaluronate (density approximately between 1.4 and 1.5 g/ml) and link proteins (density approximately less than 1.4 g/ml). Although gel permeation chromatography was performed under dissociative conditions, 4 M Gdn-HCl/0.05 M sodium acetate was removed from the eluent solution (using a hollow fiber system) just before collection of eluent fractions, it is possible that the uronic acid-positive materials eluted near the void volume of the column in proteoglycan fractions II and III might represent aggregated proteoglycans since there might be hyaluronic acid in these fractions.

Although it was not demonstrated in this study that proteoglycans occur as mixtures or as a hybrid proteoglycan with different glycosaminoglycans on the same protein core, all proteoglycan preparations (fractions I, II, and III) from fresh and cryopreserved tissues were bigger than the monomer form because K_{av} values of proteoglycan fractions (K_{av} = 0.13 and 0.50 (I), 0.23 (II), and 0.40 (III) in fresh tissue and K_{av} = 0.13 and 0.47 (I), 0.20 (II), and

0.43 (III) in cryopreserved tissue) were much smaller than that of chondroitin sulfate (K_{av} = 0.80). Even if they were from a hybrid-type proteoglycan, heterogeneity with respect to individual glycosaminoglycan and polydispersity with respect to their molecular size could be possible in these preparations. Hybrid glycosaminoglycans are present in several tissues (Fransson and Roden, 1967; Habuchi et al., 1973) and in the proteoglycans from bovine aorta maintained in culture (Kresse et al., 1971).

The similar overall K_{av} values of uronic acid positive and proteinaceous materials of proteoglycans from fresh and cryopreserved porcine aorta conduit tissues indicate that proteoglycans isolated from cryopreserved tissue have similar molecular weights to proteoglycans of the fresh tissue. The similar molecular weight proteoglycan fractions in the cryopreserved tissue presumably result from normal synthesis of the core protein with several glycosaminoglycans prior to procurement, and suggest that processing for cryopreservation does not result in enzymatic modification or loss of proteoglycans during processing and cryopreservation. Berberian and Fowler (1979) reported that there was no significant effect on the specific activities of lysosomal enzymes of human aorta up to 40 hours of postmortem interval.

Proteoglycans exert significant influence over cartilage calcification.

Loss or alteration of proteoglycans accompanies cartilage calcification or transformation of cartilage into bone. Reddi et al. (1977) found that the proteoglycans present in plaque at the time of maximum chondrogenesis are primarily in the aggregated form. However, when the hypertrophic cartilage undergoes extensive calcification, there is a decline in the synthesis of proteoglycans and a large proportion of the newly synthesized molecules are of lower molecular weight (Reddi et al., 1977).

Lohmander and Hjerpe (1987) also found that rib cartilage lost approximately half its content of proteoglycans with the onset of calcification, and the proportions of very high molecular weight proteoglycans were decreased in mineralized tissue. They suggested that the quantitative and qualitative change of the proteoglycans of cartilage during calcification resulted from the concerted actions of released lysosomal hydrolytic enzymes. The presence in cartilage of both proteases (Woessner, 1973) and a hyaluronidase (Wasteson et al., 1975) has been demonstrated. These enzymes may be released into the intercellular matrix directly from cells and/or from matrix vesicles during and after degeneration and disintegration of chondrocytes (Anderson, 1969; Thyberg and Friberg, 1970). Degeneration of chondrocytes is frequent in the hypertrophic and mineralizing parts of epiphyseal cartilage (Thyberg and Friber et al., 1970).

Lysozyme (a protein synthesized by chondrocytes) also exists in cartilage matrix (Kuettner et al., 1971; Kuettner et al., 1974) and appears to be concentrated in the hypertrophic zone of the growth plate. In vitro studies (Kuettner et al., 1974; Pita et al., 1975; Pita, Muller and Howell, 1975) showed that lysozyme reduces proteoglycan aggregates to the size of subunits and removes their inhibitory effect on mineral growth. Although the mechanism of lysozyme action on proteoglycan aggregates is unclear, the evidence from in vitro studies suggests that lysozyme disassembles aggregates or reverses aggregation by binding to hyaluronate and displacing subunits and link proteins (Tang et al., 1981). It might also prevent aggregate formation. Since the lysozyme exists throughout the matrix but apparently does not disassemble aggregates throughout the matrix, there must be some mechanism to control lysozyme activity. Howell's group (1981) demonstrated that lysozyme inhibitors exist in cartilage and that they can prevent lysozyme from reducing aggregate size of proteoglycans.

It was found that proteoglycan aggregates inhibit hydroxyapatite growth in a dose dependent fashion (Chen et al., 1984). Boskey et al (1992) demonstrated that the enzyme-mediated alteration in the size of proteoglycans can significantly enhance the amount of mineral formed in an in vitro hydroxyapatite formation and growth assay. They suggested that the degraded

proteoglycan can function as a hydroxyapatite nucleator, or may reflect the easier accessibility of calcium ions to the phosphate ions in solution because of the loss of the steric hindrance properties of the proteoglycan. It was also found that there was the parallel increase in alkaline phosphatase activity with increased protease activity in the calcification process of healing fracture callus (Einhorn et al., 1989). These data are consistent with the view that the proteoglycanases function to prepare the matrix for calcification.

In the study of explant cultures of bovine articular cartilage (Bolis et al., 1989), degraded proteoglycan subunits were lost from the extracellular matrix of the tissue into the culture medium. Proteolytic cleavage of the core protein, especially in the hyaluronate-binding region, was the initial step in the catabolism within the extracellular matrix of cartilage of the large proteoglycans. The core protein of this proteoglycan is usually cleaved in more than one place. Mok et al. (1992) suggested that there is a region that is susceptible to proteinase attack within the interglobular domain of the core protein of aggregates.

Most studies attempting to understand the relationships between proteoglycan metabolism and the mineralization process reported that once mature mineralization is established, the proteoglycans tend to fall to low levels

and reduce aggregate size to the subunit. The results obtained in this study indicate that the concentrations and molecular weights of proteoglycans were not changed in porcine aorta conduit tissue by those processes associated with cryopreservation, and we may therefore suggest that properly cryopreserved allograft valves may be less prone to calcification than valves which have alteration in proteoglycan content, for example valves with extended warm ischemic times where cell death occurs. It has been reported that allograft calcification is usually present more than 10 years after implantation. Therefore, calcification may be influenced by a number of systemic and local tissue factors (including availability of calcium and phosphate, hormone levels, cell synthetic function, death of matrix fibroblast cells, matrix protein changes, phospholipids, and/or infiltration of the graft by recipient cells) after transplantation although these factors and the relationship between them are not certain.

Proteoglycans isolated from atherosclerotic lesions have higher molecular weights than proteoglycans of the normal tissue (Dalferes et al., 1987; Rowe and Wagner, 1985). Dalferes et al. (1987) suggested that higher molecular weight proteoglycan from atherosclerotic tissue resulted from formation of complexes of proteoglycans with other arterial wall proteins, plasma lipoproteins, or peptides derived from these proteins. The lack of change of

proteoglycans sizes in cryopreserved porcine aorta conduit tissue, indicated by gel permeation chromatography profiles of proteoglycans in this study, suggests that there is no formation of higher molecular weight proteoglycan aggregates with tissue fibrous proteins during the cryopreservation processing.

The extracellular matrix of arterial wall is a complex in that it contains heterogeneous proteoglycans and the fibrous proteins (collagen and elastin). They are closely associated with one another which explains the observations of the sequential extraction study described. About 60 % of the total proteoglycans of the porcine arterial wall could be easily extracted by dissociative salt solutions, but extraction of the remainder of proteoglycans from association with the fibrous components of the tissue requires other methods for solubilization. Although it is possible to extract most of the proteoglycan material from several cartilageous tissues by dissociative techniques (Sajdera and Hascall, 1969), these methods fail to isolate all proteoglycans from aortic tissue.

The large amount of proteoglycans present in an insoluble form could represent: (a) the existence of cross-linked complexes between proteoglycans and fibrous protein; (b) the formation of more stable cross-linked complexes between several molecules of proteoglycans and hyaluronic acid with or without

involvement of link proteins; (c) the formation of insoluble complexes by interaction of proteoglycans with collagen and elastin in the presence of Ca^{2+} or Mg^{2+}; or (d) a high degree of physical entrapment in the network of fibers.

The use of specific enzymes, collagenase and elastase, elucidates the nature of the insoluble proteoglycans. The release of proteoglycans from the nonextractable residue by the enzymes indicates a general physical entrapment of proteoglycans in the collagen-elastin network, or that proteoglycan-collagen and proteoglycan-elastin cross-linkings play an important role in making proteoglycan material insoluble. If the insolubility were unrelated to specific interactions and reflected an intrinsic property of these proteoglycans alone, hydrolysis with these enzymes would not have further released proteoglycans. The specificity of these enzymes may be questioned since they may hydrolyze other components of the tissues. However, even highly purified preparations of elastase may have some non-specific protease activity. This extraneous activity can be inhibited by protease inhibitors which were used for extraction of tissue with dissociative solvent. In aorta, elastin and collagen constitute the bulk of the proteins, especially since elastin constitutes about 60 to 70 % of the tissue. Although another fibrous protein (microfibrillar protein that is closely related to elastin) is present in small amounts, Ross and Bornatein (1969) demonstrated that this protein is not hydrolyzable by elastase. Collagenase is highly specific

and does not have any nonspecific protease activity (Radhakrishnamurthy et al., 1977). It was found that sulfated glycosaminoglycans are capable of binding with serum low density lipoproteins in the presence of Ca^{2+} and Mg^{2+} to form insoluble complexes (Srinivason et al., 1970) and Ca^{2+} binds to neutral sites of elastin and collagen (Radhakrishnamurthy et al., 1977). It is, therefore, possible that proteoglycans form insoluble complexes with collagen and elastin in the presence of Ca^{2+} and Mg^{2+} and are resistant to extraction with salt solutions. The result of the sequential extraction of proteoglycans in the present study indicates that there is essentially no difference between cryopreserved and fresh tissue in the relative proportions of uronate per unit weight of wet tissue extracted in the procedure, except perhaps for the amount of proteoglycans solubilized by collagenase from cryopreserved tissue.

In the ultrastructural morphology study, dispersed collagen fibers were found in cryopreserved tissue, which may explain the result that more amounts of proteoglycans were extracted from the collagenase digestion of cryopreserved tissue than from fresh tissue. Because of the slightly more dispersed arrangement of collagen fibers in cryopreserved tissue, it might be more easy to digest the collagen with enzyme treatment which could then result in extraction of more proteoglycan from cryopreserved tissue.

Electron-microscopic observations contributed to an understanding of the distribution of proteoglycan in the fresh and cryopreserved porcine aortic conduit. The electron density of the Cuprolinic Blue dye-precipitated proteoglycan is sufficient for most histological evaluations, but the electron density of the dye can be increased by the changes to the Cuprolinic Blue dye reaction to see fine details of proteoglycan structure. Since Cuprolinic Blue is tetracationic, the four charges on the dye can bind to four negative sites on a polyanion. However, diffusible anions in solution, e.g. Cl^- from $MgCl_2$, can replace the polymer-bound anions and much more dye is then bound per unit of substrate (Scott, 1972).

It has been shown (Scott, 1972) that due to the competitive action of Mg^{+2} ions, cationic dyes are easily displaced from carboxylic groups at a relatively low concentration of $MgCl_2$ (about 0.05 M), but sulfate groups need considerably higher concentrations of $MgCl_2$ for displacement. At 0.3 M $MgCl_2$, Cuprolinic Blue can predominantly stain sulfated polyanions, where polycarboxylates and polyester phosphates remain unstained (Scott, 1980). Hence, at this concentration of $MgCl_2$ it is likely that Cuprolinic Blue-positive structures contain sulfate groups, suggesting that these anionic sites contain sulfated glycosaminoglycans. The appearance of the precipitates closely resembles that of spread proteoglycan monomers with the glycosaminoglycan

chains condensed (Buckwalter et al., 1982; Buckwalter and Rosenberg, 1982; Hascall, 1980). Therefore Cuprolinic Blue precipitates represent proteoglycans. This is in agreement with the study of Hascall (1980), who showed that the Ruthenium Red-positive granules, as observed in rat chondrosarcoma, corresponded to the condensed form of proteoglycan monomers. This observation also corresponds with the study of Scott (1980), who considered the Cuprolinic Blue-positive precipitates as observed in rat tail tendon, to be proteoglycans and the filamentous structure of precipitates indicated the collapse of the glycosaminoglycan side chains on the protein core of the proteoglycan (individual proteoglycan monomeric unit which exists as an extended "bottlebrush" structure in its native state).

The present study shows that the sulfated proteoglycans from porcine aortic conduit form differently shaped precipitates with the tetracationic dye Cuprolinic Blue and exhibit a site specific distribution within the arterial tissue. Three proteoglycan populations have been (Gardell et al., 1980; Oegema et al., 1979; Salisbury and Wagner, 1981) separated based on hydrodynamic size: a large chondroitin sulfate proteoglycan (CSPG) which is capable of forming high molecular weight aggregates with hyaluronic acid, a smaller dermatan sulfate proteoglycan (DSPG), and heparan sulfate proteoglycan (HSPG). Types I, II, and III proteoglycan-Cuprolinic Blue precipitates reported in this study may be

attributed to three individual proteoglycans having different chemical composition.

Iozzo et al. (1982) emphasized the possibility that the size and shape of the cationic dye-positive structures reflect the chemical size of individual proteoglycan monomers. They have shown a direct relationship between the number and size of the cationic dye-positive granules and the quantity and molecular size of the proteoglycan isolated from the interstitial matrix of normal and malignant colon. Similar techniques have been applied to blood vessels as well (Huang et al., 1984; Richardson et al., 1980). Quantitative morphological techniques were used in these studies to demonstrate that the number of cationic dye-positive granules increases in the interstitial space in areas of blood vessels undergoing intimal hyperplasia. Biochemical studies also demonstrated that these regions preferentially accumulated proteoglycan (Wight, 1985).

Studies (Oegema et al., 1979; Wegrowski et al., 1986) showed that dissociative agents solubilize mainly chondroitin sulfate and small amounts of dermatan sulfate proteoglycans. It was reported that the aortic chondroitin sulfate proteoglycan is a hydrodynamically large molecule (molecular weight approximately 1.4×10^6: Oegema et al., 1979), which could be represented by

the large proteoglycan-Cuprolinic Blue precipitates in soluble matrix. Studies using a monoclonal antibody against aortic chondroitin sulfate proteoglycan (CSPG) revealed that this proteoglycan is confined to regions of the arterial interstitium which contains the large cationic dye granule (Lark and Wight, 1986). Volker and coworkers (1986) found large electron dense proteoglycan-Cuprolinic Blue precipitates in soluble matrix of bovine aorta and suggested this precipitate is due to the presence of chondroitin sulfate proteoglycan. Furthermore they found that the precipitates in the soluble matrix are attached to 2 nm fibrils. They suggested that association of proteoglycan-Cuprolinic Blue precipitates in a repeating distance with 2 nm fibrils indicates the presence of a proteoglycan-hyaluronate complex. The appearance of this precipitate in soluble matrix of bovine aorta is similar to that of the type I precipitates reported in this present study. Therefore, large type I precipitates in porcine aortic conduit may represent proteoglycans mainly containing chordroitin sulfate.

Type II proteoglycan-Cuprolinic Blue precipitates associated with the collagen fibrils are separated from each other according to the major banding period of the collagen fibrils, indicating a specific interaction between proteoglycan and collagen. It is otherwise difficult to explain the very regular distribution of proteoglycan precipitates along the collagen fibrils.

Ultrastructural association of cationic dye-positive structures and collagen fibrils has also been demonstrated in a variety of tissues, for instance, predentin (Nygren et al., 1976), primate arteries (Wight and Ross, 1975), and embryonic cornea (Trelstad et al., 1974). Using either Ruthenium Red or Alcian Blue for staining, the anionic granules in these tissues were mostly observed to be next to the collagen fibrils. However, Scott (1980), using Cuprolinic Blue and the critical electrolyte concentration method, found that in tendon, electron dense precipitates were distributed on the outside of the collagen fibers, and there were also precipitates running parallel to the fibril axis. In this study, it was found that there were few precipitates running parallel to the axis in comparison to those running perpendicularly. Furthermore, precipitates connecting adjacent collagen fibrils to each other could also be detected. Hence it is possible that collagen fibrils in the porcine aortic conduit are surrounded by a network of proteoglycans and in such a way as to provide structural coherence.

Dermatan sulfate has been shown to bind specifically to collagenous fibers in a culture system (Gallagher et al., 1983). Experiments in solution also demonstrated that dermatan sulfate interacts more strongly with collagen than does chondroitin sulfate (Öbrink, 1973). Studies have shown that the proteoglycans associated with collagen mostly have dermatan sulfate and smaller amounts of chondroitin sulfates. Aortic dermatan sulfate proteoglycan

is a hydrodynamically smaller molecule than aortic chondroitin sulfate proteoglycan (Rowe and Wagner, 1985). Chondroitin sulfate proteoglycans can be solubilized both by dissociative solvents and collagenase, which suggest that at least two species of chondroitin sulfate proteoglycans may be present in the aorta. Therefore, type II Cuprolinic Blue (CB)-positive precipitates reported in this study could represent proteoglycans containing mainly dermatan sulfate. Dermatan sulfate proteoglycan have also been isolated from skin and tendon (Fuji and Nagai, 1981; Vogel and Heinegard, 1983), and they are characterized by the presence of a few (1-4) dermatan sulfate chains. In this study, less electron dense type II proteoglycan-Cuprolinic Blue precipitates, in comparison with the type I and III precipitates of the porcine aortic conduit, might be due to this limited number of glycosaminoglycan chains in the proteoglycans associated with collagen.

Two important functions probably occur at the gap zone of collagen: cross-linking of collagen fibrils, and calcification of collagenous matrices. A proteoglycan molecule may influence both functions with its high charge and considerable excluded volume. Radial growth of the fibril, by accretion of collagen molecules or fusion of fibril, could be inhibited by restricted access to the cross-linking sites, due to proximity of the proteoglycan. It appears from electron microscopy that proteoglycans must be displaced during fibril fusion

(Scott et al., 1981). Scott and Orford (1981) showed that dermatan sulfate containing proteoglycans are localized to the d-band (gap region) of the collagen I fibril in tendon, and further study (Vogel et al., 1984) indicates that a dermatan sulfate proteoglycan isolated from tendon is capable of inhibiting type I and II collagen fibrillogenesis in vitro. Whether the small dermatan sulfate proteoglycan present in blood vessel exhibits a similar activity is not yet clear, but a periodic association of a small proteoglycan precipitate to aortic conduit collagen was found in porcine aorta in this study as well as in collagen gels populated by arterial smooth muscle cells (Lark and Wight, 1986). A similar enrichment of proteoglycan was seen within collagen gels from cultures of lung (Vogel et al., 1981), skin (Gallagher et al., 1983) fibroblasts, and endothelial cells (Winterbourne et al., 1983).

Hydroxyapatite initially appears in newly calcified bone in the holes of the gap zone of collagen. Proteoglycans in the spaces later to be occupied by hydroxyapatite would probably have to be displaced or removed for calcification to occur. This would suggest that proteoglycan contents would be much lower in calcified as compared with uncalcified matrix, since disappearance of proteoglycan is a necessary prerequisite to the process of mineralization.

By connecting collagen fibrils with each other, proteoglycans in collagen may also contribute to achieve their functioning together as fibers, rather than as separated fibrils. In such a way they may provide structural coherence.

The intermediate-sized type III precipitates represent elastin-associated proteoglycan. Pasquali-Ronchetti et al. (1984) reported that glycosaminoglycans associated with lathyritic elastin include mainly dermatan sulfate and heparan sulfate. Vijayagopal et al.(1983) observed that the bulk of proteoheparan sulfate was released from aortic tissue on treatment with pancreatic elastase and suggested a close association of heparan sulfate with elastin. Dalferes et al. (1987) also found that there are greater amounts of heparan sulfate in the elastase hydrolyzed tissue extracts than in dissociative solvent extracts in human aorta, and they suggested that this proteoglycan is in large part bound to elastin of the tissue. Heparan sulfate proteoglycans are important in the maintenance of the integrity of the intimal surface of the arterial wall as well as their role in atherogenesis. A decrease in heparan sulfate proteoglycans was observed in atherosclerotic lesions (Berenson et al., 1984). This finding may be accounted for by large areas of distorted and depleted elastic fibers which may be important in the initiation of atherosclerosis. Therefore, type III proteoglycan precipitates described in this study probably contain mainly heparan sulfate.

The affinity of type III precipitate for elastin resembles similar findings by Kadar et al. (1972) who demonstrated numerous granules associated with newly formed elastic fibers in chick embryo aortas. The observation of close association of elastin and proteoglycan-Cuprolinic Blue precipitate in this study suggests that one function of arterial proteoglycan may be to hold elastic fibers together. Furthermore, the proteoglycan-Cuprolinic Blue precipitates appear to interconnect elastin with collagen, suggesting that the major intercellular matrix components are held together in a type of meshwork by proteoglycans. These linkages might also serve to keep the major aortic conduit tissue components separated and thus could help to maintain turgor in the artery wall. In this capacity, the proteoglycans might function as a type of plastic interstitial substance, important in absorbing and/or dissipating stress imposed on the aorta under various physiological conditions (Balazs and Gibbs, 1970).

The histologic status of the fresh and cryopreserved porcine aortic conduit did not differ markedly. The normal tissue architecture was not affected markedly by the cryopreservation procedure as neither alteration of elastic structure, fibrous proteins nor alteration of nuclear distribution or smooth muscle cell morphology was detected.

The size and spatial distribution of proteoglycan-Cuprolinic Blue precipitates in both fresh and cryoporeserved porcine aortic tissues appeared to

be qualitatively similar. These results correlated well with the results of a quantitative biochemical study of sequentially extracted tissue, which demonstrated that there is essentially no difference in the relative quantitative distribution of proteoglycans in fresh and cryopreserved porcine aortic tissue. It can be suggested that the functional performance of proteoglycans in fresh and cryopreserved tissue is probably similar, especially with respect to the role of proteoglycans in calcification.

Pathological studies (Maxwell et al., 1989; Miller and Shumway, 1987) in aortic allograft calcification demonstrate prominent calcification of the aortic wall and, to a lesser extent the valve leaflets. The observed aortic wall mineralization involves calcification of elastin as a prominent feature. Intrinsic calcification (calcific deposits both in cellular remnants and in collagen fibrils) of the aortic allograft was also demonstrated in rat circulatory studies.

Urist and Adams (1967) reported on factors that influence calcification in transplants of the aorta as a result of their experiments of implanting aortic fragments into the anterior chamber of the eye in rats. They demonstrated that various physical and chemical properties of the implant are important. This work on chemical factors influencing the incidence of calcification is clearly of great potential importance. In proportion to the amount of calcium, phosphate

ion association may occur such that the tissue contains a relatively soluble tripartite elastin-calcium-phosphate complex. Elastin can respond more rapidly than other tissues to hypercalcemia, can bind more calcium ion, and can calcify more rapidly than other tissues. Uptake of phosphate ion is influenced chiefly by the amount of calcium in elastin, since elastic tissue binds almost no phosphate (Eisenstein et al., 1964). Phosphorylated aorta, like phosphorylated tendon, is less, rather than more, calcifiable (Urist and Adams, 1967).

The quantitative biochemically detectable calcium in the cryopreserved porcine aorta tissue was more than that in fresh tissue. The amount of phosphorus in cryopreserved aorta tissue was similar to that in fresh aorta tissue. If the elastin structure, normally saturated with protein-calcium complexes, develops the capacity to calcify after degradation or splitting of fibers and increased rate of uptake of calcium ions, the implanted cryopreserved allograft might calcify because of a higher content of total calcium.

It has been suggested that an immunological basis exists for calcification, demonstrating significantly more severe valvar calcification in allografts to transgenic rats compared with syngeneic rats (Khatib and Lupinetti, 1990). Gonzalez-Lavin et al. (1988) also demonstrated the importance of immunologic influences on allograft valve degeneration. They found that calcium content of

allograft valve leaflet and aortic conduits was greater when the transplant was performed between unrelated dogs than when the transplant was between littermates. It was initially believed that valve allografts may be immunologically privileged since the graft does not retain cellular viability following transplantation or the cells are rapidly replaced by those of recipient origin. However, it is now acknowledged that valve grafts are antigenic and provoke host immune response (Gonzalez-Lavin et al., 1988; Heslop et al., 1973). Some investigators have proposed that ABO matching between donor and recipient should be performed if possible, or it is necessary to consider immunosuppression, at least temporarily, after implantation of homovital grafts in growing individuals (Gonzalez-Lavin et al., 1988; Yankah et al., 1988). Fresh aortic allografts are antigenic and their antigenicity is not altered by cryopreservation (Cochran and Kunzelman, 1989). Jonas et al. (1988) suggested that cryopreservation might enhance immunogenicity since they observed more lymphocytic infiltration of the cryopreserved conduits relative to the fresh grafts in a sheep model. Cellular viability of cryopreserved allograft valves is superior to that of tissues preserved by other methods and the antigenic effect may result from enhanced endothelial preservation since the endothelium of vascular structures is the most immunostimulatory of all vascular components (Pober et al., 1986).

It has been speculated that persistence of the endothelium may be desirable in grafted valve leaflets to protect the physicochemical balance of the matrix and facilitate the retention of basic structural features until new fibrous connective tissue develops (Armiger et al., 1985). However, since the endothelium is antigenic and incites host immune response, its value is in question. Processing and storage techniques that result in gentle displacement of the graft endothelium may be optimal.

The extended performance of the cryopreserved allograft valve was previously ascribed to persisting cell viability and linked to retention of the original viable cell population of the donor tissue (O'Brien et al., 1987; O'Brien, Kirklin et al., 1987). The evidence for this in human cryopreserved valves is demonstrated from cell growth that occurred on tissue cultures of explanted human valves. Chromosomal analysis showed that donor cells persisted in leaflet tissue of a transplanted allograft valve for 9¼ years. However, Allen and coworkers (1991) demonstrated that no donor cells were cultured from aortic valve leaflets in a growing sheep model and retention of viable donor cells does not appear to be essential for preservation of valve function in this model. Indeed, persistence of donor cells was more prevalent in calcified allograft wall specimens than in unaffected valve leaflets. They suggested that the retention of living donor cells may induce degenerative

changes, perhaps through an increased immune response to the graft. Maxwell et al. (1989) suggested that the development of calcification in allografts is much less extensive than in xenografts because of the different mode of initiation of calcification in the two types of graft. The persistence of donor fibroblast remnants in the glutaraldehyde-treated xenografts is not seen in the antibiotic-sterilized allografts in which all donor cells undergoes necrosis and disappear (Gavin et al., 1973).

In a study of single cusp homologous aortic valve replacement in dogs, it was demonstrated that improved leaflet mobility and pliability occurred in nonviable leaflets that had been repopulated by the ingrowth of host cells as compared with viable valves containing residual donor cells (Mohri et al., 1968). Studies in explanted fresh human allograft valves (Armiger et al., 1983; Gavin et al., 1973) suggested that freedom from valve deterioration may be related to ingrowth of host tissue onto the connective tissue of fresh homograft leaflets.

Fresh and cryopreserved human aortic valves when used as allografts on stents have a tendency to calcify whereas those used as free hand orthotopic aortic valve replacements appear to calcify only rarely and have increased longevity (Angell et al., 1989; Gavin et al., 1973). If access of recipient cells

to the leaflets is important in preserving long-term valve function, the presence of the stent may interfere with ingrowth of recipient cells onto the valve matrix, rendering the stented allograft at risk for more rapid deterioration. Alternatively, the presence of the stent may facilitate calcification by alteration of stresses placed on the valve during repeated function.

The presence of viable donor cells may be a key to extended protection from degenerative changes, but the effect may correlate more with the existence of living host cells and repopulation phenomena rather than the persistence of donor cells. Therefore, the recipient cell population may play a significant role in the protection of the allograft from degenerative changes, or they may contribute to subsequent calcification through death and release of hydrolytic enzymes. These enzymes would presumably alter the content and size of proteoglycans.

No effective therapy presently exists for removal of established cardiovascular calcific deposits and no clinically useful preventive measures are available. However, a broad range of compounds and treatment processes demonstrably reduced bioprosthetic calcification in animal models, but had potentially serious ramifications through deleterious effects on bone metabolism (Levy et al., 1987). Strategies for controlled release of antimineralization

compounds in the vicinity of the valve might serve to reduce the systemic toxicity of this approach (Golumb et al., 1986). Criteria need to be considered to evaluate both efficacy and safety for each of the anticalcification approaches considered.

Detergent pretreatment of bioprosthetic heart valve tissue inhibits subdermal bioprosthetic leaflet calcification and delays the onset of circulatory deposits in some studies (Jones et al., 1988; Thiene et al., 1986). The mechanism of action of detergent mitigation of calcification may be due to either the extraction of membrane lipids, net surface charge modification, or removal of endogenous alkaline phosphatase. Diphosphonates are synthetic analogues of pyrophosphate and can inhibit hydroxyapatite crystal growth (Fleisch, 1989). Irving et al. (1966) demonstrated that polyphosphates prevent calcification even in aorta saturated with calcium complexes produced by hypercalcemia; they postulate an acidic phospholipid of unknown composition to be a local factor in calcification of aorta. Other reagents are probably less selective than pyrophosphate and may prevent crystal growth of apatite. Fe^{3+} and Al^{3+} are said to block nucleation by inhibiting membrane-linked calcification in devitalized cells (Webb et al., 1988).

Based on the findings presented in this study in conjunction with research

presented by other groups, the following suggestions may be made:

(1) The standard preimplantation processing procedure such as utilized by LifeNet Transplant Services for human tissues does not have a significant damaging effect on the microscopic structure of porcine aorta conduit tissue.

(2) Quantitative proteoglycan content of porcine aorta conduit tissue is not changed after cryopreservation.

(3) The size distribution of proteoglycans in fresh and cryopreserved porcine aorta tissue is similar. This observation suggests that enzyme-mediated alteration in the content and size of proteoglycans during cryopreservation is minimized.

(4) Proteoglycans in porcine aorta tissue are primarily present in a form which is extractable by use of a dissociative solvent, and the remainder of the proteoglycans are associated with the collagen or elastin matrix. The results of the sequential extraction of proteoglycans in this study suggest that the relative proportions of proteoglycans in valve conduit are essentially similar in fresh and cryopreserved tissue.

(5) The topological distribution of proteoglycans within aorta conduit tissue was not affected by the cryopreservation procedure.

(6) There is more biochemically detectable calcium in cryopreserved tissue than in fresh tissue. Cryopreserved aorta conduit tissue might be more likely to calcify after implantation because of this higher content of total

calcium if this characteristic contributes to subsequent calcification.

(7) The phosphorus content of fresh and cryopreserved porcine aorta conduit tissue was similar which suggests that cryopreservation processing does not influence the tissue phosphorus content.

It has generally been assumed that retention of a viabie fibroblast cell population in a cryopreserved allograft heart valve improves long-term durability. The basis of this improved durability was thought to be due to a continued synthesis and repair of the valve matrix. As an alternative explanation, it might be suggested that retention of a viable donor fibroblast cell population in cryopreserved allograft valves is important in that a reduction in cell death limits the release of hydrolytic enzymes into the matrix space. These enzymes would degrade, to varying extents, the collagenous and noncollagenous proteins. It is suggested that degradation, i.e. alteration in quantity and size, of proteoglycans in a transplanted allograft heart valve may be responsible for the initiation of the process of mineralization as has been reported in cartilagenous tissues (bone). In addition, dead cells and their phospholipid membranes, may serve as sites for nucleation of hydroxyapatite crystallization. Previous research in this laboratory have suggested that conditions such as increased warm ischemia time results in valve conduit tissue which stimulates differentiation of a human dermal fibroblast into an osteoblast-like cell as measured by increased

levels of the enzyme alkaline phosphatase (Hu, 1992). Increased levels of this enzyme have been found to be associated with the process of mineralization, presumably by increasing the effective concentration of inorganic phosphate at essentially the same tissue location where proteoglycan degradation is altering the distribution of free and complexed calcium ion.

The present study suggests that the procedures associated with cryopreservation do not alter the content or size distribution of proteoglycans in conduit tissues. These studies, however, do not take into account the possible effects that warm ischemic times might have on changes in proteoglycan content and the effects that processing of these valves for cryopreservation might have on tissues with increased cell death. Following transplantation of a cryopreserved allograft valve with some degree of warm ischemic time and cell death, recipient mesenchymal cells may migrate into the conduit portion of the allograft where they may be induced to differentiate into an osteoblast-like cell with increased levels of alkaline phosphatase. Via a process of mineralization similar to that for cartilage (bone formation), i.e. alteration in proteoglycan quantity and size, hydroxyapatite crystallizes may nucleate on membrane fragments of dead donor cells, and mineralization may proceed along the elastin/collagen fibers until degenerative calcific deposits are formed. That recipient cells are less likely to migrate into leaflet tissues, as opposed to the

conduit tissues, may explain the greater tendency of conduit tissue to calcify following transplantation.

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AUTOBIOGRAPHICAL STATEMENT

Yun Hee Shon was born in Dae-Gu, South-Korea on 15 August 1960. She received a Bachelor of Science in Biology from Kyung-Buk National University in South-Korea in 1982. After graduation from the university, she was general science teacher at So-Sun Girls' Middle School, Dae-Gu South-Korea for one year and biology teacher at Kyung-Sang Girls' high school, Dae-Gu South-Korea for two years. She came to America and studied at Old Dominion University. In August 1987, she started research and study in Dr. Christopher Osgood's molecular biology laboratory as a graduate research assistant. Research activity was recombinant DNA technology. Study was performed to assess the effectiveness of the integration of the plasmid DNA into the drosophila's genome. Work included the reconstruction of plasmid DNA pCO-3'NEO by the cloning experiment. She received her Master of Science Degree in biology in 1989. Since August 1989 she has held a research assistant position under the direction of Dr. Lloyd Wolfinbarger, Jr. Research activity was in cancer chemotherapy and cardiovascular system. Study was performed to determine the role of nutrients in cancer chemotherapy by the dietary

changes prior to chemotherapy to improve the effectiveness of the chemotherapeutic treatment. Study also included the evaluation of the effects of cryopreservation on the proteoglycan content of porcine cardiovascular tissue.