AORTIC STRUCTURAL GLYCOPROTEINS:

FACT OR ARTIFACT?

by

Phillip R. Bach

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(Professor in charge of thesis)

[redacted]

(Chairman, Graduate Council)

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1 Historical Setting.

1.1 Introduction.

Connective tissues are made up of fibrous proteins and a variety of polysaccharides known collectively as "ground substance" (84). The two commonly known fibrous connective tissue proteins are collagen and elastin. But within the last twenty years, a third type of insoluble connective tissue protein has been described. Robert and Comte (109) have named it glycoprotéins de structure, or structural glycoproteins. Keeley, Labella and Queen (5 9) describe their preparation as alkali-soluble protein. Timpl, Wolff, and Weiser (131) speak of acidic structural protein, and there are other names as well. I shall call this class of connective tissue proteins structural glycoprotein, or SGP, even though it may be neither structural nor a glycoprotein in all cases.

It is worth noting here that connective tissue chemistry differs from most other biochemistry in one very striking respect. Connective tissue proteins are covalently crosslinked in a fibrous network. Except for small amounts of soluble precursors, they are not extractable in saline, and cannot be solubilized by the gentle techniques normally employed in biochemical studies. The task of extracting a connective tissue protein is therefore a matter of selective degradation, with the objective being to sever the bonds between molecules without severing the bonds within molecules. As this review will show, the actual seldom attains to the ideal, and any given extraction procedure is an attempt to maximize solubility while minimizing degradation.

1.2 Early Chemical Studies of SGP.

Evidence for the existence of SGP as a separate, sparingly soluble component of connective tissue has been accumulating since 1861. That year, Mueller (cited by Richards and Gies (100)) mentioned a protein which was tightly bound to elastin, removable only by boiling in dilute KOH. In 1902, Richards and Gies (100) wrote of the same material. They reported that it contained a higher percentage of sulphur than did unfractionated elastic fibers. The next report was by Lowry, Gilligan, and Katersky (68) in 1941, who noted that the sulphur in this protein was in a reduced form. With the advent of the automatic amino acid analyzer (126), many analyses of elastin appeared (6)(45)(47)(65). They reflected varying amounts of a contaminant rich in polar amino acids and cysteine. The amount of this contaminant decreased as the severity of the elastin purification schemes increased.

In the 1960's, several investigators turned their attention to the polar contaminant itself. Many of them chose methods of extraction less destructive than cold or hot alkali. Berenson and Fishkin (37) used normal saline to obtain very small amounts of an SGP-like material from bovine aorta. Gotte, Serafini-Fracassini, and Moret (44) extracted autoclaved bovine ligamentum nuchae with 2 M NaCl. Adlemann, Marquardt, and Kühn (1) treated rat skin successively with 2 M NaCl and collagenase. Robert and Comte (106) extracted a number of different tissues with hot 5% trichloroacetic acid (TCA) to remove collagen, and then with 8 M urea.

Other investigators chose alkali extractions. Barnes and Partridge (9) used cold 0.1 N NaOH to extract a glycoprotein from human aorta. They purified it by ion exchange chromatography and characterized it extensively. Keeley, Labella, and Queen (59) examined protein obtained from chick aorta with 0.1 N NaOH at 100°C.

Amino acid and carbohydrate compositions of most of these preparations were reported. Characteristically, they contained much higher amounts of polar amino acids, especially aspartic and glutamic acid, than did collagen or elastin. But the analyses also showed variable, often large amounts of glycine and hydroxyproline, indicative of collagen or elastin contamination (12). Finally, most contained small amounts of carbohydrates.

The evidence thus appeared to demonstrate the existence of a new connective tissue component very different from collagen, elastin, or ground substance polysaccharides. In 1968, Robert and Comte (109) proposed that the new class of proteins be called "structural glycoproteins", defined by the authors as having a high content of polar and aromatic amino acids, some cysteine and hydroxylysine, little if any hydroxyproline, and a unique carbohydrate moiety.

1.3 Microfibrils

While biochemists were exploiting the amino acid analyzer, other investigators were using another recent development, the electron microscope, to examine connective tissues in detail. The banded, rod-like fibers of collagen became familiar objects to electron microscopists. As useful magnifications were increased, smaller, less

regular fibers were seen. Jakus (Jakus, M.A., cited by Low (67)) first reported them in rat cornea in 1954. By 1962, they had become so well-known that Low (67) assigned them the name of "microfibrils", characterizing them as extracellular, about 80 Å in diameter, and usually crooked and branching. Three years later, Haust (51) published an excellent survey of the occurrence of microfibrils. She showed their presence in such diverse tissues as skin, conjunctiva, endocardium, tendon, arthritic knee joint, interstitium of testis, and aorta. Microfibrils were found to appear in fetal tissues earlier in histogenesis than does elastin (48)(49)(5 2). Haust, More, Bencosme, and Balis (52), and later Ross and Bornstein (113) proposed that they serve as a template for subsequent deposition of elastin. Since microfibrils are also found in tissues which have little or no elastin, such as tendon (49) or basement membrane (67), this cannot be their only function.

In 1969, Ross and Bornstein published what has become a classic paper in the field (113). Working with fetal bovine ligamentum nuchae, they first prepared intact elastic fibers by collagenase digestion and extraction with 5 M guanidinium chloride. Under the electron microscope, these fibers showed the usual fringe of microfibrils on their outer surfaces. They then extracted the fibers with 5 M guanidine containing dithioerythritol (DTE), one of the methods of purifying elastin developed as a gentler substitute for hot alkali. Returning to the microscope they saw that the elastic fibers had been stripped of microfibrils. Digestion with trypsin or chymotrypsin achieved the same

results as extraction with guanidine and DTE, while a short digestion with elastase gave a bundle of microfibrils from which the central core of elastin had been largely removed. Hyaluronidase had no effect on either fiber. The guanidine/DTE extracts had amino acid compositions showing high percentages of polar amino acids, very unlike hydrophobic elastin. The extracts were estimated to represent 10% to 15% of the dry weight of the ligament. Ross and Bornstein concluded that microfibrils are made of a protein very unlike collagen, elastin, or ground substance polysaccharides. Although Ross and Bornstein did not refer to SGP, the compositions of microfibrils and SGP were remarkably alike. In 1971, Robert, Szigeti, Derouette, Robert, Bouissou, and Fabre (104) carried out experiments prompted by the work of Ross and Bornstein. They suggested that microfibrils "are derived from or are identical with" SGP as defined by Robert and Comte (109). This meant only that the two entities, SGP and microfibrils, shared similar compositions and could be extracted from the tissues with the same solvents.

1.4 Chemical Studies of SGP.

In attempts to obtain pure, intact SGP, many extraction procedures have been employed by many different investigators. Table I lists the majority of these preparations chronologically. A few typical papers will be described to illustrate the complexity of the situation.

Keeley, Labella, and Queen (59) treated chick aorta of bovine ligamentum nuchae sequentially with chloroform-methanol, 0.2 M saline, 0.5% acetic acid, autoclaving, and finally 0.1 N NaOH at 100°C. They studied the NaOH extract. Timpl, Wolff, and Weiser (131) treated a

variety of tissues from several species with saline, 0.2 M borate (cation not specified), chloroform-methanol, 5% TCA at 90°C, 8 M urea, and 0.2 N NaOH at 4°C. They studied the urea and NaOH extracts and found them to be quite similar in composition and isoelectric pH. Barnes and Partridge (9) extracted human aorta with 0.1 N NaOH at 1°C after prewashes with saline and ethanol-ether. Ross and Bornstein (113) homogenized fetal calf ligamentum nuchae in 5 M guanidine, and digested the residue with collagenase. The collagenase residue was extracted with more guanidine containing 0.05 M dithioerythritol. They examined this last extract. Moczar and Robert developed a sequence they have called "chemical dissection" (76), and treated several tissues this way. They used acetone-butanol, 1 M $CaCl_2$ buffered with tris citrate, 2.7% TCA at 90°C, 8 M urea, 8 M urea plus 0.1 M 2-mercapteothanol, and finally 1 M KOH in 80% ethanol. The urea extracts were examined. Anderson (3) used 3 M $MgCl_2$ to obtain a crude extract from bovine tendon. He then isolated the glycoproteins from the extract by affinity chromatography. In an interesting and unusual preparative scheme, Takagi (129) fractionated chick embryo aorta homogenates on sucrose gradients by centrifugation. As seen under the electron microscope, elastic fibers precipitated with nuclei, while intact microfibrils were in the microsomal fraction.

While many investigators have continued to work with normal connective tissues, some have turned to various alternatives in their efforts to obtain SGP in less degraded form. Rajamäki and Kulonen (98) implanted viscose-cellose sponges subcutaneously in rats,

removed them after 21 days, and digested the resulting granulomas with collagenase. They then isolated and characterized a material designated as structural glycoprotein from the supernatant. Sear, Grant, and Jackson (120) purified an SGP-like protein from cultured fibroblasts, and Muir, Ross, and Bornstein (82) repeated their microfibril preparation on cultures of arterial smooth muscle cells.

Yields of SGP from these procedures vary widely. Rajamäki and Kulonen (98) reported that their purified species represented 0.03% of the dry weight of the granuloma. McCullagh, Derouette, and Robert (72) reported that the urea extract was 40.5% of the dry weight of human aorta. Values of 5% to 15% are more typical. (See Table I.)

In many preparations (Table I), a fraction of the SGP was extracted with the aid of a disulfide bond reducing agent such as 2-mercaptoethanol or dithioerythritol. The existence of such a fraction implied that SGP was crosslinked, either to itself or to other insoluble proteins, by disulfide bonds. In 1970, Furthmayr and Timple (39) extracted the crosslinked SGP fraction with the aid of mercaptoethanol, sodium borohydride, or NaOH. After lyophilization, the extracts were not soluble in denaturant alone unless more reducing agent was added. Blocking of free thiol groups with iodoacetamide before lyophilization allowed the lyophilized material to be redissolved completely in denaturant alone. Furthmayr and Timpl (39) concluded that a fraction of SGP is crosslinked to itself by disulfide bonds. Rigas[1] has suggested that the disulfide bonds may form in the

[1] D.A. Rigas, Personal Communication, 1974.

tissue during extraction with denaturant in the presence of atmospheric oxygen. Published data have not ruled out this possibility.

1.5 Immunology of SGP.

Seeking to find out if different SGPs are really the same or related proteins, a number of investigators have turned to immunochemistry. Wolff, Fuchswanns, Weiser, Furthmayr, and Timpl (138) found cross-reactivity, by gel diffusion in agarose and by passive hemagglutination, between SGP preparations from different tissues of the same species, between the same tissue of different species, and even between different tissues of different species. Calf joint capsule extracts revealed complete identity with calf skin extracts, and partial correspondence with human skin and human placenta extracts. Robert, Grosgogeat, Reverdy, Robert, and Robert (102) found cross-reactivity by gel diffusion between urea extracts of human or pig aorta and the $CaCl_2$ extracts which preceded them. The residual elastin also cross-reacted with the urea extracts. The authors suggested that there were small amounts of SGP and elastin in all three fractions. Also by immunodiffusion in agarose, McCullagh, Derouette, and Robert (72) found antigenic similarities between urea extracts of elephant and human aorta, but not between the elephant aorta extract and extracts of calf tendon and cornea. They found that the urea extracts of elephant aorta differed from subsequent urea-mercaptoethanol extracts. Wolff, et al (138) had earlier shown that some of the SGP antigenic determinants are destroyed by reduction with mercaptoethanol.

Studies have confirmed that SGPs from many species and tissues are similar, if not identical, immunologically. They have also shown that SGPs extracted with such mild buffers as $CaCl_2$ have some similarity to SGPs extracted with urea, or with urea and mercaptoethanol. These similarities led Wolff et al (138) to suggest that the more soluble SGP fractions might represent precursors of the less soluble fractions. Robert, Robert, Moczar, and Moczar (111) have made the same suggestion.

1.6 Metabolic Studies.

Although the precise nature of SGP is yet to be defined, a number of metabolic studies of SGP have been published. Robert and Parlebas (106) in 1965 showed that labelled glycine, proline, and glucose were incorporated more rapidly into SGP than into collagen of cultured calf or rabbit corneas. In a 1972 review of their work, Robert et al (111) report similar findings from tendon, sclera, and aorta. Ouzilou, Courtois, Moczar, and Robert (86) compared the rate of incorporation of ^{14}C-lysine into SGP and collagen of aorta tissue cultures from rabbits of increasing ages. They found that synthesis is highest in the newborn, decreasing greatly by six months of age. In all cases, SGP incorporated label more rapidly than did collagen or elastin. DeBacker, Pizieux, and Moschetto (27) report a decrease in the percentage of SGP-like material associated with aortic elastin in human fetal aortas as the fetus develops. This agrees with an earlier paper by Keeley and Labella (60), who examined chick embryo aorta. In 1971, Downie, Labella, and West (29) found that pregnant rat uterus contained a higher percentage of alkali-soluble protein than did the

uterus of virgin rats. This protein was shown to be synthesized and degraded rapidly (30). The data suggest that SGP is metabolically much more active than collagen or elastin.

SGP has also been examined in relation to disease processes, principally atherosclerosis. Robert et al (102) found that injections of human or pig aorta SGP produced atherosclerosis-like lesions in 50% of recipient rabbits. McCullagh et al (72) noted that SGP content and atherosclerotic lesions both increased distally along elephant aorta. They suggested that SGP may be involved in the disease process. This idea was supported by Ouzilou, Robert, Robert, Bouissou, and Pierragi (85), who noted a relative increase in the percentage of SGP in atherosclerotic over normal human aorta. Keeley and Partridge (62) have found that an amount of SGP proportional to the amount of calcium is insoluble, even in NaOH, until removal of the calcium from calcified human aortic plaques. They suggest SGP plays a role in calcification. Saito and Yosizawa (116) have reported percentage increases of sialic acid-containing glycopeptides derived from pronase-digested plaques of human aorta, as compared to unaffected tissue. They imply that these glycopeptides are from the SGP component of the tissue. These reports suggest that SGP may play a role in the etiology of atherosclerosis.

1.7 Dissimilarities Between SGP Preparations.

The studies of SGP immunology, metabolism, and pathology are difficult to interpret, because in them SGP is defined only by extraction procedure and amino acid composition. Although SGP preparations show similarities in content of polar and aromatic amino acids, and

seem to be related by immunochemical criteria, they are definitely unlike one another by such criteria as their content of glycine and hydroxyproline, or their molecular weight. For example, one SGP extracted from pulmonary tissue by Francis and Thomas (38) had 69 residues of glycine per 1000 amino acids, while the elastic fiber glycoprotein prepared from elephant aorta by McCullagh et al (72) contained 160 residues of glycine. A high content of glycine can be explained as contamination by collagen or elastin, but the authors report the complete absence of hydroxyproline, long considered a marker for collagen and elastin (12).

The molecular weights of SGP from different preparations vary over two orders of magnitude. Wolff et al (138) examined their extracts by polyacrylamide gel electrophoresis (PAGE) in sodium dodecylsulfate (SDS) and 2-mercaptoethanol. They found many bands in the range from 15,000 to 88,000 d, and speculated that a small monomer of approximately 5,000 d was polymerized to form the various bands seen. Barnes and Partridge (9) calculated a molecular weight of 80,000 d for their glycoprotein from human aorta. Moczar and Moczar (77) have reported an SGP subunit of molecular weight 35,000 d from porcine aorta. Sear et al (120) described a glycoprotein from fibroblast cultures which had a molecular weight of 200,000 to 250,000 d. All had the amino acid composition of SGP as originally defined by Robert and Comte (109).

Recently, three laboratories have characterized what they believe to be SGP monomers with molecular weights of 12,000 to 16,000 d. Davril and Moschetto (26) have identified a species from porcine aorta

which emerged from an agarose column in urea with an apparent molecular weight of 12,500 d. Serafini-Fracassini, Field, and Armitt (122) have characterized extensively a protein from bovine ligament which had a molecular weight of 14,000 to 15,000 d by three independent techniques. It had a single N-terminal residue, glycine. And Randoux, Cornillet-Stoupy, Desanti and Borel (99) have described two putative SGP subunits from rabbit skin. Both had molecular weights of 16,000 d by polyacrylamide gel electrophoresis in SDS or gel filtration in SDS. The N-terminal amino acid of one of these proteins was glycine, in agreement with Serafini-Fracassini et al. These three reports must be given more weight than many of the other reports, simply because they agree with one another, and because they have avoided many of the pitfalls of earlier papers reporting the molecular weight of SGP, pitfalls I shall now describe.

Most SGP preparations are heterogeneous with respect to size. There are several possible explanations for this heterogeneity. There may actually be many SGP monomers of widely different sizes. Or there may be a single, small monomer aggregated or polymerized into many larger species, as suggested by Wolff et al (138) or by Davril and Moschetto (26). Or there may be a single large monomer degraded into smaller fragments.

The mechanisms of possible degradation are many. Proteases in the tissue could degrade the monomer during extraction, a possibility noted by Muir et al (82). There is always the danger of bacterial degradation during workup and purification of extracts. Hot TCA,

often used to remove collagen from SGP preparations, almost certainly degrades the proteins. Anderson (5) suggests that such treatment hydrolyzes most of the peptide bonds involving aspartic acid. Collagenase, commonly used to remove collagen from SGP, is almost never completely specific as it comes from commercial suppliers (5)(73). Only Serafini-Fracassini et al (122) have dealt decisively with this problem, purifying the enzyme by affinity chromatography and applying stringent tests for specificity. Finally, dilute NaOH probably degrades protein, a warning published in 1902 by Richards and Gies (100) and no less valid today. Since nearly every SGP article in the literature fails to rule out one or more of these forms of degradation, data on the molecular weight of SGP must be viewed with caution.

Earlier, I discussed the immunological similarities among various SGP preparations. These findings also must be viewed with caution, in light of other immunological findings. As early as 1965, Robert et al (107) suggested that the similarities might be due to the carbohydrate moieties. In 1967, Goldstein, Halpern, and Robert (42) found that antiserum to Streptococcus A precipitated in double diffusion experiments with SGP prepared from bovine aorta or heart valve. Strep A is the pathogen of rheumatic fever. Later, Robert et al (111) reported that SGP cross-reacts with several cell surface antigens. Anderson (5) feels that the similarity among SGP preparations, and between SGP and Streptococcus A or cell surface antigens, lies in similar carbohydrate moieties, and not necessarily in similar polypeptide chains.

At this point it is not clear whether all SGP preparations represent the same molecule or family of molecules. As seen above, the literature is confusing and often contradictory. Nevertheless, as Table I reveals, most authors working in the field consider their preparations to be the same or related proteins. For example, Keeley and Labella (60) include the protein taken from whole human aorta with alkali by Barnes and Partridge (9), the SGP of Robert and Comte (109), the acidic structural protein of Timpl, Wolff, and Weiser (130), and the microfibrils of Ross and Bornstein (113) in a discussion comparing these proteins to the alkali-soluble protein (ALSP) they have obtained from chick embryo aorta. They refer to all the preparations as "similar proteins", and state their belief that ALSP is identical to Ross and Bornstein's microfibrils. Rajamäki and Kulonen (98) state that the purpose of their work was to isolate from a granuloma one of the proteins described as SGP by Moczar, Moczar, and Robert (75), as acidic structural protein by Timpl, Wolff, and Weiser (130), and as microfibrils by Ross and Bornstein (113). The identity of these various preparations to one another has been assumed by many investigators, but has not been proven.

The molecular characteristics of SGP are still very much in doubt. The need remains for basic, rigorous protein chemistry, but the nature of connective tissue proteins, especially their insolubility, makes the task a very difficult one. A sentence from Anderson's review (5) summarizes the current situation succinctly: "Lack of detail on the

structural characteristics and general properties of connective tissue glycoproteins has discouraged any but the most general biosynthetic studies."

1.8 Entrapment.

There is one more difficulty in the literature concerning SGP. It is not immediately apparent. In fact, one is hard-pressed to find any reference to it at all, and herein lies the problem. It becomes obvious only in retrospect, and conspicuous then only by its absence. It is the matter of entrapment.

Anderson (5) discusses briefly but pointedly the fact that serum proteins, and even cells such as macrophages, are always present in the connective tissue matrix, often entrapped between the fibers and inaccessible by mild extractions. He states that a technique such as immunoelectrophoresis should be used to compare easily-extractible connective tissue components to whole serum. He comments that many reports have not included this precaution. Those SGP preparations from saline extracts of various tissues must be assumed to be serum proteins until proven otherwise.

The problem is not restricted to serum proteins. Organs such as aorta or placenta, often used as source tissues for the preparation of SGP, are not purely connective tissue, but are about half smooth muscle (50). Muscle contains a major proportion of contractile proteins--actin, myosin, and several other lesser components (50)(66). Of all the papers in the SGP field, only one mentions the possibility of actin or myosin as a contaminant in the preparation. Moschetto and

Havez (80) acknowledged the presence of these proteins in pig aorta, but assumed they were quantitatively removed by pre-extractions with 0.2 M Na_2HPO_4. Their assumption appeared valid, since Hamoir (50) and others have reported that smooth muscle actomyosin (a somewhat stoichiometric aggregate of actin and myosin) is solubilized by 0.075 M salt. However, the assumption that actin and myosin are removed by salt extractions of connective tissue does not recognize the problem of entrapment. Finally, the problem cannot be avoided by turning to nonmuscular tissues such as ligament or tendon or skin, as a source of pure connective tissue proteins, since actin has recently been shown to be a component of such diverse tissues as lens, skin, liver, and brain (118)(18).

To summarize, the evidence points to the existence in connective tissue of a protein, or family of proteins, distinctly different from collagen or elastin. The proteins, which I have chosen to call SGP, share a common amino acid composition. At least some SGP takes the form of microfibrils, which cluster at the periphery of elastic fibers or in basement membranes. A plethora of procedures, from the most gentle to the most severe, have been used to obtain various fractions of SGP. There is considerable immunological similarity among SGP preparations from widely different sources, but this similarity may well be due to carbohydrate rather than protein moieties. The size of an SGP monomer is open to debate, with reports ranging from 5,000 to 250,000 d. Finally, SGP preparations have not been shown to be free from entrapped serum proteins or from the fibrous muscle proteins,

actin and myosin. In spite of these ambiguities, several metabolic and pathologic studies of SGP have been published.

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
RAT Granuloma	Tissue	water	0.6% of wet (purified)	13,000-20,000 (sed vel)	37 (1961)	first paper
COW Aorta	tissue	water	0.2 - 0.5% of wet tiss.		13 (1962)	granuloma extract (37)
CALF-FETAL Skin	tissue	water		3.15 S (sed vel)	16 (1962)	none
OX Lig. nuchae	fat-free autoclaved	2M NaCl			44 (1963)	microfibrils (personal observation)
COW Cornea	hot TCA res	water			105 (1963)	first paper
COW Cornea HORSE RABBIT CALF	hot TCA res	water			103 (1963)	sulfated sialo. (105)
COW Spleen	reticulin	pepsin	20% of dry reticulin	9,000 (N-terminal)	125 (1963)	none
CALF Lig. nuchae	autoclave residue	2M NaCl 2°C			79 (1964)	NaCl extract of ligament (44)

A glossary of abbreviations follows the last page of this table.

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
COW Aorta	wet tissue	.15M NaCl	0.3% of wet	60,000-70,000 (sed vel)	94 (1964)	water extracts of granuloma and aorta (13, 37)
CALF RABBIT Cornea in tissue culture	hot TCA res	used res.			106 (1965)	SGP or KGAG (103, 105, 107)
Cornea from CALF HORSE RABBIT HUMAN WHITE SALMON	hot TCA res	water			107 (1965)	sulfated sialo. (105)
RAT Skin	res after 2M NaCl	collagenase			1 (1966)	Water extract of skin (16) Water extract of granuloma (37) Pepsin digest of reticulin (125) SGP (107)
DOG glomerular basement membrane	membrane	8M urea + 2-ME	65% of dry membrane	132,000 (sed vel)	64 (1966)	no SGP-related papers

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
PIG HORSE SHEEP Aorta	hot TCA res	8M urea			75 (1967)	SGP from cornea (107)
CALF Cornea	hot TCA res	8M urea	1.7% of protein	30,000 (sed)	108 (1967)	SGP (103, 107)
CALF Sclera			0.6% of protein			
HUMAN Aorta	fat-free dry tissue	0.1N NaOH 1°C		80,000 (indirect)	9 (1968)	SGP (107) Saline extract of lig. nuchae (44)
COW Aorta Heart valve Synovial membrane Cartilage Skin Tendon Cornea	hot TCA res	8M urea			109 (1968)	SGP (75, 105, 107, 108)
SHEEP Aorta						

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
HORSE Aorta	hot TCA res	8M urea			109 (1968)	SGP (75, 105, 107, 108)
HUMAN Heart Valve						
RABBIT Cornea						
CALF Joint capsula Skin	hot TCA res or collagenase residue or hydroxyl-amine res.	8M urea or 2% SDS or 0.1M Na_2CO_3 or 6M guanidine	"similar"	heterogeneous aggregated (gel filtr'n)	130 (1968)	amyloid fibers (24) SGP (109)
RABBIT Skin						
HUMAN Amyloid						
RAT Skin						
HUMAN Skin						

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
CHICK EMBRYO Aorta	autoclaved fat-free tissue	0.1N NaOH 100°C	23% of autoclaved res.	12,000 (gel filtr'n)	59 (1969)	NaCl extract of autoclaved ligament (44) acidic structural proteins (130)
CHICK (1 Day) Aorta						
COW Lig. nuchae			3% of autoclaved res.			
CALF Cornea	hot TCA res	used res			74 (1969)	SGP (75, 103, 105, 107)
PIG Aorta	hot TCA res	8M urea	7.8% of dry tissue	hetero (gel filtr'n)	80 (1969)	SGP (75) NaOH extract of aorta (9)
FETAL CALF Lig. nuchae	res after 5M guan	5M guan + DTE	5-10% of dry lig.		113 (1969)	microfibrils (48, 49, 51, 67)
CHICK EMBRYO Aorta: nuclear, mitochondrial, microsomal, and supernatant fractions	saline and collagenase residue	used res.			129 (1969)	NaCl extract of ligament (44) microfibrils (48, 113)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
CALF Skin	hot TCA res	8M urea	3% of proteins		131 (1969)	SGP (103) microfibrils (48, 51, 67) saline extract of lig. nuchae (44)
	urea res	0.2N NaOH	0.4% of proteins			
CALF Joint capsula	hot TCA res	8M urea	3%			
	urea res	0.2N NaOH	0.6%			
RAT Skin	hot TCA res	8M urea	1%			
	urea res	0.2N NaOH	13%			
RABBIT (5mo) Skin	hot TCA res	8M urea	9%			
	urea res	0.2N NaOH	7%			
RABBIT(15mo) Skin	hot TCA res	8M urea	5%			
	urea res	0.2N NaOH	14%			
RAT RABBIT Skin	res after collagenase and 8M urea	8M urea + 2-ME performic acid		40,000-48,000 (PAGE/SDS)	39 (1970)	acidic structural protein (130, 131) maybe microfibrils (113)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
RAT RABBIT Skin	res after collagenase and 8M urea	$NaBH_4$ in 8M urea		15,000 (PAGE/SDS)	39 (1970)	acidic structural protein (130, 131) maybe microfibrils (113)
PIG Aorta	hot TCA res	8M urea	10% of dry tissue	0.8 S (sed vel)	76 (1970)	SGP (109) NaOH extract of aorta (9) NaCl extract of aorta (13, 94)
CALF Cornea	hot TCA res	used res.		34,000 (sed equil)	101 (1970)	sulfated sialo. (103, 105) SGP (74, 75, 108)
RAT Uterus CAT Uterus	fat-free res after autoclave	0.1N NaOH 95°C	1-1.5% of wet tiss.		29 (1971)	ALSP (59) NaCl extracts of ligament (44) acidic structural protein (131) NaOH extract of aorta (9)
RAT Granuloma	saline residue	collagenase	0.03% of wet tissue	3.1 S (sed vel)	98 (1971)	SGP (1, 75) acidic structural proteins (130, 131) microfibrils (113)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
CALF Skin	collag-enase res.	8M urea + 2-ME		15,000-88,000 (PAGE/SDS)	138 (1971)	acidic structural protein (39, 130, 131) maybe microfibrils (113) maybe SGP (76, 109) maybe basement membranes (63)
RAT Skin						
RABBIT Skin						
COW Tendon	tissue	0.2M NaCl	0.08% of dry	hetero (isoelectric focussing)	4 (1972)	SGP (109) NaCl extract of aorta (94) NaOH extract of aorta (9)
	NaCl res	3M $MgCl_2$	0.036%			
PIG Aorta	hot TCA res	8M urea			26 (1972)	SGP (76, 104) urea-soluble acidic structural proteins (131, 138)
	urea res	0.1N NaOH 37°C	4% of urea residue	12,500 and multiples (gel filt)		ALSP (59, 131)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
CHICK EMBRYO Aorta	autoclave residue	0.1N NaOH 100°C	60% of dry (11 day) 20% of dry (hatching)		60 (1972)	ALSP (59) microfibrils (113) NaCl extracts (13, 44) SGP (109) NaOH extracts of aorta (9) acidic structural protein (39, 130) Polar elastin contaminant (128)
COW Lig. nuchae	tissue	0.1N NaOH 98°C	15% of tiss. (wet?)		61 (1972)	ALSP (63)
PIG Aorta (intima)	fat-free dry tiss	0.3M NaCl	0.1% of dry	71,000 (sed equil)	134 (1972)	SGP (76) NaCl extract of aorta (94) NaOH extract of aorta (9)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
RAT CAT COW, and HUMAN Uterus	fat-free autoclave residue	0.1N NaOH 95°C		hetero (gel filtr)	30 (1973)	NaCl extracts of ligament (44) NaOH extract of aorta (9) SGP (76, 109) ALSP (59) acidic structural protein (131) microfibrils (113)
	fat-free tissue	8M urea +2-ME				
	urea/2-ME residue	0.2N NaOH 4°C collagenase				
	fat-free autoclave residue	trypsin				
	trypsin residue	8M urea + 2-ME				

TABLE I: CHRONOLOGICAL TABULATION OF "SGE" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
ELEPHANT Thoracic Aorta	hot TCA res	8M urea	12% of dry		72 (1973)	SGP (76, 102, 104, 107) microfibrils (104, 113)
	urea res	8M urea + 2-ME	5% of dry			
ELEPHANT Abdominal Aorta	hot TCA res	8M urea	17% of dry			
	urea res	urea + 2-ME	8% of dry			
HUMAN Thoracic Aorta	hot TCA res	8M urea	41% of dry			
	urea res	urea + 2-ME	3% of dry			
PIG Thoracic Aorta	hot TCA res	8M urea	22% of dry			
	urea res	urea + 2-ME	21% of dry			
HUMAN Aorta (normal)	hot TCA res	8M urea + 0.1M 2-ME	1% of wet		85 (1973)	SGP (26, 76, 80, 102, 104)
(severely sclerotic)			3% of wet			

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
HUMAN Aorta	autoclave residue	0.1N NaOH 100°C			62 (1974)	NaCl extract of ligament (44) ALSP (65) NaOH extract of aorta (9)
COW Aorta	tissue	saline		50,000 (gel filtr)	97 (1974)	NaCl extracts of aorta (13, 94, 95, 96)
COW Tendon	saline residue	3M $MgCl_2$	.004% of wet	60,000 (PAGE/SDS)	3 (1975)	$MgCl_2$ extract of tendon Is NOT SGP (109)
RAT Skin	fat-free tissue	0.45M NaCl	2.5% of protein	76,000 (PAGE/SDS) main band	22 (1975)	acidic structural proteins (130, 131, 138) microfibrils (51, 67, 113)
HUMAN Aorta	hot TCA res	8M urea	0.8-2.1% of dry		27 (1975)	SGP (26, 104) microfibrils (104, 113) ALSP (60)
	urea res	0.1N NaOH 37°C	0.1-1.2% of dry			

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
COW Lung (Pleura)	dry tiss	8M urea	0.5% of dry		38 (1975)	SGP (101 microfibrils (113) acidic structural proteins (131) NaOH extracts of aorta (9) NaCl extract of ligament (44)
	urea res	urea + $NaBH_4$	3% of dry			
		urea + 2-ME	1.6% of dry			
		0.1N NaOH	1.1% of dry			
(parenchyma)	dry tiss	8M urea	1.3% of dry			
	urea res	urea + $NaBH_4$	15% of dry			
		0.1N NaOH	8% of dry			
COW Heart valve	fat-free collagenase residue	8M urea	4% of dry	hetero 48,000 main band	55 (1975)	$MgCl_2$ extract of tendon SGP (42) granuloma extract (98) acidic structural protein (130, 131)
	urea res	8M urea + 2-ME	3% of dry	hetero 7,800 (PAGE/SDS)		

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
SPONGE Entire	residue after hot TCA and trypsin	8M urea		hetero (PAGE/SDS)	58 (1975)	SGP (101, 105, 109, 111)
RABBIT(birth) Aorta culture	collagenase residue	8M urea + 2-ME	0.4% of wet		86 (1975)	SGP (111)
(1 mo old)						
(6 mo old)			0.6% of wet			
COW Lig. nuchae	res after 5M guan	5M guan + DTT		15,000 (end group) 14,000 (sed equil)	122 (1975)	microfibrils (113)
PUPPY Cartilage	res after 4M guan and collagenase	used the residue		40,000-45,000 (PAGE/SDS)	124 (1975)	SGP (104, 109) NaOH extract of aorta (9) microfibrils (104, 113) acidic structural proteins (131, 138)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	EXTRACTION Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
PIG Mitral valve	wet tiss	0.3M NaCl	1.4% of wet	Two: 72,000 120,000 (PAGE/SDS)	7 (1976)	SGP (76, 107) NaCl extract of aorta (134) NaOH extract of aorta (9) NaCl extract of aorta (94)
HUMAN Epidermis	tissue	8M urea		Two: 63,000 77,000 (PAGE/SDS)	10 (1976)	No SGP literature cited
MONKEY Smooth muscle cells	res after 5M guan	5M guan + DTT		270,000 (PAGE/SDS)	82 (1976)	NaOH extract of aorta (9) SGP (urea/2ME fraction) (76) ALSP or AIP (30, 60) microfibrils (113) Cell surface glycoproteins (57)
RABBIT Skin	collagenase residue	8M urea	0.5% of wet	16,000 (PAGE/SDS)	99 (1976)	SGP (76, 109) acidic structural protein (138) granuloma extract (98) $MgCl_2$ extract of tendon (4) lung acidic structural protein (38)

TABLE I: CHRONOLOGICAL TABULATION OF "SGP" PREPARATIONS (Continued)

ANIMAL Tissue	EXTRACTION Material Extracted	Extracting Agent	YIELD	MOL. WT. (method)	REFERENCE (date)	Author compares this to: (references he cites)
PIG Aorta	tissue?	6M urea + 2-ME		35,000 (gel filtr)	77 (1976)	SGP (Same author as ref 76. This paper is an abstract without references.)
SQUID Skin	fat-free saline res	1M $CaCl_2$	1% of proteins		78 (1976)	SGP (109, 111) NaOH extract of aorta (9) $MgCl_2$ extract of tendon (4)
HUMAN Fibroblasts	culture medium		25-35% of soluble protein	250,000 (gel filtr)	120 (1976)	SGP (5) microfibrils (82)
CALF Aorta	residue after "chaotropes"	SDS/2-ME	4-6% of dry tissue	15,000-200,000. principle 50,000 (PAGE/SDS)	19 (1977)	microfibrils basement membrane proteins (no refs. cited; abstract)

ABBREVIATIONS USED IN TABLE I

AIP	alkali-insoluble protein
ALSP	alkali-soluble protein
DTE	dithioerythritol
DTT	dithiothreitol
gel filt	gel filtration
guan	guanidinium chloride
hetero	heterogeneous
KGAG	keratoglycosaminoglycan
lig. nuchae	ligamentum nuchae
2-ME	2-mercaptoethanol
MOL. WT.	molecular weight
PAGE/SDS	polyacrylamide gel electrophoresis in sodium dodecylsulfate
res	residue
SDS	sodium dodecylsulfate
sed	sedimentation (velocity or equilibrium not specified)
sed equil	sedimentation equilibrium
sed vel	sedimentation velocity
sialo	sialofucoglucosaminogalactomannosidoglycan
TCA	trichloroacetic acid
tiss	tissue

2 Experimental Sections.

The greatest need in the area of SGP research has been to define SGP as a molecular entity, with a known molecular weight, a known composition, and eventually a known sequence of amino acid residues. Consequently, the experiments to be described in Section I sought to understand the nature of SGP heterogeneity and to isolate and characterize an SGP monomer. Section II sought to determine if the proposed SGP monomer isolated in Section I was in fact different from the myofibrillar protein actin. Section III is a survey of extractable proteins from bovine aorta. These experiments sought to determine what known proteins are found in aorta extracts that were heretofore defined by the literature as SGP. They sought to find a single polypeptide, fitting the description of SGP present in a quantity sufficient to account for the large amounts of SGP reported by the literature to be present in aorta (19)(59)(60)(72)(76)(80). And these experiments sought to determine if aortic SGP reported in the literature could be denatured or degraded actin.

Bovine aorta was chosen for these experiments for several reasons. The literature shows that aorta contains large amounts of SGP. Aorta is also a principle site of atherosclerosis, currently the most frequent cause of death in the United States. SGP has been presumed to play a role in this disease (62)(85)(102). Although the aorta is a more complex organ than is ligament, for example, techniques were available for identifying and separating the many molecular species present in the whole organ. Finally, bovine aorta was easily obtainable in large amounts from animals at the slaughterhouse.

2.1 Section I: SGP Heterogeneity.

The objectives of this first experimental section were to resolve at least some of the controversy over the size of an SGP molecule, and to define SGP as a molecular species of known molecular weight and known composition. It was felt that until this had been done, no meaningful metabolic or pathologic studies of SGP were possible. One could postulate that SGP preparations were heterogeneous because they actually contained monomeric species of widely differing sizes, or because they were a mixture of aggregates and/or polymers of a small monomer, or because they were a mixture of fragments of a large monomer. For this work, a polymer was defined as being monomers held together by covalent bonds other than disulfide crosslinks. An aggregate was defined as being monomers held together by ionic, hydrophobic, or hydrogen bonds, or by disulfide crosslinks.

The experimental approach of this section was to prepare SGP from the tissue in such a way as to avoid degradation of the polypeptide chains, then to fractionate the extracts according to molecular size, and to characterize the various fractions.

2.1.1 Analytical Methods.

2.1.1.1 Lowry Protein Assay.

2.1.1.1.1 Introduction.

Protein was assayed in solution by the method of Lowry, Rosebrough, Farr, and Randall (69). This most commonly used protein assay is based on the reaction of Folin's phenol reagent with proteins and cupric ion.

In the absence of copper, the Folin reagent reacts with tyrosine and tryptophane residues only, but with cupric ion present the reaction is with the peptide bonds as well. Initially, a manual method was used, but later assays were automated for greater speed and reproducibility.

2.1.1.1.2 Manual Assay.

For the manual assay, Reagent A was made by dissolving 2.29 g of $Na_2CO_3 \cdot H_2O$ in 98 ml of water (all water used in all procedures was distilled and deionized unless specifically stated otherwise), followed by 1.0 ml of 2.7% K Na Tartrate and 1.0 ml of 1% $CuSO_4 \cdot 5H_2O$. Reagent B, made fresh daily, was a dilution of stock 2 N Folin reagent (Sigma) to 1 N with water.

Bovine serum albumin (BSA) was used as a standard. "Albumin Stock Solution", 100 mg/ml, was obtained from Sigma, stored at $4^{o}C$ and diluted in water to concentrations of 50 to 400 µg of protein per ml. Dilutions were made fresh daily.

Samples and standards were assayed in duplicate. For the manual method, 0.5 ml of protein solution and 0.5 ml of 1 N NaOH were mixed in a test tube. 5 ml of Reagent A were added and mixed well. The tubes were allowed to stand at room temperature for 10 minutes. One half ml of Reagent B was then added with rapid, continuous mixing. After an additional 30 minutes at room temperature, the samples were read at 500 nm on a Beckman model DU spectrophotometer using standard 1.0 cm cuvettes. Reference blanks were 0.5 ml of water, rather than protein solution, carried through the above procedure.

2.1.1.1.3 Automated Assay.

The automated assay was performed on a Technicon Auto Analyzer (Technicon). Reagents were made as three solutions, rather than two for the manual assay.

Copper reagent was made by dissolving 4.0 g of potassium and sodium tartrate in 1.5 liters of water, followed by 2.0 g of $CuSO_4 \cdot 5H_2O$. The solution was made up to 2.0 liters. It gave constant results for several weeks when stored at room temperature.

A 1 N sodium carbonate buffer of pH 11.6 was made by dissolving 168 g of $NaHCO_3$ and 90 g of NaOH in 1.8 liters of water. This buffer also gave constant results for several weeks at room temperature.

Folin reagent was made from Sigma stock solution, but diluted to 0.5 N with water. It was diluted fresh daily.

Standards were diluted as for the manual assay, in the range of 5 to 400 µg of BSA per ml.

A flow diagram of the Auto Analyzer manifold is shown in Figure 1. The bubble line, rather than the exit line from the flow cell, was recycled through the proportioning pump in order to reduce surging caused by bubbles in the waste line.

Samples of 0.5 ml were placed in 1 ml cups on the sampler, with one cup of water between samples. 40 cups per hour were sampled for 60 seconds each, with a 30 second water wash between cups.

2.1.1.1.4 Calculation of Results.

Results of either manual or automated Lowry assay were calculated by first plotting the absorbances of the standards versus µg of

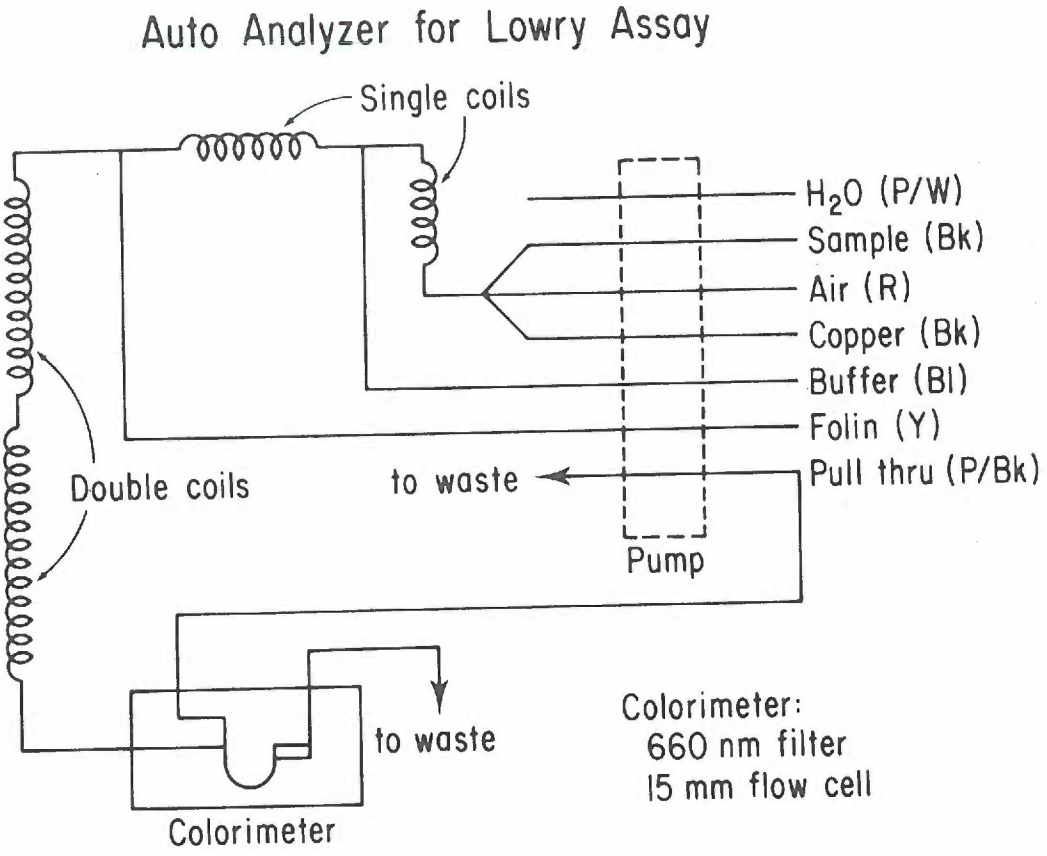
Figure 1. Auto Analyzer Manifold for Protein Assay By the Method of Lowry et al (69).

The manifold was modified from the standard Technicon configuration in that the debubbler line rather than the exit line from the flow cell was returned through the pump. This led to a reduction in surging and a more stable flow rate through the manifold. A 40-2/1 cam was used (40 samples/hr, with a 2:1 ratio between sampling time and washing time between samples). Sample cups were alternated with water blanks.

Color coding of pump tubing:

Abbreviation	Color(s)	Inside Diameter (inches)
P/W	Purple/White	.110
Bk	Black	.030
R	Red	.045
Bl	Blue	.065
Y	Yellow	.056
P/Bk	Purple/Black	.090

Text section 2.1.1.1.3, page 38.


Auto Analyzer for Lowry Assay
Single coils
H_2O (P/W)
Sample (Bk)
Air (R)
Copper (Bk)
Buffer (Bl)
Folin (Y)
Pull thru (P/Bk)
Double coils
to waste
Pump
to waste
Colorimeter
Colorimeter:
660 nm filter
15 mm flow cell

standard per ml of solution. A least-squares linear regression was used to determine the best straight line through the points. Unknowns were then calculated from the slope and intercept of this line. Duplicate samples for either manual or automated assays were accurate to within 5% for protein concentrations in the range of 50 to 400 µg/ml. The manual version was not usable below 50 µg/ml, but the automated version routinely detected 5 µg/ml with a reproducibility of about 20% at this lower limit of detection.

2.1.1.2 Kjeldahl Nitrogen Assay.

2.1.1.2.1 Introduction.

Protein was also measured as total nitrogen by a modification of the Kjeldahl procedure (17), using the phenol-hypochlorite reaction of Russell (114) for assay of the liberated ammonia. This assay is based on digestion of the sample in boiling concentrated H_2SO_4 until all nitrogen has been converted to NH_4^+, which is then quantitated by any of a number of methods. The digestion I used employed SeO_2 as a catalyst and $HClO_4$ as an oxidizer. Bradstreet (17) reports conflicting opinions as to the usefulness of these additives, and Sandberg (117) chose to avoid them. Because they reduce the digestion time from hours to minutes, I chose to try them. They proved to be completely satisfactory, as seen below. For determination of liberated nitrogen, a colorimetric assay was chosen over distillation as a matter of convenience. Sandberg (117) reported excellent results with an automated version of Russell's procedure. Since these reagents are stable and easily prepared, I also chose to use Russell's method.

The Kjeldahl assay was used in place of the simpler Lowry procedure when the material to be assayed was thought to contain something that might interfere with the Lowry assay, or when the solution was expected to be largely collagen, which is relatively unreactive in the Lowry assay (69).

2.1.1.2.2 Reagents.

The digestion reagent was made by adding 300 mg of SeO_2 in 10 ml of water dropwise with cooling to 90 ml of concentrated H_2SO_4. When cool, 2.33 ml of 60% $HClO_4$ were added to the solution. The reagent gave constant results for months when stored in glass at room temperature.

For the color reaction, three reagents were needed. NaOH solution was made from 350 g of NaOH dissolved in 700 ml of water, with cooling in ice. At or below room temperature, 30 mg of sodium nitroferricyanide ($Na_3Fe(CN)_5NO \cdot H_2O$) were dissolved in the NaOH solution. The volume was made to 1.0 liter. This reagent was pale yellow when first made, but became colorless after a few days with no effect on the assay. It gave constant results for weeks when stored at room temperature.

Sodium phenolate reagent was made by dissolving 100 g of NaOH in 250 ml of water with cooling in ice. At or below room temperature, 138.0 ml of 88% liquified phenol were added and mixed. The phenol must be colorless and without preservatives. This reagent gave constant results for 2-3 weeks when kept at room temperature, but gradually

turned brown over longer periods, resulting in higher baseline and lower color yield.

2.1.1.2.3 Sample Digestion.

For sample digestion, material expected to contain 2-80 µg of nitrogen in up to 0.4 ml of water was placed in a 10 ml Kjeldahl flask, along with two boiling chips. 1.5 ml of digestion reagent were then added. The samples were digested until a pale, greenish color had appeared and faded. This normally required 7-8 minutes of boiling. The color appeared with blanks as well as with samples. Its nature was not determined, but its appearance was found to be a consistent indicator of the completion of a digestion. Tests with bovine serum albumin standards showed that over 90% of the total nitrogen was released after only 3 minutes of boiling, and that boiling times in excess of 8 minutes did not cause any detectable loss of nitrogen.

2.1.1.2.4 Standards.

Two types of standards were used in this assay. Ammonium sulfate was weighed out, ideally 943.4 mg, into a tared, glass-stoppered weighing bottle. This bottle had previously been dried to constant weight in an oven at 100°C. The ammonium sulfate was then heated and cooled to constant weight in the oven. A standard solution containing ideally 2000 µg/ml of nitrogen was made by dissolving the contents of the weighing bottle in 100.0 ml of water. From this primary standard, dilutions were made in the range of 5 to 200 µg N/ml. 0.4 ml aliquots were digested for comparison with unknowns.

Bovine serum albumin standards were prepared from twice-recrystalized protein (Calbiochem or Sigma) by dissolving 30 mg in 25 ml of water. Dilutions were made to give 40 to 1,200 µg of protein per ml. Again, 0.4 ml aliquots were digested.

2.1.1.2.5 Manual Color Reaction.

Initially, the color reaction for released ammonia was done manually. Digested samples were diluted with 3.0 ml of water and transferred to 50 ml test tubes. With cooling in an ice bath, 9.0 ml of NaOH reagent were added slowly. Much heat was liberated, and this process was very tedious because of the cooling required. Next, 2.5 ml of Na phenolate reagent were added and mixed well. Samples were not allowed to remain in the ice bath after they had cooled to room temperature, since on further cooling a heavy precipitate, presumably sodium sulfate, formed. On a strict time schedule, 1.5 ml aliquots of NaOCl reagent were added to each tube. Tubes were read on a spectrophotometer in a 1 cm cuvette, at 630 nm, exactly 9 minutes after the addition of the hypochlorite.

2.1.1.2.6 Automated Color Reaction.

Because of the time required for the manual assay, and because of the possibility of loss of ammonia during the addition of NaOH to the sample in an open tube, a Technicon Auto Analyzer was constructed according to Sandberg (117) and used to automate the color reaction. Figure 2 shows a schematic diagram of this manifold. Several alterations were made from Sandberg's apparatus.

Figure 2. Auto Analyzer Manifold for Kjeldahl Color Reaction.

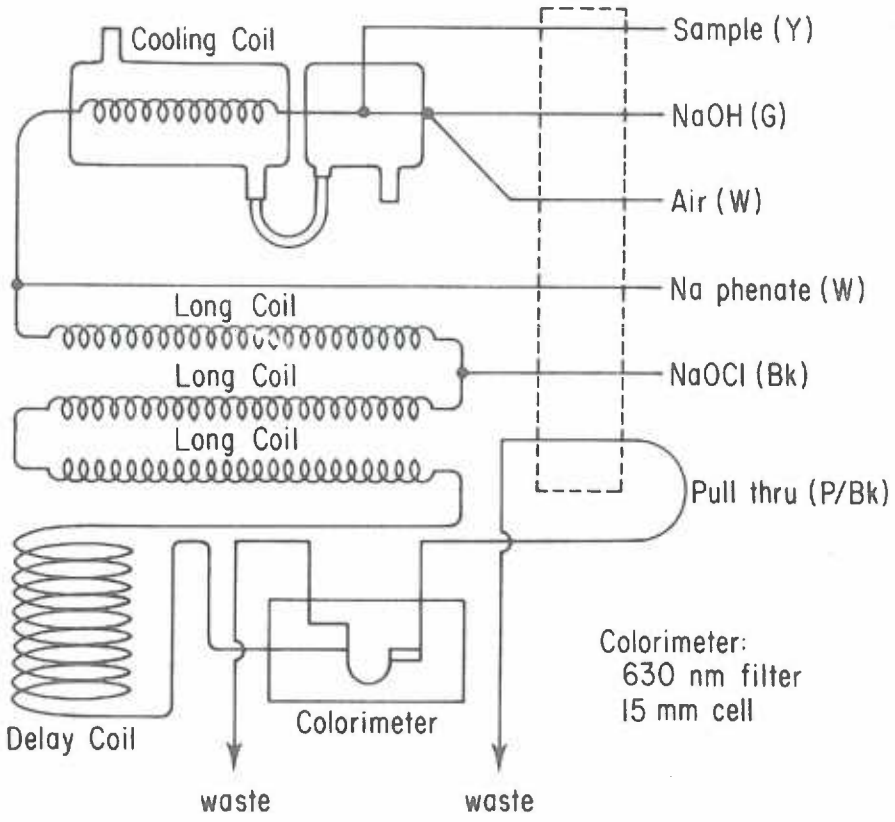
The reaction was the phenol-hypochlorite reaction of Russell (114) adapted for the Auto Analyzer by Sandberg (117). Rectangle in dashed lines represents proportioning pump as in Figure 1. Manifold was modified from Sandberg's design by inclusion of the jacketed "tee" where sample and NaOH meet, and by addition of the 1250 cm Delay Coil. Sampler used a 40-2/1 cam (40 samples/hr with a 2:1 ratio between sampling time and washing time between samples.) Sample cups were alternated with water blanks.

Color coding of pump tubing:

Abbreviation	Color(s)	Inside Diameter (inches)
Y	Yellow	.056
G	Green	.073
W	White	.040
Bk	Black	.030
P/Bk	Purple/Black	.090

Text section 2.1.1.2.6, page 44.

Auto Analyzer for Kjeldahl Assay



First, a coil of glass tubing 1250 cm long and having an inside diameter of 1.6 mm was inserted between the last mixing coil and the colorimeter. The color produced by the reaction was unstable, rising to a maximum at 9 minutes, then decaying rapidly. In the apparatus as described by Sandberg, the sample reached the colorimeter several minutes prior to completion of color development. With the additional coil, the reading time was 9.0 minutes, at the point of maximum color development. Second, the sampler was modified to tolerate the 12 N acid from the sample cups. This was accomplished by substituting a 50 µl glass capillary for the stainless steel dipping tube of the sampler, and by installing acidflex and Teflon connection tubing to the manifold. Third, the heat of neutralization of the 12 N acid sample with 35% NaOH was excessive. The two streams were therefore brought together in a jacketed "tee" connected to the input end of the cooling coil. Cooling water was run through both jackets at a rate of 1 liter per minute or greater.

In operation, the analyzer sampled 40 cups per hour with one water wash between sample cups. Samples of 2 ml were applied. With a 15 mm flow cell, this configuration gave an absorbance at 630 nm of 1.0 for an 80 µg sample digested and diluted as described above. A 2 µg sample was readable immediately after an 80 µg sample with the single water wash in between. An acid blank gave a depression of 0.01 absorbance unit from the baseline. Since samples were in acid, the depression was taken as baseline.

2.1.1.2.7 Results.

Samples were digested in duplicate, and each digest was assayed at least in duplicate. The data were calculated as described for the Lowry assay. Duplicate digests were reproducible within 0.01 absorbance unit, or 2% at an absorbance of 0.500. Ammonium sulfate standards were used in assessing the completion and reproducibility of digested protein standards. Sandberg (117) reported that the digestion as described above gave incomplete recovery of nitrogen, and irreproducible results. I found it to be both accurate and reproducible for proteins in solution. Sandberg's analyses were on protein hydrolyzates, and on solid residues, and these differences may have led to the spurious results he described.

2.1.1.3 Hydroxyproline Assay.

Hydroxyproline was measured in protein hydrolyzates either on the amino acid analyzer, to be discussed later, or by the Technicon Auto Analyzer method of Grant (46). This procedure requires prior hydrolysis of the hydroxyproline-containing protein. The free hydroxyproline is oxidized with chloramine T. Excess chloramine T is then destroyed with perchloric acid. Reaction of the oxidized derivative of hydroxyproline with p-dimethylaminobenzaldehyde develops a pink color which is then read at 550 nm. Hydroxyproline was determined either by this method or as part of amino acid analysis, described below.

For hydrolysis, protein samples of two milligrams each were placed in 15 ml test tubes equipped with screw caps and Teflon inserts. 2 ml of redistilled, constant-boiling 6 N HCl were added to each tube.

The tubes were swept with nitrogen and capped. They were heated to 105°C for 24 hours. Hydrolyzates were evaporated to dryness under a stream of nitrogen at 50°C, and made up to a volume with water such that the resulting concentration of hydroxyproline was between 0.2 and 10 µg/ml.

Reagents, and the Auto Analyzer, were as described by Grant. Chloramine T and p-dimethylaminobenzaldehyde were from Matheson, Coleman, and Bell. A 0.025 inch inside diameter Solvaflex tubing was inserted between the cooling coil and the colorimeter as a pulse suppressor. Standard hydroxyproline (K and K Laboratories) was dissolved in water, at concentrations of 0.2 to 10 µg/ml. In operation, samples and standards were run in duplicate. 1 ml portions were alternated with water washes on the sample tray. Calculation of results was as described for the Lowry determination.

2.1.1.4 Amino Acid Analysis.

2.1.1.4.1 Apparatus.

Analysis of the hydrolyzates was performed on a Beckman Model 116 Amino Acid Analyzer. As described by Spackman, Stein, and Moore (126), this apparatus separates amino acids in protein hydrolyzates by ion exchange chromatography on columns of sulfated polystyrene resin. The column effluent is then mixed with ninhydrin reagent, which reacts with free amino groups to give an intense purple color at 570 nm, or with imino acids (proline and hydroxyproline) to give a yellow chromophore (440 nm). The absorbances at these wavelengths are monitored on a multi-channel chart recorder.

2.1.1.4.2 Sample Preparation.

Protein samples for amino acid analysis were hydrolyzed in redistilled, constant-boiling 6 N HCl. 1-2 mg of each protein were weighed into 5 ml glass ampoules. Alternatively, solutions or suspensions of protein in water, shown by Lowry or Kjeldahl assay to contain 1-2 mg of protein, were pipetted into the ampoules and lyophilized. 2 ml of the redistilled HCl were added to each ampoule. The ampoules were then evacuated with a water aspirator until the HCl began to bubble vigorously. The evacuated ampoules were sealed with a flame. Hydrolysis was performed at 105°C for 24 hours. After cooling, the hydrolyzates were dried rapidly at 40°C in a rotary evaporator under 5-10 mm Hg pressure. They were made up to 2.0 ml with water. Particles of humin, if present, were filtered out by passing the hydrolyzate through a plug of glass wool in a Pasteur pipet. Hydrolyzates in water were stored for 2-3 days at 4°C, or indefinitely at -10°C.

2.1.1.4.3 Analysis of the Sample.

Analyses of the different preparations were performed over the space of four years, during which time several changes were made on the instrument, for reasons often unrelated to the work described in this thesis. These changes make it impractical to describe here the exact conditions of each individual chromatogram. These conditions will be reported in the appropriate methods section for each experiment involving amino acid analysis.

2.1.1.4.3.1 Columns.

Earlier analyses employed the two-column system described in the operating manual for the instrument (11). The long column, used for acidic and neutral amino acids, had a bed height of 55 cm. The short column, used for basic amino acids, had a bed height of 20 cm. Both columns were 9 mm in diameter. The long column was packed with Beckman type UR-30 resin, and the short column with Beckman PA-35 resin. Later, a single-column system was adopted, using only the long column. Still later, the single column was repacked with Technicon Chromo-beads type C-2.

2.1.1.4.3.2 Buffers.

A-buffer was 0.2 N Na citrate prepared by dissolving 78.4 g of Na citrate·$2H_2O$, 50 ml of 12 N HCl, 10 ml of thiodiglycol (Pierce), 0.4 ml of n-Caprylic (Octanoic) acid, and 40.0 ml of 30% Brij 35 nonionic detergent (Pierce), in 3.5 liters of water. The solution was titrated to the desired pH, in the range of 3.00 to 3.30, with HCl, and made up to 4.0 liters.

B-buffer was 0.2 N Na citrate, prepared similarly to A-buffer except that 33 ml of 12 N HCl were used, rather than 50 ml. This was titrated to pH 4.25.

C-buffer was made by dissolving 149 g of Na citrate·$2H_2O$, 0.4 ml of caprylic acid, 99.2 g of NaOH, and 40 ml of 30% Brij 35 solution in 3.5 liters of water. The buffer was titrated to pH 6.40 with HCl and made up to 4.0 liters.

D-buffer was 0.38 N Na citrate, prepared by dissolving 137.3 g of Na citrate·$2H_2O$, 26 ml of 12 N HCl, 0.4 ml of 30% Brij 35 in 3.5 liters of water, titrating to pH 5.25, and making to 4.0 liters with water.

Pre-A-buffer was A-buffer to which 3% n-propanol had been added (v/v), and which was then titrated with HCl to a slightly lower pH than A-buffer. When Pre-A was used, A-buffer also contained 3% n-propanol.

2.1.1.4.3.3 Operation of the Two-Column System.

For all analyses, the columns were pumped at 68 ml/hr. The long column was equilibrated for 1 hour with A-buffer. The sample was applied to the column in a volume of 0.25-1.0 ml depending on its concentration and on experimental objectives. Change to B-buffer was made after 95-115 minutes, again depending on experimental objectives. If a temperature change was used, the column was equilibrated and loaded at 47.5°C. Change to 55°C was begun 40 minutes after sample application. The water bath feeding the column jackets required 22 minutes to warm from low to high temperature. After the emergence of phenylalanine, the column was washed for 15 minutes with 0.2 N NaOH containing 10 mM EDTA and 10 ml of 30% Brij 35 per liter. It was then re-equilibrated with A-buffer for the next run. The short column was run entirely at 55°C, and entirely in D-buffer.

2.1.1.4.3.4 Operation of the Single-Column System.

The column was eluted with A-and B- buffers as above, then with C-buffer in place of the NaOH wash. The change to C was made 145-150 minutes after sample application. C-buffer was pumped until arginine had been eluted, 300-315 minutes after sample application. The column was then re-equilibrated for 1 hour with A-buffer. For analyses in which hydroxyproline was to be measured, Pre-A buffer was used in place of A-buffer for column regeneration, and the switch was made to A-buffer when the sample was applied.

Because of the volume of the buffer lines between the buffer bottles and the column, buffer changes actually arrived at the upper end of the column 27 minutes after the indicated time of the change-over. The change from Pre-A to A occurred at time zero, but A-buffer actually reached the column 27 minutes later. Changes from A to B and B to C also involved this delay.

2.1.1.4.3.5 Ninhydrin Reagent.

Ninhydrin reagent was prepared according to the Beckman technical manual for the instrument (11). 1.461 g of anhydrous $SnCl_2$ was used in place of 1.600 g of the monohydrate called for in the manual. For the analysis, ninhydrin reagent was pumped at 34 ml/hr. A 4000 ml preparation of reagent lasted for twenty single-column analyses. Even when stored in a dark bottle under nitrogen, some deterioration of the ninhydrin over a period of several weeks was unavoidable. For earlier analyses, the deterioration was not taken into account. For experiments in Section II, the standard was run within 2-3 days of the

unknown analysis. For the large number of analyses in Section III, compensation for ninhydrin deterioration was accomplished by running standards at the beginning and end of a series of analyses. Color yield per micromole as a function of time was plotted for each amino acid. Standards for each unknown were calculated from these plots and the date of the unknown analysis. The assumption of this method, that the deterioration is linear with time, was found to be reasonable, since there was no difference between the results of duplicate samples run at widely separate times.

2.1.1.4.3.6 Standards.

Routinely, the analyzer was calibrated with a standard mixture containing Asp, Thr, Ser, Glu, Pro, Gly, Ala, ½Cys, Val, Met, Ile, Leu, Tyr, Phe, His, Lys, and Arg, plus ammonia. They were obtained as pre-mixed solutions from Pierce. To these routine standards, any or all of a number of other standards were added to meet the needs of individual experiments. These included 4-Hydroxyproline (Hypro, Nutritional Biochemicals), DL-allo-4-Hydroxylysine (Hylys, Calbiochem), L-3-Methylhistidine (3M-His, Calbiochem), and S-Carboxymethyl cysteine (CM-Cys, Sigma or Aldrich). Sample chromatograms produced by these standards are shown in Figures 3 and 4.

2.1.1.4.4 "Ammonia" Plateau.

As can be seen in Figure 4, the baseline of single column chromatograms had a large, flat plateau between 180 and 260 minutes after sample application. The plateau was caused by ammonia and other

Figure 3. Amino Acid Analysis. Elution Profiles of Standard Mixtures of Amino Acids from Long and Short Columns, 2-Column System.

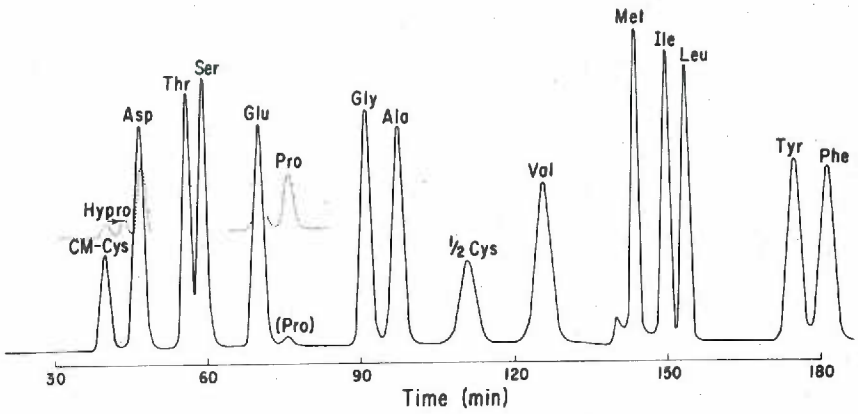
Tracing is percent transmission versus time after sample application. Solid line 570 nm, dotted line 440 nm. Only proline and hydroxyproline portions of 440 trace are shown.

Nonstandard Abbreviations

CM-Cys	S-Carboxymethyl cysteine
Hypro	Hydroxyproline
Glu-N	Glucosamine
Gal-N	Galactosamine
Hylys	Hydroxylysine

Small peak ahead of Met is buffer change artifact.

Text section 2.1.1.4.3.6, page 54.


Met
Ile
Leu
Ser
Thr
Asp
Glu
Gly
Ala
Pro
Tyr
Phe
Val
Hypro
CM-Cys
½ Cys
(Pro)
30
60
90
120
150
180
Time (min)

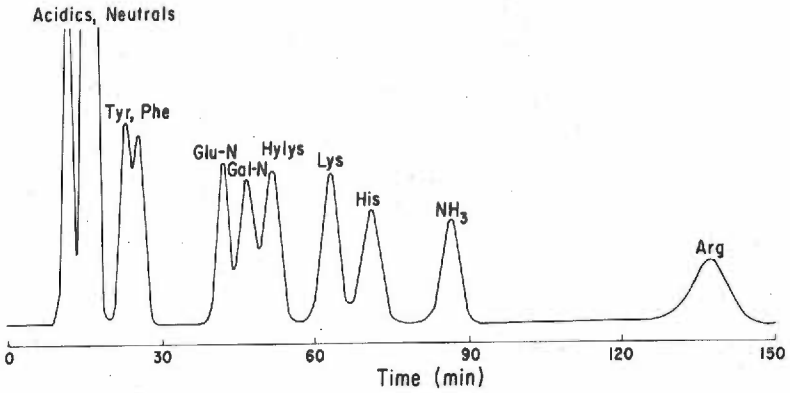

Acidics, Neutrals
Tyr, Phe
Glu-N
Gal-N
Hylys
Lys
His
NH3
Arg
0
30
60
90
120
150
Time (min)

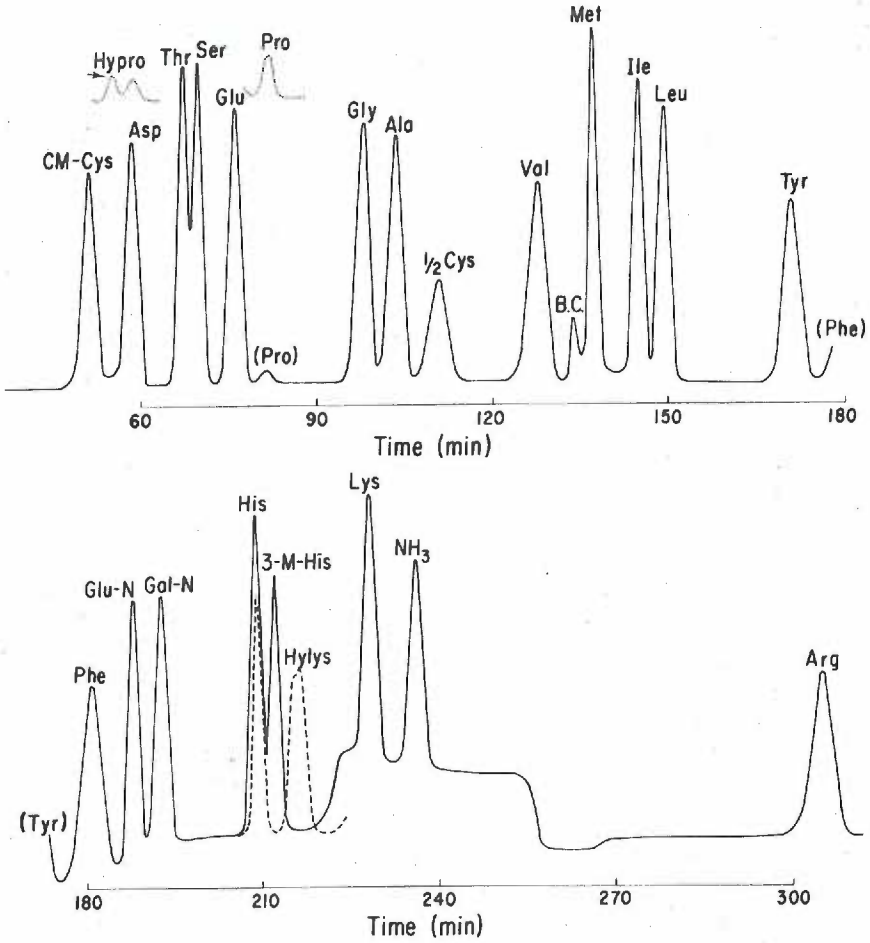
Figure 4. Amino Acid Analysis. Elution Profile of Standard Amino Acids from Single Column System.

Tracing is percent transmission versus time after sample application. Solid line 570 nm; dotted line 440 nm. Only proline and hydroxyproline portions of 440 trace are shown. Dashed line on second half of chromatogram shows histidine and hydroxylysine from a separate run. Note separation of histidine, 3-methyl histidine, and hydroxylysine.

Nonstandard Abbreviations

CM-Cys	S-Carboxymethyl cysteine
Hypro	Hydroxyproline
B.C.	Buffer change artifact
Glu-N	Glucosamine
Gal-N	Galactosamine
3-M-His	3-Methyl histidine
Hylys	Hydroxylysine

Text sections 2.1.1.4.3.6 and 2.1.1.4.4, pages 54 and 59.


Hypro
Thr
Ser
Pro
Glu
Asp
CM-Cys
Gly
Ala
Met
Ile
Leu
Val
Tyr
½Cys
B.C.
(Pro)
(Phe)
60
90
120
150
180
Time (min)
His
Lys
3-M-His
NH3
Glu-N
Gal-N
Hylys
Phe
Arg
(Tyr)
180
210
240
270
300
Time (min)

cationic species present in the A and B buffers. Bound to the column while these buffers were in use, the contaminants were then eluted by the C-buffer. Others in the laboratory have found that passing the A- and B-buffers through a cation exchange column removes the "ammonia plateau". However, since it did not seriously interfere with quantitation of the amino acids, no steps were taken to remove it.

2.1.1.4.5 Calculation of Results.

Briefly, the peak areas were measured as the product of the net height of the peak multiplied by the width of the peak at the point halfway between its baseline and its maximum absorbance. Asymmetric peaks and partially resolved peaks were divided into small time intervals and integrated by summing the absorbances at each interval. Peak areas of standards were used to obtain an area-per-micromole figure for each amino acid. Unknowns were then quantitated using these standard factors. Compositions were reported as residues per 1000 amino acid residues. In some instances, Hypro content from the Auto Analyzer method of Grant, described above, was incorporated into this calculation.

2.1.1.5 Hexuronic Acid Analysis.

Earlier analyses for hexuronic acid were by the automated carbazole method of Balazs, Berntsen, Karossa, and Swann (8). Based on the method of Dische (28), this colorimetric assay digests uronic acids, free or in polysaccharides, with concentrated H_2SO_4 at 100°C. The chromogen formed in this reaction then reacts with carbazole to give a pink color which is measured at 535 nm. The reaction gives smaller amounts of color, at somewhat different wavelengths, for hexoses and pentoses. Color yield of uronic acids in polysaccharides is variable.

Standard glucuronolactone (K + K Laboratories) was diluted as needed from a 100 µg/ml standard in water. In operation, unknowns and standards were run in duplicate, with two water wash cups between each sample cup on the sampler tray.

Later analyses for hexuronic acid were by the automated m-hydroxydiphenyl (m-HDP) method of Rosenthal, Bentley, and Albin (112). This is also a colorimetric assay, based on the reaction of uronic acid derivatives in H_2SO_4 with m-hydroxydiphenyl. The assay is preferable to the carbazole method because of its much lower response to hexoses, and its greater sensitivity to uronic acid. Standard was as for the carbazole method. Meta-hydroxydiphenyl was obtained as m-phenyl phenol from Eastman Organics. The "macro" method, as described in the paper, was used for all analyses.

Assays, by the carbazole method or by the m-HDP method, were calculated as described for the Lowry protein assay.

2.1.1.6 Sialic Acid.

Sialic acid was determined by the method of Warren as described by Tuppy and Gottschalk (132). This method was chosen over the resorcinol reaction of Svennerholm, also described by Tuppy and Gottschalk, because of its greater sensitivity. This, too, is a colorimetric assay, based on the oxidation of sialic acid with periodate to give a chromogen thought to be β-formypyruvic acid (135). Excess periodate is then destroyed with arsenite, and the chromogen is combined with 2-thiobarbituric acid to give the color. The chromophore is extracted into cyclohexanone for reading at 549 nm.

Sialic acid must be hydrolyzed from glycosidic linkages in order to be detected by the assay. This was accomplished by heating material expected to contain 2-20 μg of sialic acid at 80°C for 1 hour in 0.2 ml of 0.1 M H_2SO_4. The color reaction was then carried out exactly as in the literature. Standard was N-acetylneuraminic acid (Calbiochem). 2-Thiobarbituric acid was from Sigma.

2.1.1.7 Hexosamines.

Glucosamine and galactosamine were estimated in protein hydrolyzates as part of routine amino acid analysis. (See section 2.1.1.4.) The elution positions are shown on Figures 3 and 4. No modification of the analyzer operating procedure was necessary to resolve these amino sugars. Standard glucosamine and galactosamine were obtained from K and K Laboratories and included in the standard amino acid solutions. Hexosamines were calculated during the calculation of amino acid analyses, by the method of Spackman et al (126). Results were

reported as residues of hexosamine per 1000 amino acid residues. Since hexosamines are known to be degraded by the conditions used for protein hydrolysis (71), the values obtained from this determination were assumed to be low. 25% of the galactosamine present after a 6 hour hydrolysis was found to be lost by 24 hours. A 4% loss of glucosamine was seen over the same interval. Hexosamine determination was considered incidental to the experiments, and accurate quantitation was not attempted.

2.1.1.8 Sugars by the Phenol-H_2SO_4 Reaction.

The content of sugars in a sample was estimated by their reaction with phenol in concentrated sulfuric acid. This assay was described by Dubois, Gilles, Hamilton, Rebers, and Smith (31). The samples contained 5-25 µg of sugar in 1 ml of water. To each was added 25 µl of 80% liquified phenol. Concentrated H_2SO_4, 2.5 ml, was then added with rapid stirring and without cooling. After 10 min, the tubes were cooled and read on a spectrophotometer at 485 nm. Sucrose was used as a standard. Triplicate analyses of samples varied as much as 10% from the mean value of the three readings. Unknowns were calculated as described for the Lowry assay.

2.1.2 Extraction of Tissues.

2.1.2.1 Introduction.

Specimens of bovine aorta were extracted at several different times during the investigation. These extractions will now be described in detail, together with the results of analyses performed on whole extracts. Analyses performed on various fractions of these extracts will be described following each individual fractionation.

2.1.2.2 Extraction Procedures.

2.1.2.2.1 Experiment 1: Extraction with 4 M Guanidinium Chloride.

This extraction was performed by J. Peter Bentley. It is described here because some of the preliminary experiments described in this thesis were carried out on material from this extraction. The objective of this extraction was to obtain aortic SGP without use of saline pre-washes which would allow tissue proteases to attack the material, and without use of hot trichloroacetic acid to remove collagen, a treatment known to destroy peptide bonds, (5).

2.1.2.2.1.1 Extraction of the Tissue.

Three adult bovine aortas were obtained from animals at the slaughterhouse. They were stored in ice for transportation to the laboratory. After being stripped of fat and adventitia, the intima and media together were cut into 2-3 cm sections, frozen in liquid nitrogen, and crushed in a stainless steel mortar and pestel pre-cooled in liquid nitrogen.

Wet, crushed tissue (355 g) was stirred overnight at 4°C with 1 liter of 4 M guanidinium chloride. The suspension was centrifuged 30,000 x g for 30 minutes. The supernatant was dialyzed overnight against cold, running tap water. A massive precipitate formed. The precipitate was pelleted by centrifugation at 30,000 x g for 1 hour, and washed seven times with 400 ml of cold, distilled water by resuspension in water and centrifugation as above. The precipitate was then lyophilized.

2.1.2.2.1.2 Collagenase Digestion.

An 8-gram portion of the washed, lyophilized precipitate was suspended in 300 ml of 0.05 M tris HCl, 0.4 M NaCl, 1 mM $CaCl_2$, pH 7.4, preparatory to collagenase digestion by the method of Peterkofsky and Diegelmann (90). 4.8 mg of N-ethyl maleimide (Pierce) were added and stirred to dissolve. 12 mg of collagenase (Worthington), purified by gel filtration (90) in Sephadex G-200 (Pharmacia) were added. The suspension was incubated at 37°C for 24 hours with gentle stirring. After centrifugation at 30,000 x g for 30 minutes, 200 ml of supernatant were decanted. An additional 250 ml of the above buffer, and 3.2 mg of N-ethyl maleimide, were added, followed by 8 mg of purified collagenase, and the suspension was digested for an additional 24 hours at 37°C. After centrifugation, 260 ml of supernatant were removed and combined with the first 250 ml supernatant. The precipitate was dialyzed for 3 hours versus distilled water and lyophilized.

This preparation will be referred to in later sections as being from "Experiment 1".

2.1.2.2.2 Experiment 2: Extraction with Guanidine and Dithiothreitol.

This extraction was done to obtain more material after the material prepared in the previous extraction had been used for preliminary experimentation. It was also done to obtain the fraction of SGP crosslinked by disulfide bonds (39)(113), which remains in the tissue after extraction with denaturant alone. Guanidine was used in preference to urea or SDS as a denaturant because it gave the most complete disaggregation of extracted material in gel filtration experiments to be described below (section 2.1.3), and because SDS was difficult to remove quantitatively from proteins. To extract the crosslinked fraction, dithiothreitol was chosen over 2-mercaptoethanol because of its ability to reduce disulfide bonds more quantiatively than mercaptoethanol (23), and because of its relatively slight odor.

2.1.2.2.2.1 Extraction Procedure.

Guanidinium chloride, technical grade, was obtained from Matheson, Coleman, and Bell. This was found to be of better quality than reagent grade material from many other suppliers. It dissolved instantly and completely to give a nearly colorless solution of low ultraviolet absorbance at 280 nm. It was stirred at 40°C for 30 minutes with activated charcoal and filtered before use. Dithiothreitol, reagent grade, was purchased from Calbiochem or Sigma.

All procedures were carried out at 4°C unless otherwise noted. A thoracic aorta from an adult steer was obtained from the slaughterhouse and placed on ice within 30 minutes of the death of the animal. It was

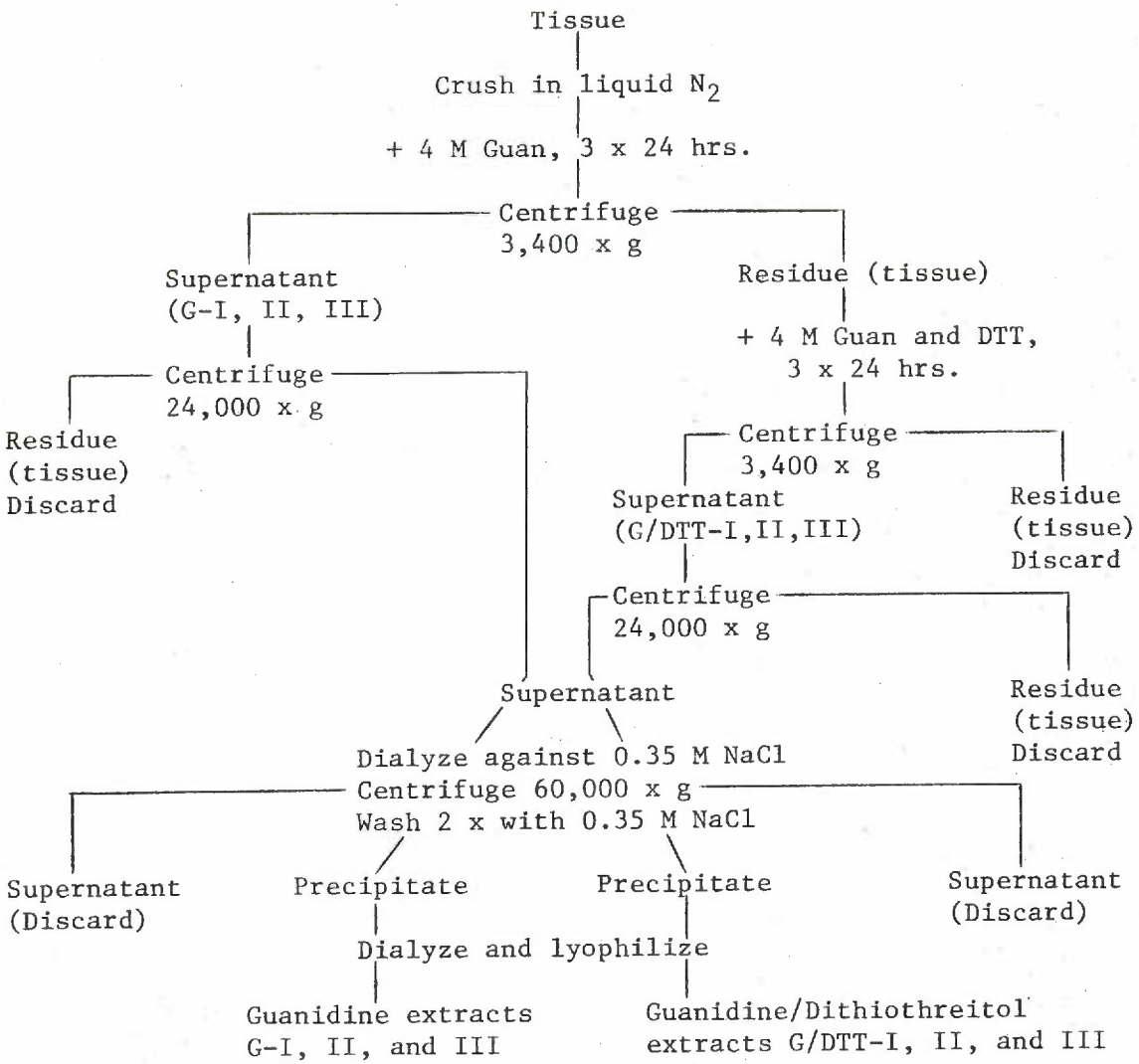


Figure 5. Flow Diagram for Aorta Extraction Experiment 2.

Text section 2.1.3.3.2, page 68.

frozen pending homogenization. The partially thawed tissue was stripped of fat and adventitia, cut into 2-3 cm. sections and crushed at liquid nitrogen temperature as described above. The resulting pulverized tissue, 96.5 grams, was extracted three times with 200 ml of 4 M guanidinium chloride, 0.05 M tris Cl, 0.1% Na_4 EDTA, pH 8.0. Each extraction was as follows. The tissue was stirred in this buffer for 24 hours in a 700 ml centrifuge bottle, then centrifuged at 3,400 x g for 20 minutes. The supernatants were removed and stored, labelled G-I, G-II and G-III. After three extractions, the tissue was suspended in another 200 ml of buffered guanidine and bubbled with nitrogen for 20 minutes. 2.70 g of dithiothreitol were added, and the suspension was capped tightly and stirred magnetically for 24 hours. The tissue was centrifuged as before. Three Guanidine/Dithiothreitol extracts were prepared in this manner, labelled G/DTT-I through G/DTT-III.

2.1.2.2.2.2 Workup of Extracts.

The six extracts were divided into 30 ml centrifuge tubes and centrifuged at 24,000 x g for 30 minutes to clear finely-divided tissue which was not removed by the earlier centrifugations in the large bottles, and then dialyzed against 10 liters of 0.35 M NaCl. The dialysis medium was changed at 48 hour intervals for a total of four dialyses. The dialyzed extracts were spun at 60,000 x g for 30 minutes. Pellets were washed twice with 50 ml of fresh .35 M NaCl by suspension and centrifugation as before, then resuspended in 50 ml of water,

dialyzed exhaustively against distilled water, and lyophilized. Figure 5 summarizes these operations.

These preparations will be referred to in later sections as being from "Experiment 2".

2.1.2.2.3 Sequential Aorta Extracts.

These extractions were performed primarily to answer the questions posed in Experimental Section III. The extraction sequence therefore will be discussed in detail there. However, since a few of the experiments dealt with matters of SGP heterogeneity and molecular weight, a brief description of the extraction procedure is presented here.

Bovine thoracic aorta was stripped of adventitia. Removal of adventitia was verified histologically. The media was homogenized and washed with 0.15 M NaCl containing diisopropylfluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA), then with 0.05 M $NaHCO_3$, then with 1 mM EDTA, and finally with acetone, to remove soluble proteins and lipids. The dried residue was extracted with nine changes of 0.2 mM ATP, 0.2 mM ascorbate. The residue was next extracted with three changes of 5 M guanidinium chloride (extracts G-I, G-II, and G-III) followed by four changes of guanidine containing dithiothreitol (extracts G/DTT-I through G/DTT-IV). The tissue residue was finally extracted twice with 0.1 N NaOH at 5°C, neutralized, rinsed and dried. Extracts were assayed for total protein by the method of Lowry et al (section 2.1.1.1) or by the Kjeldahl nitrogen determination (section 2.1.1.2), for hexuronic acid by the m-HDP method (section 2.1.1.5.), and for amino acid composition (section 2.1.1.4). Details and results of all these operations are presented in Section III. Results pertinent to Section I are also given below.

These preparations will be referred to later as being from "Experiment 3".

2.1.2.3 Results of the Tissue Extraction Experiments.

Table II shows the yields from the three tissue extractions. The yield from Experiment 1 was the weight of material which precipitated upon dialysis of the guanidine extract against water. Yields from Experiment 2 were weights of the precipitates after dialysis against 0.35 M saline. Yields from Experiment 3 were from Kjeldahl assay of exhaustively dialyzed suspensions.

Hexuronic acid was determined in the crude extracts of experiments 1 and 3, as shown in Table III.

Table IV shows the amino acid compositions of a number of these extracts, and, for comparison, the compositions of two SGPs and ox tendon collagen from the literature.

TABLE II: YIELD OF TISSUE EXTRACTIONS.

Experiment	Starting Wt. of Tissue	Extract	Yield (mg)	% of Starting Weight Wet	% of Starting Weight Dry
1	355 g wet	G-I	9350	2.6	-
2	96.5 g wet	G-I	1438	1.5	-
		G-II	47	.05	-
		G-III	19	.02	-
		Total G	1504	1.6	-
		G/DTT-I	145	.15	-
		G/DTT-II	17	.02	-
		G/DTT-III	13	.01	-
		Total G/DTT	175	.18	-
3	97.1 g wet 20.0 g dry	G-I	1995	2.1	10.0
		G-II	415	.43	2.1
		G-III	120	.12	0.6
		Total G	2530	2.6	12.7
		G/DTT-I	594	.61	3.0
		G/DTT-II	122	.13	0.6
		G/DTT-III	114	.12	0.6
		G/DTT-IV	39	.04	0.2
		Total G/DTT	869	.89	4.4

Abbreviations: G - guanidine extract
G/DTT - guanidine/dithiothreitol extract

Text section 2.1.2.3, page 70.

TABLE III: HEXURONIC ACID IN AORTA EXTRACTS.

Experiment	Extract	Hexuronic Acid (mg)	Percent of Wet Tissue (x 10^{-3})
1	G-I (crude)	80.13	23
	G-I (after collagenase)	53.20	15
3	G-I	14.1	
	G-II	5.6	
	G-III	1.1	
	Total G	20.7	21
	G/DTT-I	5.4	
	G/DTT-II	1.2	
	G/DTT-III	.4	
	G/DTT-IV	.4	
	Total G/DTT	7.4	7.6
	NaOH-I	1.8	
	NaOH-II	.4	
	Total NaOH	2.2	2.3

Abbreviations: G - guanidine extract
G/DTT - guanidine/dithiothreitol extract

Text section 2.1.2.3, page 70.

TABLE IV: AMINO ACID COMPOSITION OF AORTA EXTRACTS (RESIDUES PER 1000 RESIDUES).

Amino Acid	Experiment 1		Experiment 2		Experiment 3		Human[4] Aorta Extract	Cow[5] Aorta SGP	Ox[5] Tendon Collagen
	Note 1	Note 2	G-I	G/DTT-I	G-I	G/DTT-I			
Hypro[3]	6	0	3	26	3	26	--	tr	95
Asp	93	99	88	76	93	91	99	91	48
Thr	56	63	52	46	57	56	51	64	18
Ser	65	69	64	58	66	58	61	61	29
Glu	125	134	117	114	130	117	113	130	74
Pro	61	57	52	71	59	75	70	50	121
Gly	108	82	90	168	93	152	84	80	349
Ala	85	81	77	98	82	73	71	92	91
½Cys	17	15	19	9	16	7	12	15	0
Val	54	51	63	46	54	53	64	67	26
Met	17	21	22	16	21	16	21	10	6
Ile	35	37	43	32	42	36	50	55	14
Leu	71	80	78	72	82	70	86	97	26
Tyr	31	31	31	24	30	25	42	17	5
Phe	33	35	35	27	33	32	42	35	14
Hylys[3]	--	--	6	2	1	6	--	0	33
Lys	66	66	74	48	63	39	53	67	34
His	21	21	25	13	21	18	20	23	5
3M-His[3]	--	--	--	--	tr	--	--	--	--
Arg	55	59	61	55	56	52	48	53	47
Trp	--	--	--	--	--	--	14	--	--

[1]Before collagenase
[2]After collagenase
[3]Hypro: hydroxyproline. Hylys: hydroxylysine. 3M-His: 3-methyl histidine
[4]Barnes and Partridge (9)
[5]Robert and Comte (109)

Text section 2.1.2.3 page 70

2.1.2.4 Discussion of Tissue Extraction Results.

A fraction was extracted from adult bovine thoracic aorta with guanidine. A subsequent fraction was extracted from the residue with dithiothreitol added to the guanidine. These fractions were comparable, in yield and amino acid composition, to many structural glycoprotein preparations from aorta reported in the literature. As expected, the extracts contained variable amounts of hydroxyproline and small amounts of hexuronic acid, suggestive of contamination by collagen and acid mucopolysaccharides, respectively.

Many of the SGP preparative schemes in the literature (Table I) can be generalized as extractions with a chaotropic agent such as guanidine, urea, or detergent, followed by extractions with more chaotrope in the presence of a reducing agent such as dithiothreitol or 2-mercaptoethanol. Alternatively, SGP can be extracted from the tissue with 0.1 N NaOH. Timpl, Wolff, and Weiser (131) and Keeley and Labella (60) have shown that NaOH extracts SGP more completely than do reducing agents in chaotropic buffers. It is quite probably more degraded, however. The SGP described in this thesis was prepared by the gentler procedure of chaotropic agent with reducing agent. Yields were comparable in all three experiments. Yields were also comparable to the yields of SGP reported in the literature from pig aorta (26)(72)(76)(80), from human aorta (85), from chick aorta (60), and from calf aorta (19). The yields cannot be compared to those obtained by extraction with dilute buffers only (94)(97)(134).

The amino acid compositions of all preparations were generally similar, and very unlike collagen. It was noted in the historical section that amino acid composition has been the principal basis of comparison of SGPs, even though by itself such a comparison means very little. These data allow estimation of contamination by collagen. The G and G/DTT extracts (Table IV) from all three extraction sequences had compositions which fell within Robert and Comte's definition of SGP (109), showing "the presence of hydroxylysine and cysteine, the absence or low levels of hydroxyproline, (and) high amounts of polar and aromatic amino acids." More recent papers consistently show that hydroxyproline and hydroxylysine in SGP preparations are due to collagen or elastin contamination, rather than to the presence of these residues in SGP (82)(99)(122). The compositions of G and G/DTT extracts from all three experiments were consistent with this finding. After collagenase digestion, the material from the first experiment showed complete removal of hydroxyproline. A reduction of glycine content from 108 to 82 res/1000 was also seen, consistent with the removal of collagen, which contains over 300 residues of glycine per 1000 residues (Table V). The extracts from Experiments 2 and 3 showed varying amounts of hydroxyproline, and increased amounts of glycine in proportion to the amount of hydroxyproline, both indicative of the presence of collagen in the extracts.

As expected, hexuronic acid was found in the aorta extracts, the largest amount being in the initial guanidine extracts. The uronate was quite tightly bound to the precipitate which formed upon removal of

the guanidine by dialysis, since in Experiment 1 it was still present after seven water washes. It was apparently also quite tightly bound to the insoluble tissue residue, since small amounts of uronic were seen even in the NaOH extracts of Experiment 3. Although the m-HDP assay is quite specific for hexuronic acid (15)(112), it is possible that the low levels of uronic seen were caused by an unidentified interfering substance.

The G and G/DTT extracts precipitated heavily upon removal of the guanidine by dialysis, either against water or against 0.35 M NaCl. The saline supernatant from Experiment 2 gave very little additional precipitate when dialyzed against water. From the solubility observations, it is reasonable to suggest that the substances isolated in the three different experiments represented the same fraction of the tissue. Their compositions, and other data yet to be presented, support this conclusion.

It should be noted that the precipitates isolated in the first two experiments presumably contained some albumin and other soluble proteins. There were two reasons for this. First, the tissue was not washed with dilute buffers prior to extraction with guanidine. Second, these proteins, being denatured by the guanidine during extraction, would precipitate upon dialysis to lower salt concentration.

The next series of experiments will further characterize the aorta extracts.

2.1.3 Experiments Using Column Chromatography.

2.1.3.1 Introduction.

The objectives of Experimental Section I were to extract SGP from aorta tissue without degrading it, and to fractionate the extracts. The question to be answered was whether the reported heterogeneity of SGP was due to aggregation, polymerization, or fragmentation. It was hoped that an SGP monomer could be isolated. After preparation of the crude extracts as described above, the next step was to fractionate them.

The methods of fractionation applicable to these extracts were limited by the nature of the extracts. The proteins were denatured by the guanidine used to extract them from the tissue. As such, they were insoluble in water or dilute buffers. Fractionation by ammonium sulfate precipitation was impossible. Similarly, many other common techniques could not be used because of the insolubility of the extracts except in the presence of chaotropic agents. In view of the problem of solubility, gel filtration was adopted as the primary technique for fractionating the aorta extracts.

This section describes the experiments which led to an understanding of the solubility problem, and other experiments which provided useful information on the nature of aortic SGP.

2.1.3.2 Materials and Methods.

2.1.3.2.1 Gel Filtration.

Gel filtration, or gel permeation chromatography, is a common technique in biochemistry. It can be carried out in virtually any

solvent in which the material to be fractionated is soluble, an advantage in view of the insolubility of the material to be fractionated in these experiments. Fischer (36) has discussed all aspects of this technique. Briefly, gel filtration provides a separation of molecules on the basis of their hydrodynamic size. Very large molecules are eluted first, at the void volume (V_o) of the column. Very small molecules are eluted last, at the total volume (V_t). Molecules of intermediate size emerge at a characteristic elution volume (V_e) between V_o and V_t. Relative elution volumes are often expressed as the K_{av}, defined as:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

I will use this convention in reporting the results of gel filtration experiments in this thesis.

For these experiments, Bio-Gel P-200 and P-300 were purchased from Bio-Rad. Sepharose 2B, 4B, and 6B were from Pharmacia. Technical grade guanidinium chloride was obtained from Matheson, Coleman, and Bell, and treated as described in section 2.1.2.2.2.1. Tris-hydroxymethylaminomethane (tris) was purchased as "Trizma Base" from Sigma. Other common laboratory chemicals were reagent grade.

Unless otherwise stated, all material to be fractionated was reduced with dithiothreitol to break disulfide crosslinks and alkylated with either iodoacetate or iodoacetamide to block the free thiol groups as described by Ross and Bornstein (113). Reduced, alkylated material was dialyzed free of guanidine. It was then either

lyophilized, or dialyzed against another buffer, or made up in another buffer by the addition of a suitable volume of buffer concentrate. Many variations of these three manipulations were used, with equal success. However, it was necessary to avoid combining SDS and guanidine in the same solution. When this was done, a heavy precipitate formed immediately. It was assumed but never proven to be guanidinium dodecylsulfate.

Individual column systems are now described.

2.1.3.2.1.1 Bio-Gel P-300 in Dilute Tris.

Buffer for this system was 0.05 M tris HCl, pH 6.85. A 2.5 x 25 cm column was prepared. (Column dimensions will always be given as diameter x length.) After sample application, the column was eluted at 4.6 ml/hr using a peristaltic pump. Fractions of 3 ml were collected, either in graduated test tubes or in tared test tubes. The void volume of the column was 16.2 ml as determined by Blue Dextran (Pharmacia). Column effluent was monitored by reading the absorbance of collected fractions at 280 nm on a spectrophotometer. Fresh buffer was used as a reference blank.

2.1.3.2.1.2 Sepharose 2B in Dilute Phosphate.

Buffer for this system was 0.05 M sodium and potassium phosphate, pH 6.75. A 2.5 x 35 cm column was filled to a height of 30 cm with 6 mm glass beads, then filled with buffer to 32 cm. A slurry of Sepharose 2B in buffer was then allowed to settle among the beads until a firm bed was obtained. The use of glass beads as a support for

low-percentage gels has been described by Sachs and Painter (115). This column was eluted at 25 ml/hr. Fractions of 2 ml were collected and read manually at 280 nm on a spectrophotometer. Void volume by Blue Dextran was 32 ml.

2.1.3.2.1.3 Columns in Guanidinium Chloride.

Several columns in guanidinium chloride were used. Buffer for all these was 4 M guanidinium chloride, 0.05 M tris HCl, pH 8.

A 2.5 x 25 cm column of Bio-Gel P-300 was eluted at 3.3 ml/hr. Fractions of 2 ml were collected and read manually at 280 nm. The void volume of this column was 41 ml.

A 2.5 x 25 cm column of Bio-Gel P-200 eluted at 3 ml/hr. Fractions of 3 ml were collected. Void volume was 40 ml. The fractions were assayed for carbohydrate by the phenol-sulfuric acid method of Dubois et al (2.1.1.8). This column was used in an attempt to separate undialyzable carbohydrate from samples eluted from Sepharose columns in guanidine.

A 2.5 x 31.5 cm column of Sepharose 2B was eluted as described for the 2B column in dilute phosphate just above, using glass beads for gel support. Void volume was 31 ml.

Two columns of Sepharose 6B were prepared in guanidine, one analytical column measuring 0.9 x 15 cm and another preparative column measuring 3.1 x 95 cm. The smaller column was eluted at 2.0 ml/hr and had a void volume by Blue Dextran of 2.75 ml, which included the volume of the flow cell used to monitor the effluent. Effluent absorbance at 280 nm was followed using a flow monitor with a 5 mm flow cell.

The instrument used was a Model UA-4 Absorbance Monitor, manufactured by ISCO. A strip chart recorder attached to the flow monitor was used to obtain plots of effluent absorbance versus time. Fractions from the small column were not collected.

The larger 3.1 x 95 cm column was eluted at 6 ml/hr. Faster flow rates were found to broaden peaks and decrease resolution severely, a phenomenon also noted by Bryce and Crichton (20). Void volume of this column varied slightly with use, being 245 ml when the column was freshly prepared and dropping to 235 ml as the gel settled and compressed. Fractions of 6 ml were collected. Effluent was monitored at 280 nm by use of the flow cell described above. Samples were applied to this column in buffer containing 5% sucrose by layering directly onto the top of the settled gel. Peaks from this column were used in a number of experiments. They were dialyzed free of salt and lyophilized.

2.1.3.2.1.4 Columns in Sodium Dodecylsulfate (SDS).

Buffer for these columns was 0.2% SDS, 0.05 M tris HCl, pH 8. It was normally made as a 25-fold concentrate and diluted with water as needed.

A 1.5 x 60 cm bed of Sepharose 2B was prepared in a column equipped with flow adaptors. It was eluted upward, at 6.5 ml/hr, to minimize bed compression. Void volume was 26.5 ml. Fractions of 2.7 ml were collected. Absorbance of effluent at 280 nm was followed by the flow monitor as described.

Small and large columns of Sepharose 6B were also prepared in SDS buffer. The smaller 0.9 x 15 cm column was eluted at 2.0 ml/hr. Void volume was 3.0 ml including the volume of the flow monitor cell as described earlier. No fractions were collected from the smaller column. The larger 3.1 x 95 cm column was eluted at 10 ml/hr. Its void volume was 260 ml. The effluent absorbance at 280 nm was monitored using the flow cell as described. Fractions of 5 ml were collected.

2.1.3.2.1.5 Sepharose 6B in 8 M Urea.

A 1.5 x 60 cm column of Sepharose 6B was prepared in a buffer of 8 M urea, 0.05 M tris HCl, pH 8. Void volume was 25 ml. The column was eluted upward at 3 ml/hr, effluent absorbance at 280 nm being followed by the flow monitor described above. No fractions were collected.

2.1.3.2.2 Other Column Chromatographic Procedures.

A number of other column chromatographic procedures were attempted in an effort to fractionate the aorta extracts on a basis other than size. Since none of them was successful, they are described here only in summary.

Several columns of DEAE-cellulose or of ECTEOLA-cellulose were run in dilute tris or dilute phosphate buffer. Gradients of NaCl with concentrations between zero and 1 M were employed, followed by elution of the entire sample with 0.1 N NaOH. Later, a column of DEAE-cellulose in 8 M urea was used. It employed a NaCl gradient from

0 to 1 M followed by a pH gradient in 1 M NaCl from 8.8 to 2.8 using tris and citrate buffers. Finally, a column of hydroxylapatite in SDS was attempted, according to the procedure of Moss and Rosenblum (81). Results of these attempts are described in the next section.

2.1.3.3 Results.

2.1.3.3.1 Experiments in Dilute Buffers.

A collagenase-digested sample of the guanidine-extractable material from Experiment 1 (section 2.1.2.2.1) emerged entirely with the void volume from a column of Bio-Gel P-300 in 0.05 M tris buffer. The same material, when fractionated by a column of Sepharose 2B in 0.05 M phosphate buffer, gave a distribution of unresolved species occupying the entire volume from V_o to V_t. The solid line in Figure 6 shows the elution profile of the Sepharose 2B experiment.

2.1.3.3.2 Experiments in Guanidine.

The collagenase-digested, guanidine-extractable material from Experiment 1 emerged at the void volume from Bio-Gel P-300 in 4 M guanidine. When chromatographed on Sepharose 2B in guanidine, this same sample gave the elution profile shown by the dashed line in Figure 6. Nearly all the material was contained in a single, symmetric peak with a K_{av} of 0.7. A small amount of absorbing material was seen at the void volume. Thus guanidine was seen to reduce the aggregation of the material.

Figure 7 shows a chromatogram of 50 mg of the first guanidine extract (G-I) from Experiment 2 (2.1.2.2.2) on the 3.1 x 95 cm column of Sepharose 6B in guanidine. Two major fractions were seen. Peak I emerged at the void volume as an asymmetric peak with considerable tailing. Peak II was seen at a K_{av} of 0.33 as a symmetric peak. A third small peak was also seen, with a K_{av} of 0.43. The

Figure 6. Gel Filtration on Sepharose 2B of First Guanidine Extract.

Plots of absorbance at 280 nm versus elution volume. Sample for both experiments was Guanidine extract of aorta (Experiment 1, section 2.1.2.2.1, page 63) after collagenase.

Solid line: Column in 0.05 M phosphate buffer, pH 6.75.

Dashed line: Column in 4 M guanidine.

Text section 2.1.3.3.2, page 84.

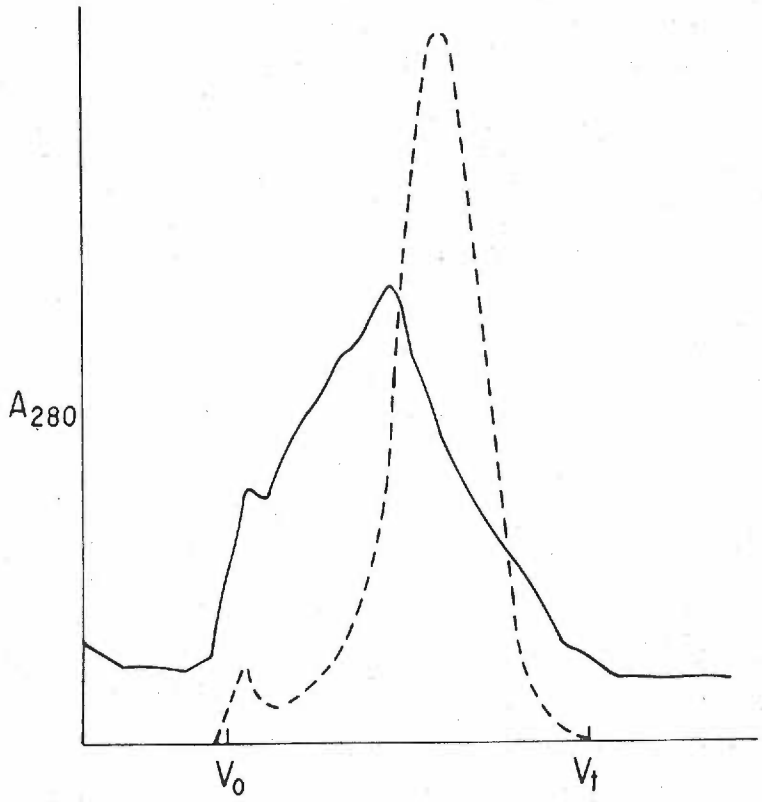

A_{280}
V_o
V_t

Figure 7. Profile of Extract G-I Eluted From Sepharose 6B in 4 M Guanidine.

Aorta extract G-I from Experiment 2 (section 2.1.2.2.2, page 65) after reduction and alkylation, on 3.1 x 95 cm column.

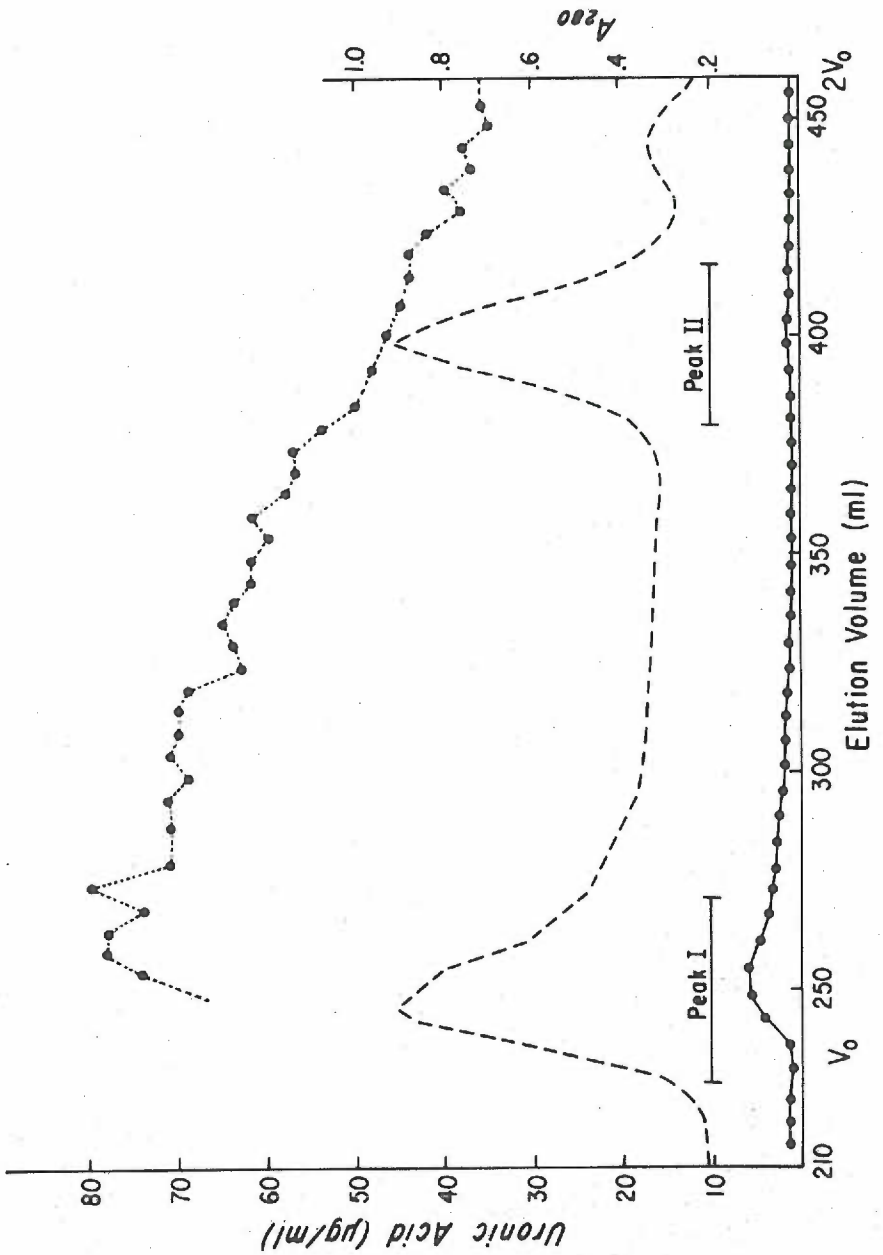
Dashed line: A_{280} from strip chart recording.

Solid line: Hexuronic acid by the m-HDP method.

Dotted line: Color yield of carbazole assay for hexuronic acid.

The dotted line represents degraded Sepharose rather than hexuronic acid. Peaks I and II as shown here were analyzed in the experiments to follow. Note that some material emerged between the peaks, and also as a small peak after Peak II. There was no absorbing material between 2 V_o and V_t.

Text section 2.1.3.3.2, page 84.


Uronic Acid (μg/ml)
80
70
60
50
40
30
20
10
A_{280}
1.0
.8
.6
.4
.2
Peak I
Peak II
V_o
$2V_o$
210
250
300
350
400
450
Elution Volume (ml)

first guanidine/dithiothreitol extract (G/DTT-I) from Experiment 2 gave the same peaks, but Peak II was much smaller in relation to Peak I. When Peak II from G-I was returned to the same column, it eluted as a single peak at a K_{av} of 0.33, showing that Peak I and Peak II did not represent an equilibrium between monomer and aggregate. Peaks I and II, as prepared from G-I on this column, were studied extensively.

Amino acid compositions of Peak I and Peak II, and of the crude G-I extract from which the two peaks were derived, are given in Table V. These were obtained using the two-column system of amino acid analysis described in section 2.1.1.4. Cysteine in these alkylated samples was measured as S-carboxymethyl cysteine. Free cysteine in Peak I was determined to be present in minute traces if at all, indicating essentially complete alkylation by iodoacetamide. Peak height on the chromatogram was estimated to be less than ½ millimeter. Hexosamines were also calculated from the chromatograms, and are included in Table V.

A profile of hexuronic acid from this column, as determined by the m-HDP assay (2.1.1.5), is shown as the solid line in Figure 7, with the absorbance at 280 nm plotted as the dashed line for comparison. Earlier attempts to measure hexuronic acid in the column effluent by the carbazole method (2.1.1.5) resulted in the dotted line also shown in Figure 7.

Peaks I and II were found to contain 6.4 µg and 2.6 µg, respectively, of sialic acid (2.1.1.6) per mg of protein.

TABLE V: AMINO ACID COMPOSITIONS OF PEAK I, PEAK II, AND THE CRUDE EXTRACT FROM WHICH THEY WERE OBTAINED (RES PER 1000 RES).

Hypro[1]	6	0	3
Asp	91	91	88
Thr	54	61	52
Ser	66	67	64
Glu	136	115	117
Pro	62	54	53
Gly	96	82	90
Ala	76	78	77
½Cys[2]	19	17	19
Val	59	56	63
Met	5	14	22
Ile	37	61	43
Leu	78	77	78
Tyr	26	39	31
Phe	32	36	35
Hylys[1]	tr	--	6
Lys	74	66	74
His	22	24	25
Arg	60	62	61
Glu-N[3]	7	3	9
Gal-N[3]	8	--	6

[1]Hypro: hydroxyproline. Hylys: hydroxylysine.
[2]as S-carboxymethyl cysteine.
[3]Glucosamine and galactosamine are lower limit estimates only.

Text section 2.1.3.3.2, page 89.

After exhaustive dialysis against water, the buffer collected from columns of Sepharose 6B in 4 M guanidine contained 60-70 µg/ml of carbohydrate as measured by the phenol-sulfuric acid assay of Dubois et al (2.1.1.8). In an effort to develop a way of removing this contamination from peaks isolated from the column effluent, a 70 ml portion of the effluent was dialyzed, lyophilized, and made to 4 ml in 4 M guanidine. It was then applied to a Bio-Gel P-200 column in 4 M guanidine (2.1.3.2.1.3). Most of the carbohydrate emerged at the void volume of the column, as detected by the phenol-sulfuric acid assay after dialysis of the fractions to remove guanidine.

2.1.3.3.3 Experiments in Sodium Dodecylsulfate (SDS).

In general, aorta extract fractionated by gel filtration in SDS showed the same type of elution profiles as those shown in guanidine. Figure 8 shows the results of an experiment in which the first guanidine extract from Experiment 1 (2.1.2.2.1) was fractionated on the 3.1 x 95 cm column of Sepharose 6B in SDS. The solid line represents the content of protein in each fraction as determined by the method of Lowry et al (2.1.1.1). Peaks I and II, as shown in Figure 8, emerged at the same K_{av} values as the corresponding peaks in guanidine (see Figure 7). Resolution was not as good in SDS, however, suggesting that disagregation was not as complete in SDS as in guanidine. Protein assay confirmed that the A_{280} profile in fact represented protein rather than SDS micelles or other artifacts.

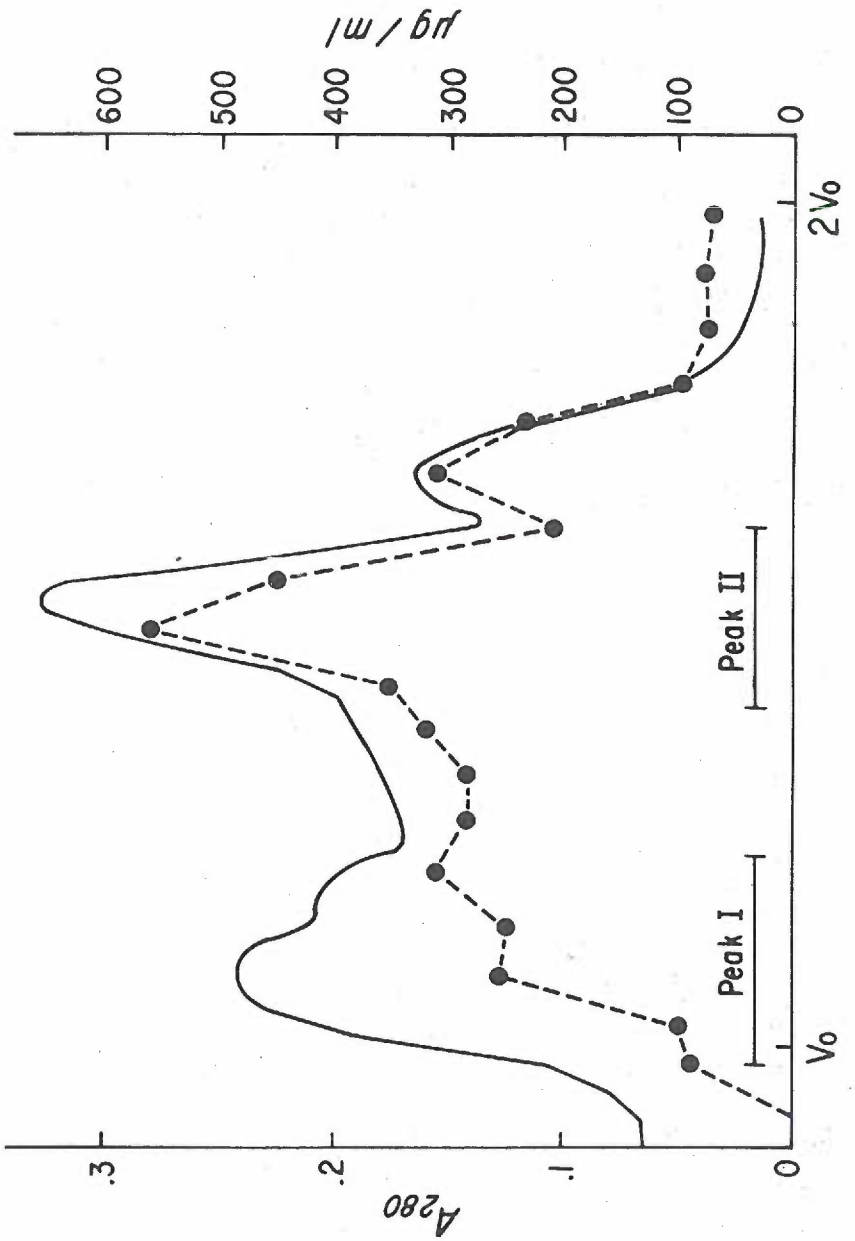
Figure 8. Profile of Extract G-I Eluted from Sepharose 6B in 0.2% Sodium Dodecylsulfate (SDS).

Guanidine extract from Extraction Experiment 1 (section 2.1.2.2.1, page 63) after collagenase digestion, reduction, and alkylation (113), on 3.1 x 95 cm column.

Solid line: A_{280} from flow cell.

Dashed line: protein (µg/ml) by the method of Lowry et al (69).

Peaks I and II were not recovered from this column. No absorbing material emerged after 2 V_o. Text section 2.1.3.3.3, page 91.


μg / ml
600
500
400
300
200
100
0
2V0
Peak II
Peak I
V0
A280
.3
.2
.1
0

2.1.3.3.4 Experiments in Urea.

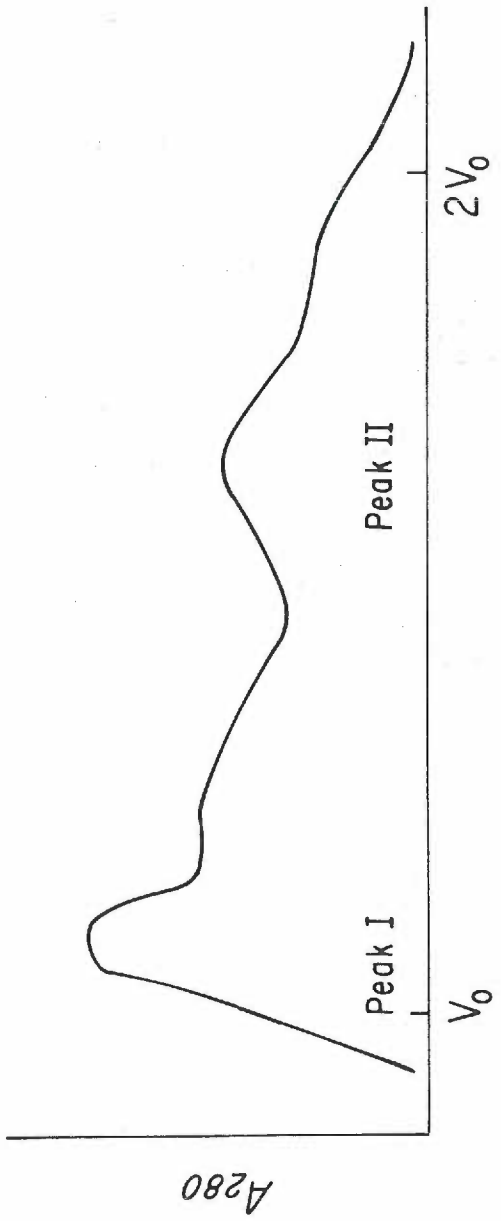
The collagenase-digested guanidine extract from Experiment 1 was fractionated on Sepharose 6B in 8 M urea. This sample was not alkylated to block free thiol groups. Formation of disulfide bridges was inhibited by the inclusion of 0.1% 2-mercaptoethanol in the column buffer. The resulting chromatogram is shown in Figure 9. Like SDS, urea did not disrupt the aggregation as completely as did guanidine (compare to Figure 7).

2.1.3.3.5 Observations on Solubility.

During the course of the column chromatographic experiments just described, a number of observations were made concerning the solubility of the aorta extracts and standard proteins exposed to denaturing buffers or reducing agents. Once precipitated or lyophilized, the guanidine or guanidine-dithiothreitol extracts could not be redissolved in guanidine, 0.2% SDS, or 8 M urea, unless a reducing agent, either 2-mercaptoethanol or dithiothreitol, was added to the solution. Once reduced, the protein dissolved in the chaotropic solutions. If the reduced protein in solution was treated with iodoacetate or iodoacetamide as described by Ross and Bornstein (113), it could subsequently be dialyzed, lyophilized, and redissolved in guanidine, SDS, or urea without the use of more

Figure 9. Profile of Extract G-I Eluted from Sepharose 6B in 8 M Urea.

Guanidine extract from Extraction Experiment 1 (section 2.1.2.2.1, page 63) after collagenase digestion. 3.1 x 95 cm column. Sample was not alkylated with iodoacetamide. Buffer for column contained 2-mercaptoethanol to reduce, and prevent reformation of, disulfide crosslinks. No absorbing material emerged after 2 V_o. Text section 2.1.3.3.4, page 94.


A280
Peak I
Peak II
V_o
$2V_o$

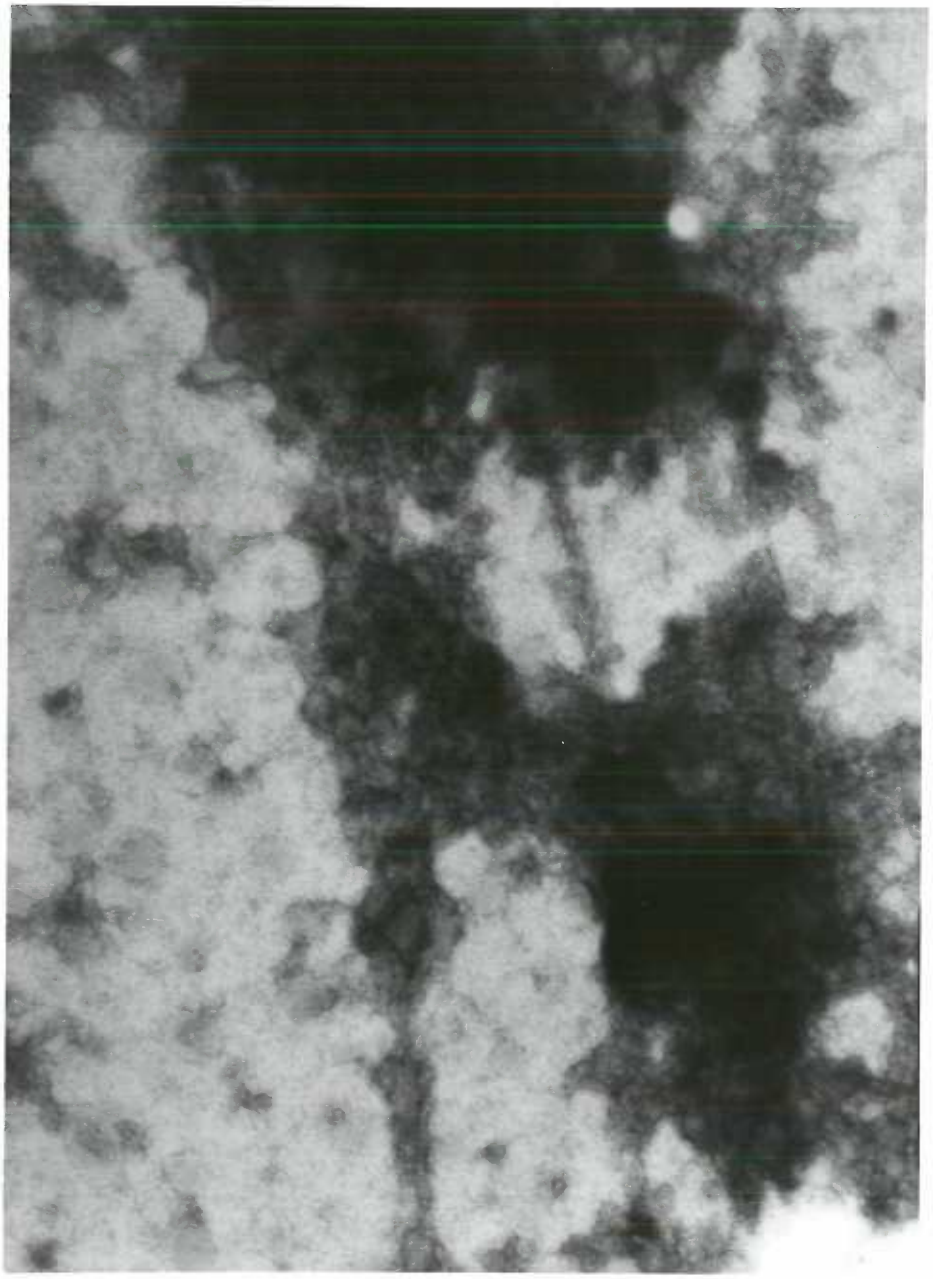
reducing agent. Similar observations led Furthmayr and Timpl (39) to conclude that intermolecular disulfide crosslinks were responsible for the insolubility of lyophilized acidic structural proteins in chaotropic solutions.

Upon dialysis of the peaks eluted with guanidine from the Sepharose 6B column, a large portion of the protein did not precipitate. During preparation of the aorta extracts in Experiments 1 and 2, this material had precipitated heavily when dialyzed from guanidine into distilled water. The concentration of protein in the column effluent was much lower than the concentration in the original tissue extracts, however. This led to an attempt to renature a portion of guanidine-extractable material by dialysis from guanidine into a buffer of 0.1 M tris HCl at a very low protein concentration (20 μg/ml). No precipitate formed after this dialysis. The solution was concentrated to 5 mg/ml by placing it in dialysis tubing and suspending the tubing in a chamber containing a partial vacuum (450 mm Hg). It precipitated heavily. A sample of the precipitate was examined with the electron microscope and seen to be an amorphous mass (Figure 10). No fibers or other structures were seen. Earlier, McCullagh et al (72) had reported fibers resembling microfibrils (113) in urea extracts of elephant aorta after lyophilization.

An attempt was made to dissolve 2 mg of alkylated, lyophilized

Figure 10. Electron Micrograph of a Precipitate of Extract G-I After Attempted Renaturation.

Extract G-I from Experiment 2 (section 2.1.2.2.2, page 65) was dialyzed from very dilute protein solution and then concentrated in an attempt to obtain renatured material. Precipitate stained with Uranyl acetate. x 110,000. Text section 2.1.3.3.5, page 97.



Peak I and Peak II material (2.1.3.3.2) in 1 ml of non-ionic detergent, either 1.5% Brij 35 (Pierce) or 5% Triton X-100 (Sigma). The objective was to perform ion exchange chromatography or electrophoresis in non-ionic detergent in order to effect a fractionation of the material on the basis of charge. Neither sample was soluble in Triton X-100, after an hour of stirring at 20°C or after subsequent heating to 95°C for 5 minutes. Peak I dissolved in the Brij solution after 5 minutes at 95°C, but Peak II remained as a solid. Other portions of the same Peak II sample were soluble in 8 M urea. Nonionic detergents were not effective in dispersing the aggregates.

Samples of bovine serum albumin (Sigma) and bovine skeletal muscle actin (prepared as described in section 2.2.3.1) were reduced with dithiothreitol in 5 M guanidine and alkylated with iodoacetamide in like manner to the treatment of aorta extracts (113). Both proteins precipitated heavily upon dialysis against water. By this treatment, these normally soluble proteins had been rendered as insoluble as the proteins extracted with guanidine from the aorta.

2.1.3.3.6 Results of Column Techniques Other Than Gel Filtration.

Collagenase-digested material from Experiment 1 was not satisfactorily fractionated by DEAE- or ECTEOLA-cellulose columns in the absence of chaotropic agents. Some material emerged as a broad plateau during the NaCl gradients. Most of it could not be

eluted from the columns except with 0.1 N NaOH, which brought the entire load out at once. Peak II from the Sepharose 6B column in guanidine emerged from DEAE cellulose in 8 M urea as a continuous, low plateau beginning at 0.5 M NaCl and continuing throughout the pH gradient. Peak II also gave a low, broad peak from the hydroxylapatite column in SDS. A mixture of bovine serum albumin (Sigma) and ovalbumin (Mann) also emerged as a single, broad peak from this column.

2.1.3.4 Discussion of Column Chromatography Results.

Column chromatographic experiments established the following characteristics of the aorta extracts:

1. They were extensively aggregated and insoluble in dilute buffers such as 0.05 M tris HCl.

2. The precipitated aggregates showed none of the microfibrillar structure reported by Ross and Bornstein (113) and by McCullagh et al (72).

3. The aggregates were dispersible and soluble in chaotropic solutions -- 4 M guanidinium chloride, 0.2% SDS, or 8 M urea.

4. The precipitated aggregates formed intermolecular disulfide crosslinks *in vitro* unless the free thiols were blocked by alkylation with iodoacetate or iodoacetamide.

5. In the presence of chaotropic agents, the material was separated into two principal peaks by gel filtration on Sepharose 6B. Peak I, at the void volume, was heterogeneous, while Peak II, at a K_{av} of 0.33, appeared homogeneous with respect to size.

6. Peaks I and II were similar in amino acid and carbohydrate composition, but Peak I could not be shown to be a polymer or aggregate of Peak II monomers.

2.1.3.4.1 Aggregation and Insolubility.

The aorta extracts were extensively aggregated and quite insoluble in the absence of chaotropic agents.

After collagenase digestion and alkylation with iodoacetate,

the guanidine extract from Experiment 1 could not be recovered from DEAE or ECTEOLA cellulose column by eluting with 1 M NaCl. It could be removed only with 0.1 N NaOH. This behavior suggested extensive aggregation or precipitation of the protein in the column. The same material, in a buffer of 0.05 M phosphate, emerged from a column of Sepharose 2B as a broad, irregular peak indicative of extensive heterogeneity (Figure 6, solid line). Since bovine serum albumin and bovine skeletal muscle actin could be rendered similarly insoluble by exposure to chaotropic agents, it was concluded that the insolubility of the aorta extracts might be due to their denatured condition. This led to the attempt to renature the proteins in a crude aorta extract by dialysis of a very dilute protein solution from 4 M guanidine into 0.1 M tris HCl. Upon concentration of the dialysate, a precipitate formed. McCullagh et al (72) had reported seeing fibers resembling microfibrils, described by Ross and Bornstein (113), in dialyzed, lyophilized material extracted from elephant aorta with 8 M urea. However, the precipitate I obtained had no appearance of microfibrils; indeed, it appeared almost entirely amorphous (Figure 10).

2.1.3.4.2 Intermolecular Disulfide Crosslinks.

The aorta extracts formed intermolecular disulfide bonds when the extracts were dialyzed free of salt and lyophilized.

The evidence for this is the same as that presented by Furthmayr and Timpl (39). The lyophilized solid could not be redissolved in

any chaotropic agent unless a reducing agent was included, or unless the free thiols had previously been blocked by alkylation with iodoacetic acid or iodoacetamide. It should also be noted that reducing agents alone did not dissolve the solid. The solid was therefore shown to be held together by disulfide bonds as well as by hydophobic or ionic interactions.

2.1.3.4.3 Aggregates Dispersed by Chaotropic Agents.

In the presence of 4 M guanidine, 0.2% SDS, or 8 M urea, the alkylated aorta extracts were soluble, and aggregates were dispersed. Under these conditions, fractionation of the extracts by gel filtration on Sepharose produced an excluded peak (Peak I) and an included peak (Peak II), as shown in Figures 7, 8, and 9.

These two principle peaks were seen in both guanidine and guanidine/dithiothreitol extracts of the aorta, although the latter contained relatively less Peak II. Peak I was judged to be heterogeneous by its lack of symmetry and by the fact that it could be resolved into a smaller V_o peak and a "tail" of unresolved material by chromatography on Sepharose 2B. Peak II appeared to be homogeneous with respect to size, since it was symmetrical and appeared within the fractionation range of the gel, between V_o and V_t. (A peak at V_o includes all molecules excluded by the gel, while a peak at V_t includes all molecules totally included by the gel. Neither peak is necessarily composed of molecules of a single molecular weight. However, peaks within the fractionation range of

the gel must be composed of molecules of the same, or nearly the same, size. See Fischer (36).)

2.1.3.4.4 Relationship Between Peak I and Peak II.

Peaks I and II were found to be quite similar in amino acid and carbohydrate composition, but Peak I could not be shown to be a polymer or aggregate of Peak II monomers.

Fractions, collected from the fractionation on Sepharose 6B in SDS of a sample of collagenase-digested guanidine extract from Experiment 1, were assayed for protein by the method of Lowry et al (2.1.1.1), with the results shown in Figure 8. The solid line (A_{280}) and dashed line (protein) showed similar results except in the region of Peak I, which showed a lower protein content relative to the absorbance at 280 nm. These findings suggest that the A_{280} profiles reflected actual protein, but that Peak I contained protein with a low color yield by the method of Lowry et al.

Amino acid analysis of Peaks I and II, prepared from the first guanidine extract of Experiment 2 by gel filtration on Sepharose 6B in guanidine, showed many similarities between the two fractions. However, Peak II was free of hydroxyproline, indicating separation from collagen. The similarity in composition between Peak I, Peak II, and the total G-I extract raised the possibility that Peak I was an aggregate or polymer of Peak II. However, the virtual absence of free cysteine in Peak I showed that there could be few if any disulfide crosslinks present in the material after reduction

and alkylation. If Peak I was a polymer of Peak II monomers, disulfide crosslinks could not account for the polymerization. A sample of Peak II in guanidine was rechromatographed on Sepharose 6B. The elution profile (not shown) revealed no trace of Peak I. The entire load emerged at a K_{av} of 0.33, the same K_{av} as the original Peak II. In experiments to be described later, Peak I could not be disaggregated to produce any additional Peak II. From this evidence, Peak I was judged not to be in a monomer-aggregate equilibrium with Peak II.

2.1.3.4.5 Carbohydrate Content of Peaks I and II.

Fractions from the column used to obtain Peaks I and II for amino acid analysis (just above) were assayed for hexuronic acid by the m-HDP method of Rosenthal et al (section 2.1.1.5). The results are shown as the solid line in Figure 7. Earlier attempts to measure hexuronic acid by the carbazole method of Balazs et al (section 2.1.1.5) gave the profile shown by the dotted line in Figure 7. This profile reflected the lower specificity of the carbazole method compared to the m-HDP method. The profile was attributed to fragments of degraded Sepharose (galactose and 3, 6-anhydro-galactose (20)) washed out by the guanidine. These fragments were not dialyzable, nor could they be separated from the recovered peaks by chromatography on Bio-Gel P-200. Bio-Gel, being polyacrylamide, contains no carbohydrate. Both protein and degraded Sepharose were excluded from the Bio-Gel. The presence of

small amounts of hexuronic acid in Peak I did not necessarily mean that the protein was a glycoprotein containing hexuronic acid. It most probably indicated the presence in the column effluent of a trace of acid mucopolysaccharides of high molecular weight. Hexuronic acid has come to be considered as a marker saccharide for these ground substance components (15).

The small amount of hexosamines and sialic acid found in Peaks I and II would be expected if these peaks did indeed represent glycoproteins. However, there was no proof that these sugars were actually bound to the major protein component(s) in these peaks.

2.1.3.4.6 Summary.

Gel filtration had resolved the crude aorta extracts into two major peaks which appeared not to be related via a monomer-aggregate equilibrium. Other attempts to fractionate the extracts by column chromatography were unsuccessful. The resolution possible by gel filtration is rather limited when compared to that of electrophoresis in polyacrylamide gels. The next section, therefore, deals with the use of polyacrylamide gel electrophoresis to further investigate the nature of the aorta extracts.

2.1.4 Experiments Using Electrophoresis and Isoelectric Focussing in Polyacrylamide Gel.

In the previous section (2.1.3) the techniques of column chromatography, especially gel filtration, were used to gain a partial understanding of the mixture of proteins extracted from bovine aorta in the presence of 5 M guanidine. However, because of the limitations of these techniques, several questions remained unanswered. In order to pursue those answers, a technique was needed which would have much greater resolving power than had gel filtration. And, since several attempts to fractionate the extracts on the basis of charge using column chromatography had failed, a usable technique was needed for resolving proteins on the basis of their charged groups rather than their sizes alone.

Polyacrylamide gel electrophoresis (PAGE) and polyacrylamide gel isoelectric focussing (PAGIF) are currently the most powerful techniques available to the biochemist for separating mixtures of proteins. Potentially, PAGE and PAGIF offered the resolution, and the ability to effect separations on the basis of charge, needed to continue the investigation of the aorta extracts.

An excellent presentation of the principles and procedures of PAGE and PAGIF has been compiled by Allen and Maurer (2). Both techniques are based upon the migration of proteins in an electric field. The molecules move at rates governed by their size, shape, and net charge. In PAGE, they continue to move as long as the electric field is applied. In isoelectric focussing, a stable pH gradient is

established between positive and negative electrodes. Proteins in PAGIF migrate to a point on the gradient where their net charge is zero, at which point they stop. If proteins are dissolved in sodium dodecylsulfate detergent (SDS), the total negative charge of all the SDS molecules bound to proteins is much greater than the charges of the amino acids in the protein. Since most proteins are denatured in SDS, and bind the same stoichiometric amount of SDS per amino acid residue, PAGE in SDS separates proteins principally on the basis of size alone. The resolution potentially obtainable using PAGE, PAGE in SDS, or PAGIF was far greater than that obtained in section 2.1.3 using column chromatography. This present section was devoted to the application of these three techniques to the characterization of aortic SGP.

The specific goals of section 2.1.4 were to determine:

1. If Peak II was actually a single molecular species, as the data from gel filtration had suggested.

2. If Peak I was made up of a number of discrete molecules, or of a continuous spectrum of large polypeptide fragments.

3. If corresponding peaks from Guan and G/DTT extracts were made up of the same or different species.

4. The molecular weights of the different species in the extracts.

5. If Peak I could be induced to separate and give Peak II as a monomer.

2.1.4.1 Materials and Methods.

2.1.4.1.1 Recrystallization of Acrylamide.

Acrylamide (Baker or Eastman) was recrystallized from chloroform as described by Bickle and Traut (14).

2.1.4.1.2 PAGE in Glass Tubes.

Earlier PAGE experiments were run in tubes 16 cm long with an inside diameter of 6 mm. Tubes were cleaned thoroughly, then dipped in 2% "Photo-Flo" (Eastman Kodak) and rinsed once with water before drying. Photo-Flo facilitated gel removal from the tubes after the run. Up to 12 such tubes were mounted in a Buchler "Polyanalyst" electrophoresis cell. For the gel casting process, they were capped at the bottom with rubber septa.

2.1.4.1.2.1 Casting the Tube Gels.

Separating gel solution was mixed as follows:

Separating Gel (5%) in SDS

1 M tris HCl, pH 8.8	3.75 ml
10% SDS	0.3 ml
TEMED*	15 µl
30% acrylamide, 0.8% BIS**	5.0 ml
Water to total	29.7 ml
5% ammonium persulfate	0.3 ml

*N, N, N', N' - tetramethylethylenediamine (Matheson, Coleman, & Bell)
**N, N' - Methylene bis acrylamide (Eastman)

For percentages other than 5% acrylamide, the volume of acrylamide solution was changed. Immediately prior to pouring, the gel was catalyzed by the addition of the ammonium persulfate. Gels were poured to a height of 13.0 cm and overlayered with 0.2 ml of water. Polymerization was complete in one hour.

Stacking gels were mixed as follows:

Stacking Gels (3%) in SDS

0.5 M tris HCl, pH 6.8	1.5 ml
30% acrylamide, 0.8% BIS	1.0 ml
10% SDS	0.1 ml
TEMED	10 µl
Water to	9.8 ml
5% ammonium persulfate	0.2 ml

When separating gels had polymerized, the water overlayer was removed with a Pasteur pipet. Stacking gel solution was then catalyzed by addition of the ammonium persulfate solution. Stacking gels were poured to a height of 7 mm and overlayered with water as before. Polymerization was for 30 minutes. The gels were used immediately.

Some gels were run in urea rather than in SDS. For these gels, dry urea was added to the gel solutions (separating and stacking) to give a concentration of 8 M urea. Also, 10% Brij 35 nonionic detergent (Pierce) was used in place of 10% SDS to aid in wetting the gel tubes and forming flat menisci when the gels were poured. Gels in urea were overlayered with 8 M urea in 1% Brij 35 rather than with water.

Samples for tube gels were dissolved to a concentration of 2 mg/ml in:

Sample Buffer for Tube Gels in SDS

Dry SDS	0.2 g
Dry sucrose	3.0 g
0.5 M tris HCl, pH 6.8	1.25 ml
2-mercaptoethanol	0.5 ml
Water to total	10.0 ml

The samples in solution were heated to 100°C for 2-3 minutes prior to use. For samples in 8 M urea, 4.8 g of urea replaced the SDS in the above recipe. Samples in urea were not heated.

Running buffer for tube gels, both upper and lower electrode chambers, was 0.05 M tris, 0.384 M glycine, pH 8.8 (no titration was needed). For gels in SDS, it also contained 0.1% SDS. The upper chamber (cathode) buffer contained 0.005% bromphenol blue as a tracking dye.

2.1.4.1.2.2 Loading, Running, and Staining Tube Gels.

The rubber septa were removed from the lower ends of the polymerized gels. Upper and lower running buffer compartments of the electrophoresis cell were filled. Finally, samples were layered on top of the stacking gel using a graduated section of Teflon tubing attached to a 1 ml syringe. Loads were normally 5-50 µl depending on the concentration of the sample, the number of components in the sample, and the objectives of the individual experiment.

The gels were then run at 100 volts, which gave an initial current of 2 milliamps per tube. Power supply was a Type 2541, from Consolidated. The current decreased during the course of the run. Electrophoresis was continued until the tracking dye was a few millimeters from the lower end of the gel. Position of the dye varied slightly from tube to tube. With the running buffer at 4°C at the outset, no cooling water circulation was needed during the run.

As quickly as possible after the end of the run, the position of the marker dye and the length of the separating gel were measured with a ruler and noted. The gels were then removed from the tubes. To loosen them, 0.2% SDS was injected between the gel and the glass tubing with a smooth-tipped #22 needle. The gels were allowed to slide out of the tubes and into 30 ml test tubes containing 20 ml of 0.1% Coomassie Brilliant Blue dye in 50% methanol, 7% acetic acid.

The gels were fixed and stained for two hours with gentle agitation. They were then destained with two changes of 50% methanol and one change of 5% methanol, 7% acetic acid. Gels were stored in 7% acetic acid.

2.1.4.1.2.3 Measurement of Band Migration Distances in Tube Gels.

The R_f of a given band is defined as the quotient of the distance moved by the band divided by the distance moved by the tracking dye. Determiniation of the R_f values for bands on tube gels was complicated by two factors. First, the tracking dye was removed during gel fixation and staining. Second, the gel itself swelled

slightly in 7% HAc relative to its size when poured. Positions of the bands and the tracking dye could not be measured on the gel at the same time under the same conditions. Consequently, the positions of both the tracking dye and the stained bands were measured with respect to the ends of the separating gel itself. Positions of bands and tracking dye relative to each other could then be calculated using the ratio of lengths of the gel before and after staining as a correction factor.

2.1.4.1.3 PAGE in Slabs.

PAGE using flat slabs of gel rather than individual tubes was carried out in two different apparatus. Earlier experiments employed an Ortec Model 4200 electrophoresis cell, using eight sample wells per gel. Later experiments employed a Bio-Rad Model 220 electrophoresis cell, using slabs 1.5 mm thick and having 20 sample wells per slab. Power for all slab gel experiments was supplied by an Ortec Model 4100 Pulsed Constant Power Supply. The apparatus was assembled and operated according to detailed instructions furnished by the manufacturers.

2.1.4.1.3.1 Casting the Slab Gel and Applying the Samples.

All solutions used in these experiments were prepared following the guidelines given by Ortec, Inc., in the instrument instruction manual. The buffer system was based on the work of Hjerten, Jerstedt, and Tiselius (53) and of Poulik (93).

The separating gel was mixed according to the following recipe:

Separating Gel, 5% Gel	
1.2 M tris sulfate, pH 8.00	7.5 ml
2% SDS	1.5 ml
0.1 M EDTA	0.3 ml
19.5% acrylamide/0.5% BIS (20% total)	7.5 ml
Water	11.7 ml
TEMED	18 µl
1% ammonium persulfate (made fresh)	1.5 ml
Total volume	30.0 ml

The ammonium persulfate catalyst was added immediately before pouring the gel. For other percentages of gel, the volumes of acrylamide/BIS solution and water were adjusted accordingly. After addition of catalyst, the gel was poured to a height of 11.0 cm (Bio Rad) or 6.5 cm (Ortec), overlayered with 1 ml of water, and allowed to polymerize for 30 minutes. For the 1.5 mm Bio-Rad slab, the above recipe gave ample solution. For the 3 mm thick Ortec slabs, all volumes were increased proportionately to give a total volume of 50 ml.

After polymerization of the separating gel, the water overlayer was removed by aspiration. The well-forming gel was then poured according to the following recipe:

Well and Cap Gel, 5% Gel

0.12 M tris sulfate, pH 8.00	2.5 ml
2% SDS	0.5 ml
0.1 M EDTA	0.1 ml
19.5% acrylamide/0.5% BIS	2.5 ml
Water	3.4 ml
TEMED	6 µl
Total	9.0 ml

One-half of this solution (4.5 ml) was mixed with 0.5 ml of 1% ammonium persulfate. This solution was poured on top of the separating gel to a depth of 5 mm. The well-forming comb was inserted into the well gel solution before polymerization had begun, and lowered gently to rest on the upper surface of the separating gel. This caused the well gel to rise between the teeth of the comb to a height of about 15 mm. No water overlay was necessary. The well gel was allowed to polymerize for 15 minutes.

The well-forming comb was removed very slowly so as not to destroy the wells. A few ml of water were run into the wells to rinse them free of traces of acrylamide monomer. This water was then removed with a syringe, leaving 2-3 mm of water at the bottom of each well.

Samples were then loaded into the wells with a 10 µl microsyringe (Hamilton). They were layered under the water remaining in the wells. This technique was necessary in order to prevent samples from creeping up the sides of the wells by capillary action and spilling over into adjacent wells. Sample volumes were between 1 µl and 15 µl per well for Bio-Rad slabs, and between 5 and 50 µl per well for Ortec slabs.

Samples were dissolved to a concentration of from 1 mg/ml (single pure protein) to 5 mg/ml (complex mixtures of proteins) in the following solution:

Sample Buffer for Slab Gels in SDS

5 mM tris sulfate, pH 8.00

1.5% SDS

20% sucrose

0.5 mM EDTA

1% 2-mercaptoethanol

All samples for gels in SDS were heated for 2-3 minutes in this solution at 100°C prior to application to the gel.

After sample loading, the cap gel was mixed by addition of 0.5 ml of 1% ammonium persulfate to the remaining 4.5 ml of the well and cap gel solution described above. It was layered carefully over the samples to avoid mixing, and filled to within 3 mm of the top of the cell. It was overlayered to a depth of 3 mm with water and allowed to polymerize for 15 minutes. The gel was then ready for electrophoresis.

A few slabs were done in both SDS and urea. For these experiments, dry urea was added to all the solutions (separating gel, well and cap gel, and sample buffer) to a final urea concentration of 8 M. Correspondingly less water was needed to bring the solutions to the desired total volume. To avoid decomposition of the urea and degradation of the sample (127), samples in urea were not heated to 100°C prior to electrophoresis. Since urea is uncharged and does not

migrate out of the gel during electrophoresis, the running buffer (described below) for gels in 8 M urea did not contain urea.

2.1.4.1.3.2 Modifications for Agarose-Acrylamide Composite Gel.

A gel was run in 3% polyacrylamide using 0.5% agarose as a support, according to the method of Peacock and Dingman (88). In this technique, agarose is used as a support for low percentage polyacrylamide gel. There were many problems associated with the technique, which was performed in the Bio-Rad apparatus. Because of them, many deviations from the above procedure for acrylamide gels were necessary. The separating gel was poured to a height of 11.5 cm, and the well-forming comb was inserted into this. No well or cap gel was used. For the agarose component, 0.147 g of agarose (Electrophoresis Grade, General Biochemicals) was dissolved by reflux in 14.7 ml of water, then stored at 40°C until use. The gel was mixed according to the above recipe for separating gels, using the solution of agarose in place of the water in the preparation. The electrophoresis cell was warmed to 40°C in a water bath. The gel solution was then catalyzed by addition of the ammonium persulfate and poured into the cell. The cell was cooled rapidly by immersion in running buffer at 4°C. The agarose and acrylamide gelled normally in 30 min. Samples were loaded, and the cap gel was replaced by a solution of separating gel buffer in 10% sucrose layered over the samples. From this point, the gel was treated similarly to other gels, as described in the next subsection.

2.1.4.1.3.3 Performing the Electrophoresis (Slabs).

Both upper (cathode) and lower (anode) buffer chambers were filled with running buffer at 4°C.

Slab Gel Running Buffer

0.05 M tris acetate, pH 8.00

0.1% SDS

1 mM EDTA

0.015 % 1,1,1 - trichloro-2-methyl-2-propanol (Mallinckrodt) as a preservative.

The upper buffer was discarded after each use. To perform the electrophoresis, the Ortec cell was filled with running buffer according to the manufacturer's directions. The lower chamber of the Bio-Rad cell was filled with buffer to a height of 1 cm above the level of the top of the gel while the upper chamber received 250 ml of running buffer. No tracking dye was used in either system, since the boundary between sulfate and acetate ions formed a sharp line which could be followed as it moved down the gel.

The Ortec power supply used for all slab gels applied very high voltage to the cell in a series of pulses. This mode of power application enabled more rapid electrophoresis with less heating than with constant-current power supplies. The power supply could be programmed for the desired voltage, discharge capacitance, and pulse rate. The significance of these parameters is fully discussed in the technical literature accompanying the instrument.

After the electrophoresis cells containing the slabs had been filled with running buffer and connected to the power supply, electrophoresis was begun. For a single slab in the Ortec cell, the power supply was set to 325 volts and 1 mfd discharge capacitance. The initial pulse rate was 75 pulses per second (pps). Pulse rate was increased to 300 pps after 20 min. The electrophoresis was terminated when the sulfate-acetate boundary reached the bottom of the gel, after 110 minutes of power application. Running time was longer when two slabs were done simultaneously. For a slab in the Bio-Rad cell, the settings were also 325 volts and 1 mfd discharge capacitance. Initial pulse rate was 50 pps. The pulse rate was increased to 250 pps stepwise according to the following schedule:

Power Supply Pulse Rate for Bio-Rad Slabs

After:	Increase to:
30 min	75 pps
45	100
55	150
65	200
75	250

This gradual increase in power improved band sharpness and decreased "tailing" when compared to the results after a sudden increase from 50 to 250 pps after 30 minutes. Power was applied until the sulfate-acetate boundary reached the bottom of the gel, after 160 min of power application.

During electrophoresis in the Bio-Rad cell, the lower chamber buffer was stirred gently by use of a magnetic stirring bar. Cooling water was circulated through the cell at a rate of 1-2 liters per minute.

2.1.4.1.3.4 Staining and Destaining of Slabs.

After electrophoresis, slabs were quickly removed from the cells according to the manufacturer's instructions. They were fixed, stained, and destained by immersion in the following series of solutions. Each step was carried out with 250 ml of solution at room temperature for at least 3 hours with gentle agitation:

1. 0.05% Coomassie Blue (Sigma), 25% isopropanol, 10% acetic acid.
2. Repeat solution 1.
3. 5% isopropanol, 10% acetic acid.
4. Repeat solution 3.
5. 10% acetic acid.

Any step could be prolonged up to at least 24 hours with no effect on the stained bands. Gels were stored in 10% acetic acid.

2.1.4.1.4 Estimation of Molecular Weights from Gels.

Molecular weights of all the bands appearing on gels in SDS were estimated essentially as described in the reference (2). However, no attempt was made to determine absolute R_f values on slab gels, relative to the ion boundary. Instead, relative migration values were calculated with respect to standard bovine skeletal muscle actin.

The slab gels were calibrated with a series of standard proteins from various sources:

Rabbit skeletal muscle myosin, heavy chains. 200,000 d.
(Prepared by the method of Perry (89) by I. Gipson)

Bovine serum albumin. 68,000 d (Sigma)

Ovalbumin. 45,000 d (Mann)

Bovine skeletal muscle actin. 42,000 d. (Prepared by the method of Carsten and Mommaerts. See section 2.2.3.1)

Actin dimer. 84,000 d

Pepsin. 35,000 d (Worthington)

Chymotrypsinogen. 25,000 d (Pharmacia, prepared by Worthington)

Apoferritin subunits. 19,000 d (Mann)

Human hemoglobin, subunits. 16,000 d (gift of R. T. Jones)

Relative migration values of each of these standards were calculated as the quotient of the distance migrated by the standard divided by the distance migrated by actin monomer. All distances were measured from the top of the separating gel to the most darkly staining point of the band, corresponding to the apex of the peak when the gel was scanned as described in section 2.3.2.5.1. Plots of relative migration (abscissa) versus logarithms of the molecular weights (ordinate) were constructed for each concentration of acrylamide as shown in Figure 15. The solid line is from 8% gels, while the broken line is from 5% gels. The two plots were very nearly identical, hence the abscissa for the 5% line has been doubled in Figure 15 in order to separate the two lines. Data from Ortec and Bio-Rad cells were superimposable, and

Figure 15 represents data from both types. Molecular weights of unknown bands were estimated empirically by calculating their relative migration and obtaining the corresponding molecular weight value from Figure 15.

It is well established that certain characteristics of different proteins cause them to give anomalous apparent molecular weights by this technique. A carbohydrate moiety adds much more than its actual weight to the apparent molecular weight of a glycoprotein (121). Similarly, collagen and collagen peptides appear abnormally large on PAGE in SDS (40), probably due to the stiffness of the polypeptide chains caused by their high content of the imino acids proline and hydroxyproline. Therefore molecular weight estimates of unknown bands on PAGE in SDS must be confirmed by other means, such as gel filtration or ultracentrifugation, if rigorous characterization of molecular weights is needed. Such rigorous treatment of the large number of bands involved was beyond the scope of this thesis.

2.1.4.2.5 Polyacrylamide Gel Isoelectric Focussing (PAGIF) in Tube Gels.

PAGIF was carried out in the tube gel apparatus described earlier (section 2.1.4.1.2). The process was essentially as described by Wrigley (139). Ampholytes, under the trade name of "Ampholine", pH range 3-10, were obtained from LKB.

A gel containing 4.2% acrylamide was used for PAGIF.

Gel Solution for PAGIF

10 M urea, freshly prepared	16.0 ml
30% acrylamide, 0.8% BIS	2.8 ml
40% ampholytes, pH 3-10	1.0 ml
TEMED	10 µl
5% Ammonium persulfate	0.2 ml
Totals: 4.2% T, 8 M urea	20.0 ml

The gels were poured to a height of 11.2 cm and allowed to polymerize for 2 hours. No water overlayer was used, as a flat gel surface was not necessary.

After gel polymerization, the cell was filled with cathode solution (1 N NaOH) and anode solution (1 M H_3PO_4) at 4°C. The gels, their upper ends beneath the surface of the anode solution, were overlayered with 200 µl of 2% ampholytes in 7 M urea. Prior to sample application, current was passed through the gels at 1 milliamp per gel for 30 minutes to remove residual persulfate and establish a pH gradient.

The sample for PAGIF was dissolved to a concentration of 1.75 mg/ml in 2% ampholytes, 8 M urea. After the 30 minute pre-focussing, 50 µl of sample solution were layered on top of each gel using a #22 hypodermic needle and a 1 ml tuberculin syringe. The sample was layered beneath the covering layer of ampholytes in 7 M urea.

After the samples had been loaded, the gels were run at a constant 1 milliamp per gel for 3 hours, during which time the voltage had increased from 50 to 350 V. The gels were run an additional 2½ hours

at a constant 350 volts, after which the current had decreased to 0.4 milliamp per gel.

The gels were removed from the tubes and washed seven times for at least two hours each with 30 ml of 5% trichloroacetic acid. The repeated washings were necessary to remove all traces of ampholytes, which stain heavily according to Wrigley (139). The gels were then stained with Coomassie Blue as described above for PAGE gels in tubes.

2.1.4.2 Results of Electrophoresis and Isoelectric Focussing Experiments.

This subsection presents detailed results of each individual experiment, arranged in logical sequence in which the results of one experiment indicated the design of the next. The following discussion section (2.1.4.3) summarizes the important conclusions drawn from these results.

2.1.4.2.1 Electrophoresis in the Absence of SDS or Urea.

The results of this experiment were as expected on the basis of the results of column chromatographic experiments in dilute, non-chaotropic buffers (see 2.1.3.3.1). The sample used was the collagenase-treated material extracted from bovine aorta with 4 M guanidine (see 2.1.2.2.1). Samples were placed on 5% gels in tubes. Neither SDS nor urea was present.

Most of the material did not penetrate the 3% stacking gel. A lesser amount of material stopped at the interface between the 3% stacking gel and the 5% separating gel. Small amounts of stainable material entered the separating gel as vertical streaks, indicative of slow release of smaller particles from a large, stationary deposit. No discrete bands were seen, other than a faint band at $R_f = 1.00$ (co-migrating with the tracking dye and hence of very low molecular weight and quite probably heterogeneous), and the "bands" formed on the upper surfaces of the stacking and separating gels.

These results support the conclusion from the column chromatographic section, that the aorta extracts are extensively aggregated in dilute buffers.

2.1.4.2.2 Results of Electrophoresis on Tube Gels in SDS.

In the previous section (2.1.3), it was shown that the aorta extracts could be fractionated by gel filtration through a column of Sepharose 6 B in 4 M guanidine, and that this fractionation produced two major peaks, I and II (see Figure 7). Samples of these fractions were placed on 8% tube gels in SDS. Again, the results were consistent with those from the column.

Figure 11 shows the results of the electrophoresis. The first tube was loaded with Peak I material. Most of it did not penetrate the 8% separating gel, although it did pass through the 3% stacking gel. Few discrete bands were seen, and there was considerable streaking. On this gel, Peak I appeared to be of high molecular weight and heterogeneous. The second tube contained Peak II material, which formed a single, heavy band at an R_f of 0.68. The third tube contained bovine serum albumin, while the fourth contained a mixture of albumin and Peak II. From comparisons to serum albumin, of molecular weight 68,000 d, and to ovalbumin (not shown), of molecular weight 45,000 d, Peak II appeared to have a molecular weight of 40,000 to 50,000 d. The tube gels were not accurately calibrated.

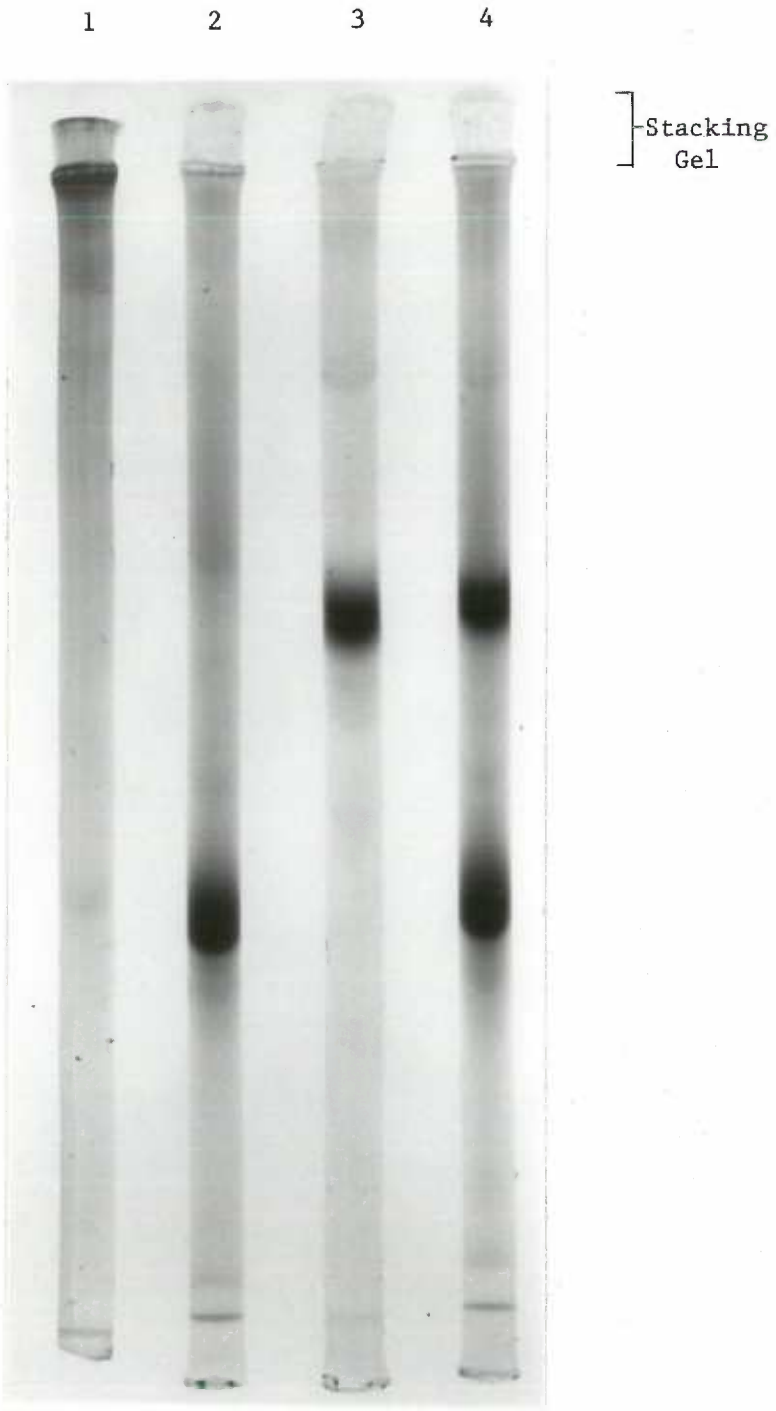
Peak I gave results similar to those on 8% gels when placed on 4% gels in SDS. Much did not enter the separating gel (not shown).

Figure 11. Polyacrylamide Gel Electrophoresis (PAGE) in Sodium Dodecylsulfate (SDS) of Peaks I and II on Tube Gels.

PAGE was carried out on 8% polyacrylamide gels in 0.1% SDS. Gels were contained in glass tubes. Staining was with Coomassie Blue. All gels were from the same run.

Tube 1: Peak I (Figure 7)
Tube 2: Peak II (Figure 7)
Tube 3: Bovine serum albumin (BSA)
Tube 4: Peak II plus BSA

Text section 2.1.4.2.2, page 127.


1
2
3
4
Stacking
Gel

2.1.4.2.3 Harsh Treatment of Peak I.

The objective of this experiment was to determine if Peak I could be broken down to give Peak II monomers. The methods chosen were sufficiently harsh to disrupt any aggregate, and in one case to degrade the material by hydrolysis of some covalent bonds in addition to relatively labile disulfide bonds.

Samples of Peak I material were treated in three ways. The first was heated to 90-100°C for ten minutes in 7 M guanidinium chloride containing 0.05 M dithiothreitol. The second sample was shaken for 2 hours at room temperature in 0.1 N NaOH. The third was heated to 95-100°C for 10 min. in 0.1 N NaOH. Samples were dialyzed against water and dissolved in sample buffer for SDS-PAGE in tube gels.

Electrophoresis showed that the treatment with hot 7 M guanidine had not changed Peak I detectably when compared to untreated material. The sample treated in NaOH at room temperature was also unaltered. The sample treated in hot NaOH showed a moderate increase in stainable material moving with the tracking dye, indicating degradation of the sample. But in none of the samples was any band seen corresponding to Peak II.

These observations demonstrated that Peak I was not an aggregate of Peak II monomers. The possibility remained, however, that it could be an extensively crosslinked polymer. Section 2.1.5 deals with this question.

2.1.4.2.4 Comparison of Results From Tube and Slab Gel Systems.

Figure 12 shows results from the three different gel apparatus and buffer systems used. From left to right, the figure shows Peak I and Peak II on 8% tube gels in SDS, then Peak I on a 5% slab in SDS using the Ortec apparatus, and finally the whole guanidine extract on 5% slab gels in SDS, first on the Ortec apparatus and then on the Bio-Rad apparatus.

Resolution on the slabs far surpassed that on the tubes. However, the slab apparatus produced an artifact band at an R_f of about 0.1 (it varied with the percent of acrylamide). The artifact band is shown in Figure 12, and is very prominent in Figure 25 below. It was visible on all gels of samples containing heterogeneous material of high molecular weight. On 8% gels, it contained myosin, and collagen beta and gamma chains, but these proteins moved ahead of the artifact band on 5% gels. Although it was not proven, it was suspected that this band consisted of "stacked" proteins which did not migrate after the passage of the sulfate-acetate boundary (2)(53).

The improved resolution of the slab systems was most apparent near the upper end of the gels, where Peak I appeared to be little more than a smear on tube gels, but was resolved into a number of sharp bands on the slabs. However, on the slabs as on the tubes, Peak I contained material which would not enter the gel, as well as some which produced a broad smear surrounding the discrete bands.

Figure 12. Composite Photograph Comparing Resolution of Tube, Ortec Slab, and Bio-Rad Slab Systems for Polyacrylamide Gel Electrophoresis in Sodium Dodecylsulfate.

All Gels were in 0.1% SDS. The purpose of this figure is to compare the sharpness of bands and the resolution between bands in the three systems. No comparison of migration distances of individual bands was intended. Migration from top to bottom of photo. Staining was with Coomassie Blue.

8% Polyacrylamide Gels in Tubes

Tube 1: Peak I, same photo as Figure 11, tube 1
Tube 2: Peak II, same photo as Figure 11, tube 2

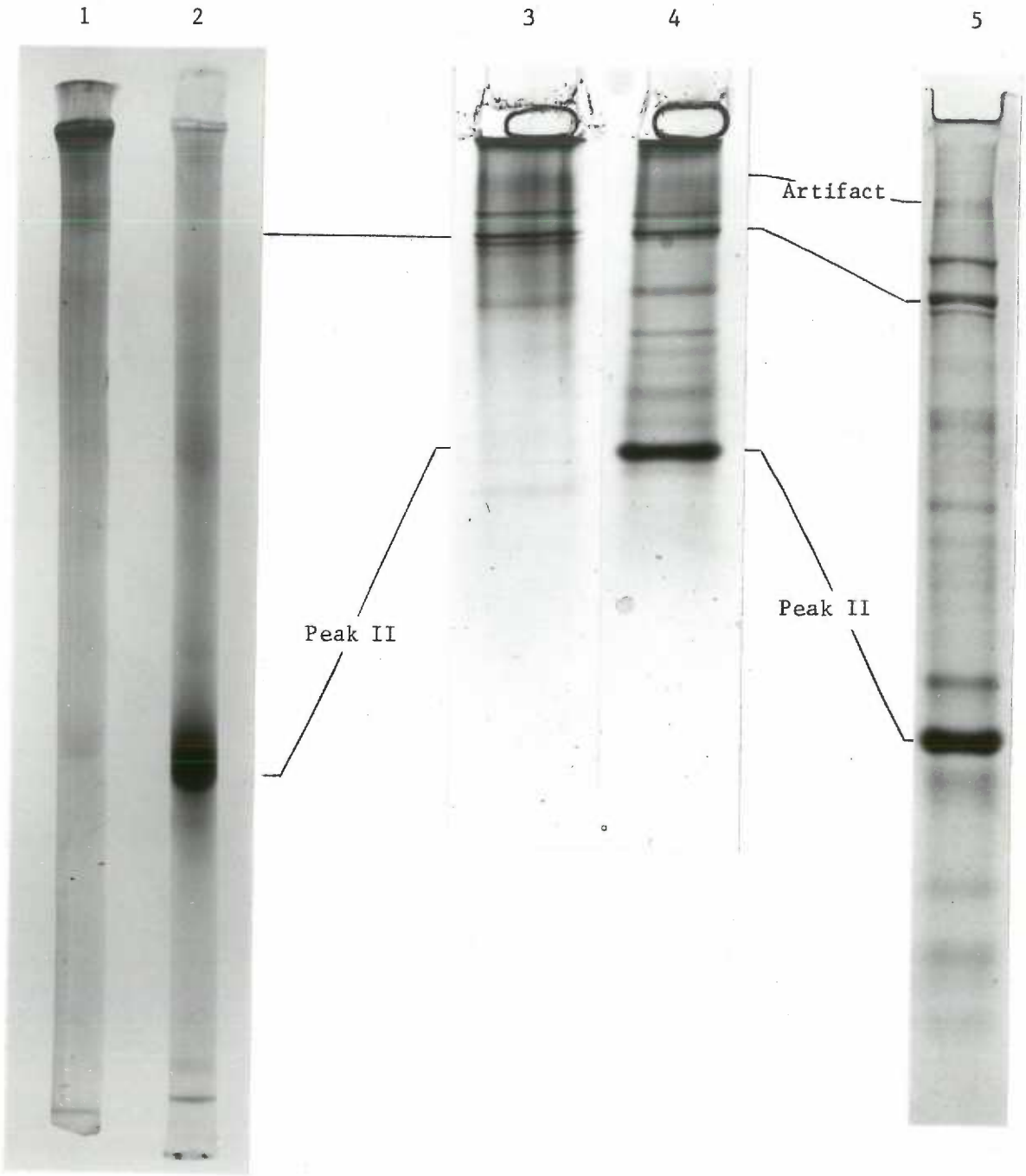
5% Polyacrylamide Gel Slabs (Ortec)

Well 3: Peak I (see Figure 7)
Well 4: Whole G-I extract from Experiment 2, section 2.1.2.2.2, page 65

5% Polyacrylamide Gel Slabs (Bio-Rad)

Well 5: Whole G-I extract from Experiment 3 (Sequential), section 2.1.2.2.3, page 69

Text section 2.1.4.2.4, page 131.


1
2
3
4
5
Artifact
Peak II
Peak II

The high resolution of the slab gel systems showed the aorta extracts to be very complex mixtures, as seen by the number of different species resolved. In all the crude extracts examined, except the last extract with guanidine and dithiothreitol, the band corresponding to Peak II was the predominant single species present. It was examined closely in the experiments to be described below, in consideration of the possibility that it represented an SGP monomer. From calibrated slab gels in SDS, its molecular weight appeared to be 42-43,000 d.

2.1.4.2.5 Comparison of Results in SDS with Those in SDS and Urea.

The whole aorta extracts were fractionated on a 5% slab gel in both SDS and 8 M urea. This was done to see if SDS and urea together would break up any aggregates not disrupted by SDS alone. The results are shown in Figure 13, and should be compared to Figure 12. Wells one and two are guanidine and guanidine/dithiothreitol extracts, respectively. Several differences were seen between this gel and gels in SDS alone. Migration distance of all species was decreased in urea to about half of the corresponding distance in SDS alone. The relative positions of bands near the top of the gel, representing Peak I material, were altered somewhat. Positions of bands farther down the gel were unchanged by the addition of urea. Most significantly, however, was the fact that no pronounced shift of stainable material from higher to lower molecular weights was seen in the gel containing urea. Such a shift would be expected if urea had disrupted aggregates stable in SDS alone. It was concluded that no such aggregates were present in detectable amounts in any of the extracts.

Figure 13. Electrophoresis of Aorta Extracts G-I and G/DTT-I in Sodium Dodecylsulfate Plus 8 M Urea.

Electrophoresis in 5% polyacrylamide gel using Bio-Rad slab apparatus.

Well 1: Extract G-I from Experiment 3 (sequential), as in Figure 12.

Well 2: Extract G/DTT-I from Experiment 3 (sequential) from section 2.1.2.2.3, page 69)

Text section 2.1.4.2.5, page 134.

2.1.4.2.6 Results from Acrylamide/Agarose Composite Gels in SDS.

This experiment was undertaken using 3% acrylamide, 0.5% agarose, with the goal of fractionating that part of Peak I which did not penetrate 5% gels. Nevertheless, the stained gel showed a large amount of material in the guanidine/dithiothreitol extracts which did not enter the gel. Peacock and Dingman (88) reported successful manipulation of a gel using as little as 1.5% acrylamide with the agarose support. An experiment using 1.5% acrylamide was attempted, with the goal of finding a gel with pores to permit migration of all of the Peak I material. However, the gel was so weak that removal of the well-forming comb pulled the gel apart. A 2% acrylamide gel was no better. No further experiments with agarose-acrylamide composite gels were attempted.

2.1.4.2.7 Electrophoresis of Guanidine/Dithiothreitol Extract After High Speed Centrifugation.

A sample of guanidine/dithiothreitol extract still in 5 M guanidine and 0.05 M dithiothreitol was centrifuged at 105,000 x g for one hour. A small pellet was discarded. This treatment has been shown to pellet particles as small as ribosomes and small fragments of the rough endoplasmic reticulum (136). After alkylation with iodoacetamide (113) and dialysis to remove the guanidine, this sample was placed on a 3% slab gel for electrophoresis in SDS. No reduction was seen in the amount of material unable to migrate in the gel. These observations indicated that this material was not composed of tissue fragments left over from the extraction process.

2.1.4.2.8 Molecular Weights of Bands on Gels in SDS.

Figure 14 shows representative electrophoresis patterns of all standard proteins used for calibration of 5% and 8% gels in SDS. In the absence of 2-mercaptoethanol, standard actin formed a series of monomers, dimers, trimers, and higher multiples, as seen in the third column of Figure 14. Because of difficulty in determining the position of the higher multiples, only the monomer and dimer were used for calibration. As seen in the photographs, the standards were not completely homogeneous in all cases. The most prominent band of each was used for calibration.

Figure 15 shows the standard curves for 8% (solid line) and 5% (broken line) gels, plotted as described in section 2.1.4.1.4.

Calibration of the 8% gels was considered accurate for molecular weights as low as 19,000 d (apoferritin subunits). Hemoglobin (16,000 d) deviated considerably from the line. Cytochrome C (12,000 d) migrated at the same R_f as hemoglobin. Because of this unusual result, which was quite reproducible, estimates of bands below 19,000 d must be viewed with caution. Although 8% gels appeared useful for molecular weights between 68,000 d (serum albumin) and 200,000 d (myosin), data for species in this interval were taken from 5% gels. Similarly, data for species smaller than 42,000 d (actin) were taken from 8% gels. Calibration of 5% gels was considered accurate for molecular weights up to 200,000 d (myosin). Although bands were seen beyond this limit, they were within a few millimeters of the artifact band which migrated at 0.13 relative to actin on 5% gels (see 2.1.4.2.4).

Figure 14. Standards for Molecular Weight Calibration of Polyacrylamide Gel Electrophoresis Gels in Sodium Dodecylsulfate.

5% polyacrylamide gel slab (Bio-Rad)

Well 1: Myosin heavy chain (200,000 d)
Well 2: Actin (42,000 d)

5% polyacrylamide gel slab (Ortec)

Well 3: Actin without 2-mercaptoethanol, showing monomers, dimers, trimers, and tetramers.

8% polyacrylamide gel slab (Bio-Rad)

Well 4: Bovine serum albumin (68,000 d)
Well 5: Ovalbumin (45,000 d)
Well 6: Actin (42,000 d)
Well 7: Pepsin (35,000 d)
Well 8: Chymotrypsinogen (25,000 d)
Well 9: Apoferritin subunits (19,000 d)
Well 10: Hemoglobin subunits (16,000 d)
Well 11: Actin (42,000 d)

Text section 2.1.4.1.4 and 2.1.4.2.8, pages 121 and 137.

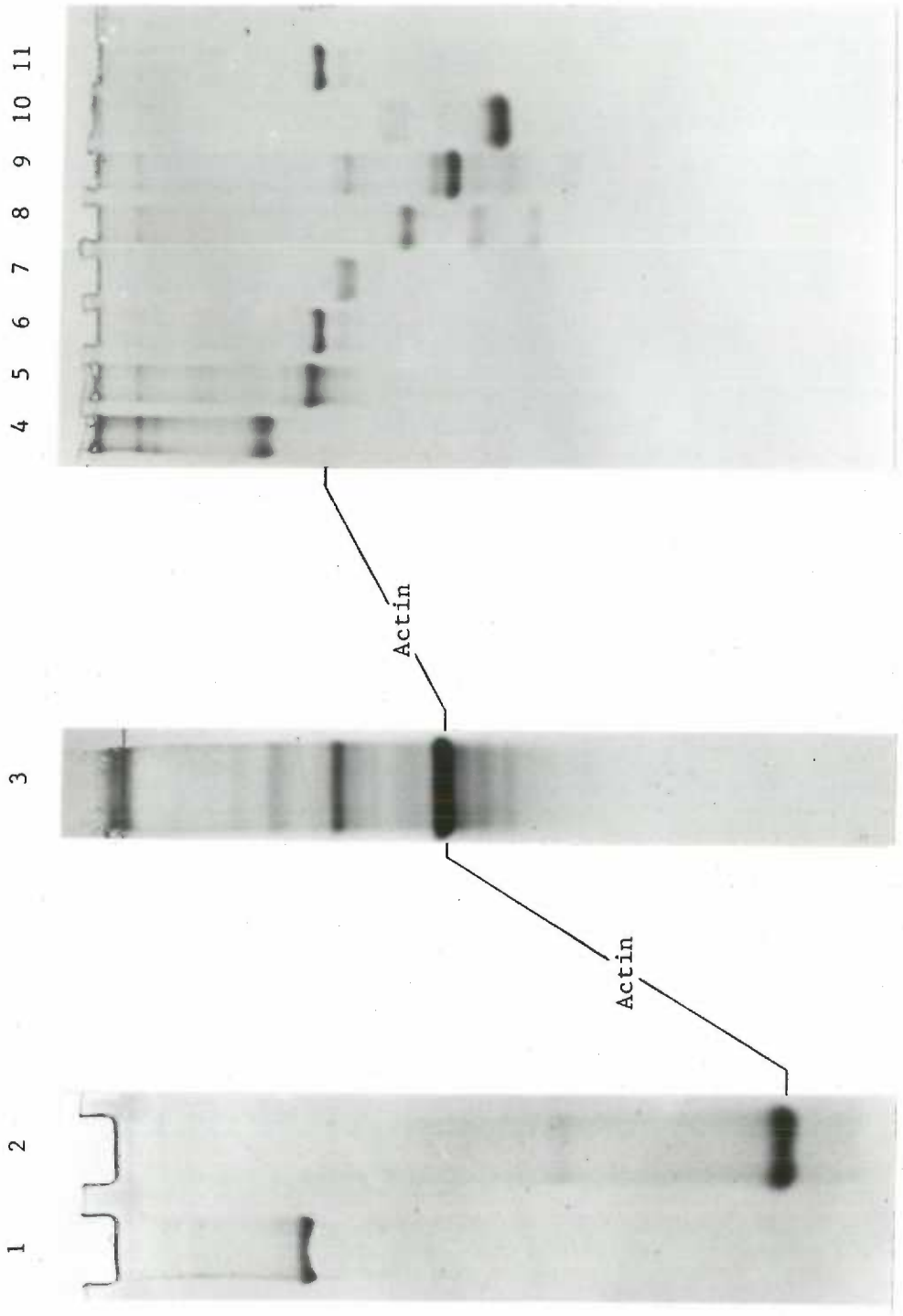

1
2
3
4
5
6
7
8
9
10
11
Actin
Actin

Figure 15. Calibration of Polyacrylamide Gels in Sodium Dodecylsulfate.

Relative migration of each standard (Figure 14) was plotted versus the logarithm of its molecular weight. All migration distances were relative to actin.

Solid line: 8% gel

Dashed line: 5% gel

Note that the relative migration scale used in this Figure for 5% gel is twice the length of the scale used for 8% gel. This was done in the figure to separate the two lines, which were very nearly superimposable when plotted on the same scale.

1	Myosin
2	Actin dimer
3	BSA
4	Ovalbumin
5	Actin
6	Pepsin
7	Chymotrypsinogen
8	Apoferritin
9	Hemoglobin

Text sections 2.1.4.2.8 and 2.1.4.1.4, pages 137 and 122.

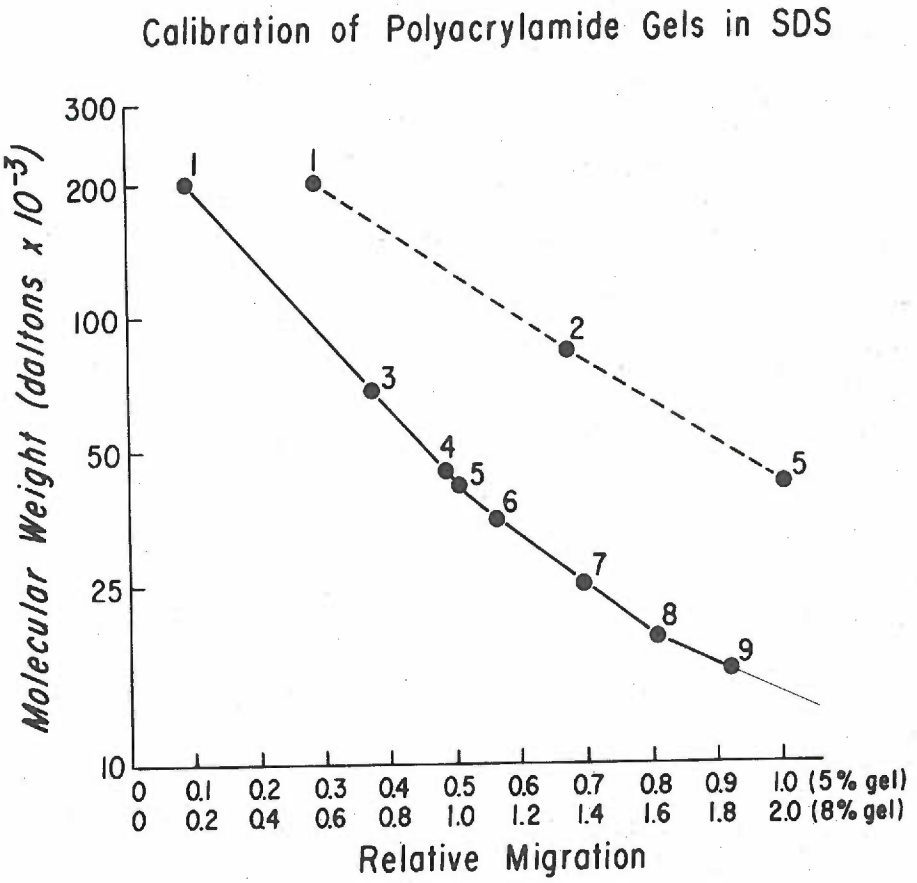

Calibration of Polyacrylamide Gels in SDS
Molecular Weight (daltons x 10^{-3})
300
200
100
50
25
10
1
2
3
4
5
6
7
8
9
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 (5% gel)
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 (8% gel)
Relative Migration

Figure 16 shows the aorta extracts on a 5% gel together with the letter and number assigned to each band for purposes of reference. Peak II becomes Band E in this reference system. Other bands were seen in the NaCl, $NaHCO_3$, EDTA, and ATP/ascorbate pre-washes of Experiment 3 (sections 2.1.2.2.3 and 2.3). These bands will be discussed in "Section III - Survey of Aorta Extracts." Molecular weights of the bands labelled in Figure 16 are given in Table VI, to the nearest 1000 daltons. These values were in very close agreement among the many gels from which they were taken. In many cases, all measurements of a given band led to the same molecular weight. In no case, except as described just below, did the variability between determinations exceed 5%. The exceptions to this conclusion, Bands F, H, J, and K, migrated relatively more slowly on 8% gels than on 5% gels, leading to the two different molecular weights shown in Table VI. The nature of these bands will be examined further in Section III.

2.1.4.2.9 Heterogeneity of Peak II Seen on Gels in 8 M Urea.

Peak II (Band E) appeared to be a single species on gels in SDS, indicating homogeneity with respect to size. It was next asked whether this material was also homogeneous with respect to charge. To answer this question, Peak II was placed on gels in 8 M urea rather than in SDS. The results of these experiments are shown in Figure 17. From left to right, these tubes contained the same sample in 7%, 5%, 4%, and 3% gels. A number of evenly-spaced bands were seen. The relative intensities of these bands were greatest at the center of the group, with the fastest and slowest-moving bands being the least intense.

Figure 16. Polyacrylamide Gel Electrophoresis in Sodium Dodecylsulfate of Extracts G-I, G/DTT-I, and G/DTT-IV.

All extracts from Experiment 3 (sequential) after reduction and alkylation (section 2.1.2.2.3, page 69). 5% polyacrylamide gel using Bio-Rad apparatus. Molecular weights of bands are given in Table VI.

Well 1: G-I
Well 2: G/DTT-I
Well 3: G/DTT-IV

Text section 2.1.4.2.8, page 142.

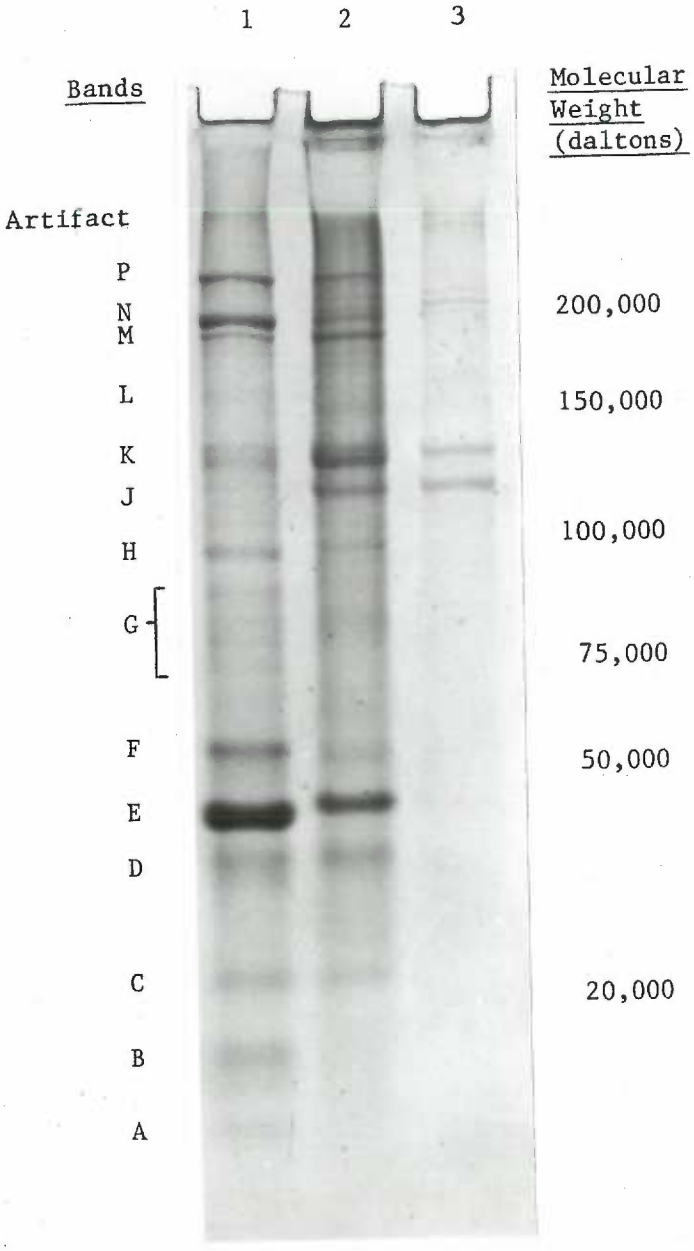

1
2
3
Bands
Molecular Weight (daltons)
Artifact
P
N
M
L
K
J
H
G
F
E
D
C
B
A
200,000
150,000
100,000
75,000
50,000
20,000

TABLE VI: MOLECULAR WEIGHT OF COMPONENTS OF G AND G/DTT EXTRACTS. SEE FIGURE 16.

Band	Molecular Weight (daltons)
A	16,000
B1	17,000
B2	18,000
C2	23,000
D1	30,000
D2	33,000
E1	37,000
E3	42,000
E4	43,000
F	51,000 on 5% gel, 59,000 on 8% gel
G1	75,000
G2	89,000
G3	95,000
G4	102,000
H	94,000 on 5% gel, 116,000 on 8% gel
I	128,000
J	116,000 on 5% gel, 132,000 on 8% gel
K1	124,000
K2	130,000 on 5% gel, 142,000 on 8% gel
M	188,000
N	200,000
P	230,000

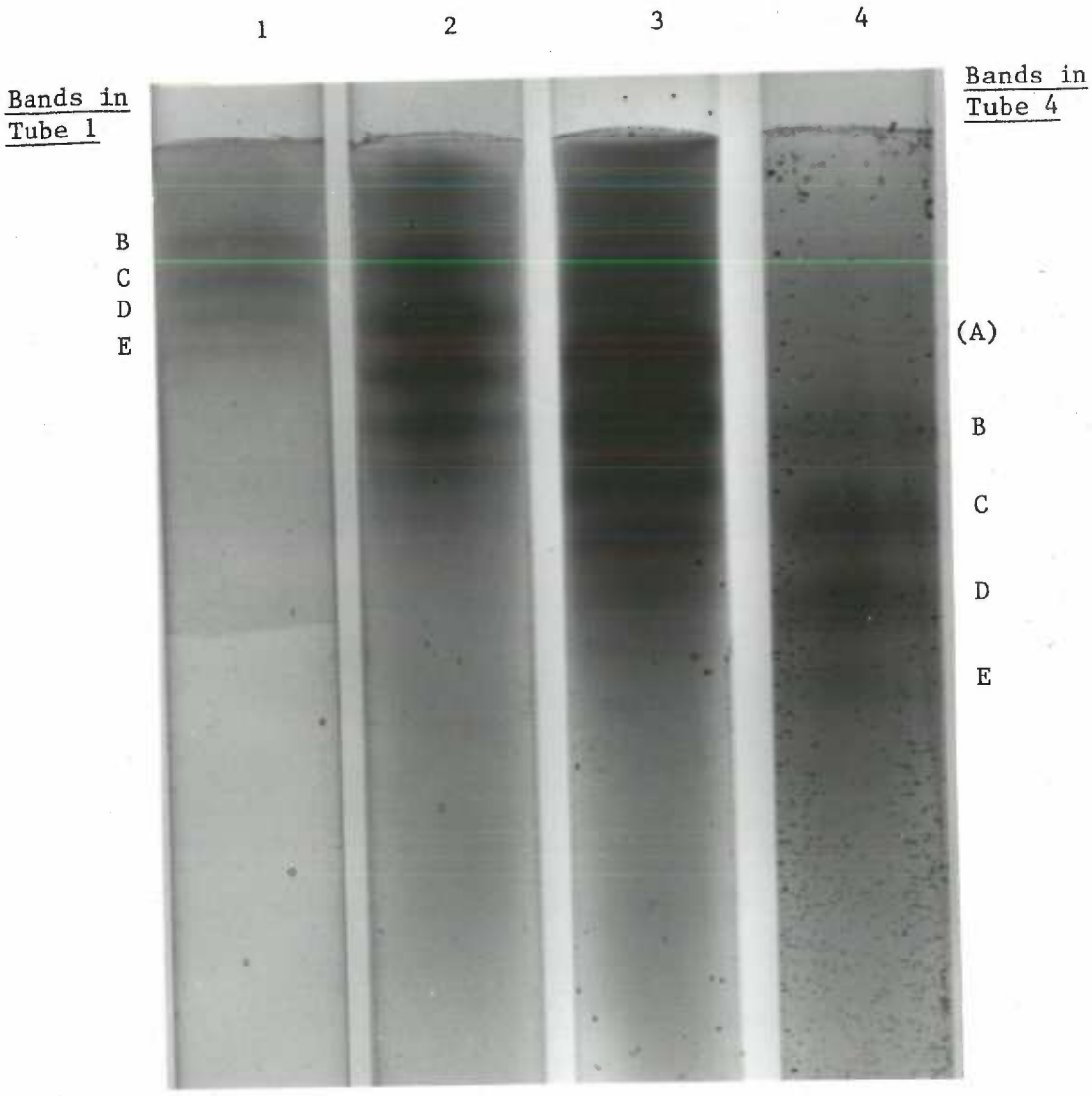
Text section 2.1.4.2.8, page 142.

Figure 17. Polyacrylamide Gel Electrophoresis in 8 M Urea of Peak II (Band E).

Peak II was the material recovered from Extract G-I as shown in Figure 7. Tube gel apparatus using varying acrylamide concentrations.

Tube 1: 7% gel
Tube 2: 5% gel
Tube 3: 4% gel
Tube 4: 3% gel

Data from these gels was used to construct the Ferguson Plot shown in Figure 18. Text section 2.1.4.2.9, page 142.


1
2
3
4
Bands in Tube 1
B
C
D
E
Bands in Tube 4
(A)
B
C
D
E

The remaining sample in 8 M urea was immediately diluted with an equal volume of sample buffer for tube gels in SDS and re-examined on 8% gels in SDS. Peak II material not exposed to urea was run on adjacent tubes as a control. All the gels were identical, showing only the single Peak II band. The multiple banding in urea had not been the result of breakage of peptide bonds to give smaller polypeptide fragments. However, it was still possible that the bands in urea represented aggregates not formed in the presence of SDS.

2.1.4.2.9.1 Nature of Peak II Heterogeneity. Ferguson Plot.

According to Ugel, Chrambach, and Rodbard (133), the mobility of a species in gel electrophoresis is given by:

$$\log M = \log M_o - K_r T \qquad \text{(eq. 1)}$$

where M = mobility in the gel

M_o = mobility in free solution

K_r = the retardation coefficient

T = the total concentration of the gel

Ugel et al further state that for denatured proteins, M_o is essentially constant throughout a series of monomers, dimers, trimers, and higher oligomers. In other words,

$$M_o \text{ (monomer)} = M_o \text{ (dimer)} = \ldots M_o \text{ (any oligomer)} \qquad \text{(eq. 2)}$$

Finally, the authors state that K_r is proportioned to the surface area of the molecule, hence under ideal conditions

$$K_r \text{ (monomer)} = \tfrac{1}{2} K_r \text{ (dimer)} = 1/3\ K_r \text{ (trimer)} = \ldots \qquad \text{(eq. 3)}$$

Equation 1 is of the form

$$y = b + m\,x \qquad \text{(eq. 4)}$$

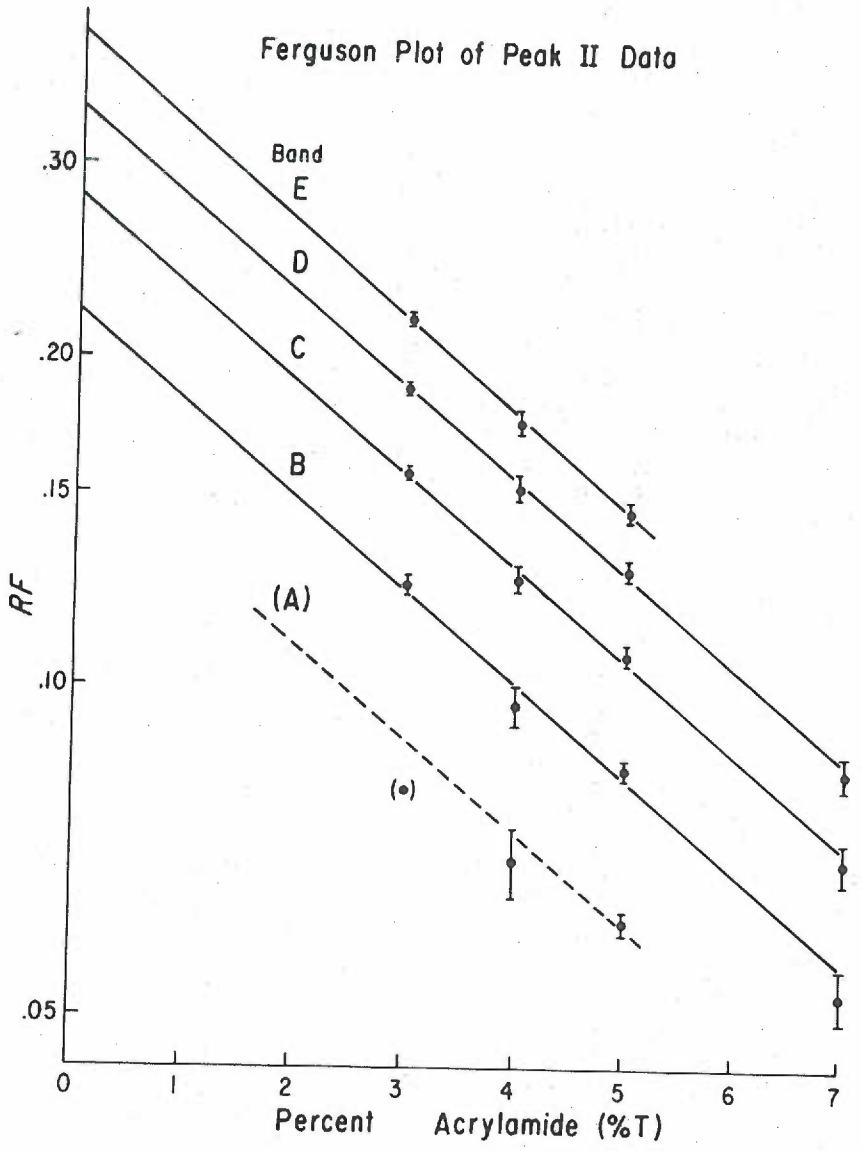
which is the equation of a straight line of slope m and y-intercept b. Since the mobility, M, of a molecule in a gel is measured by its R_f, it follows that a plot of log R_f (or y from eq 4) versus T (or x from eq 4) should give a straight line for any single species under ideal conditions. Such a plot is generally known as a Ferguson plot (133). Finally, from equations 2 and 3, a series of aggregates should give a series of straight lines with a common y-intercept, since M_o (or b from eq 4) is essentially constant while K_r (or m from eq 4) varies. Similarly, a series of equal-sized molecules of differing charge should give a series of lines with a common slope (i.e. parallel lines), since M_o varies while K_r remains constant.

These relationships were used to determine whether the bands given by Peak II in urea represented different sized aggregates, or charge differences between molecules of equal size. Four sets of twelve tube gels were run with Peak II material in 8 M urea. The sets were made with 3, 4, 5, and 7% total acrylamide. A Ferguson plot was constructed from the R_f data for each band. The plot is shown in Figure 18. The lines were very clearly parallel, having a common slope and different y-intercepts. It was concluded that the bands of Peak II in urea represented charge differences between molecules of the same size, or very nearly the same size.

Figure 18. Ferguson Plot from Bands Shown by Peak II on Polyacrylamide Gel Electrophoresis in 8 M Urea.

Plot of percent total acrylamide (%T) versus logarithm of the R_f of each band (Figure 17), as described by Ugel et al (133). Bands formed by a polymeric series lead to lines with a common y-intercept, while bands from a series of differently charged molecules of the same physical size lead to parallel lines (see text). Intervals around each point are ± 1 standard deviation. Each point from 10-12 gels. Line (A) was very faint on gels (Figure 17). Point on line (A) at 3% T was from a single observation. Letters for these bands are not related to the letters on Figure 16.

Text section 2.1.4.1.9.1, page 149.


Ferguson Plot of Peak II Data
Band
E
D
C
B
(A)
(•)
.30
.20
.15
.10
.05
RF
0
1
2
3
4
5
6
7
Percent Acrylamide (%T)

2.1.4.2.9.2 Nature of the Charge Differences.

There were several possible explanations for the charge differences between molecules comprising Peak II:

1. Differences in amino acid composition (e.g. isozymes).

2. Differences in carbohydrate composition.

3. Carbamylation of lysine residues by CNO^- formed by the decomposition of urea, by the mechanism:

$$NH_2CONH_2 \rightleftarrows CNO^- + NH_4^+$$

and then

$$CNO^- + Lys\text{-}NH_3^+ \rightarrow Lys\text{-}NHCONH_2 \quad \text{(homocitrulline)}$$

which neutralizes the positive charge on the ε-amino group (127).

4. Alkylation (113) of - SH groups by impure iodoacetamide containing iodoacetic acid. The reactions are:

$$Cys\text{-}SH + ICH_2CONH_2 \rightarrow Cys\text{-}S\text{-}CH_2CONH_2 + H^+ + I^-$$

and

$$Cys\text{-}SH + ICH_2COO^- \rightarrow Cys\text{-}S\text{-}CH_2COO^- + H^+ + I^-$$

Reaction with iodoacetate adds a negative charge to the cysteine residue.

5. Bacterial or enzymatic degradation of the preparation.

6. Partial de-amidation of Asn and Gln residues:

$$RCONH_2 + H_2O \rightarrow RCOO^- + NH_4^+$$

which would add negative charges to the molecules.

7. Any combination of 1-6.

These possibilities could be tested by a series of experiments.

Possibilities 1 and 2 could be tested by isolation of the bands by preparative electrophoresis, followed by amino acid analysis, carbohydrate analysis, or partial sequencing of the individual bands. If terminal sialic acid were wholly or partly responsible for the charge differences, digestion with neuraminidase would be expected to reduce the number of bands seen on gels in urea.

Since both decomposition of urea and carbamylation of lysine are very slow reactions at room temperature (127), possibility 3 could be tested by prolonged exposure of Peak II, and of homogeneous controls such as BSA, to "aged" urea, in equilibrium with CNO^-. Carbamylation would be expected to create additional bands on gels, and to increase the mobility of all species by giving them greater net negative charges. Homocitrulline, the reaction product of lysine with cyanate, could also be detected by amino acid analysis.

Possibility 4 could be tested by electrophoresis of Peak II, and of standard proteins, before and after alkylation with the suspect iodoacetamide. This would lead to an increase in the number of bands if iodoacetate were present. All the resulting bands would be expected to migrate faster than the original material in the gel system used, because of their greater net negative charge.

Possibility 5 could be tested by preparation of more Peak II under aseptic conditions, and comparison of this preparation to the

original Peak II on gels. Enzymatic alterations would be difficult to prove, however.

Possibility 6 could be tested by measurement of amide nitrogen in individual bands isolated by preparative electrophoresis.

For reasons to be discussed later, many of these experiments were never completed. Two preliminary results were worth reporting, however.

Standard bovine serum albumin (BSA) was incubated for 40 hours at room temperature in 8 M urea which had been previously heated to 98°C for 40 minutes. This pre-treatment of urea allows an equilibrium concentration of cyanate to be formed (127). A second sample of standard BSA was alkylated with iodoacetamide reagent as described earlier. These two samples were then compared to untreated BSA on gels in urea. Control BSA showed bands presumed to be monomer, dimer, etc., because of their similarity to the pattern seen for actin in SDS (sec. 2.1.4.2.8), and because of prior observations of the behavior of BSA. The sample exposed to pre-heated urea showed no additional bands, but the entire pattern showed greatly increased mobility, as expected for a decrease of free amino groups on lysine. If the carbamylation reaction affected all lysine residues, no additional bands would be expected. Alkylated BSA showed no increase in either band number or mobility with respect to the control. These results suggested that the alkylation process did not add free carboxyl groups to the protein, and that carbamylation was not a problem in fresh urea solution, at or below room temperature, for short periods of time.

A sample of Peak II was digested with neuraminidase, resulting in removal of about half the sialic acid originally in the sample, but no alteration whatsoever in the banding pattern on gels in urea. The results suggested that the charge heterogeneity was not due to sialic acid residues, but more rigorous experiments would be needed to prove this point.

At the conclusion of these experiments, the nature of the charge heterogeneity of Peak II material had not been determined.

2.1.4.2.10 Results of Isoelectric Focussing of Peak II in 8 M Urea.

This experiment was originally planned as a preliminary test of the applicability of preparative isoelectric focussing as a means of obtaining the individual bands seen on PAGE in urea, for purposes of amino acid and carbohydrate analysis. PAGIF of Peak II was carried out as described in section 2.1.4.1.5 The results (not photographed) confirmed the results of the PAGE gels in urea. The same number of equally-spaced bands were seen, with the intensities greatest at the center of the pattern. This finding agreed with the conclusion from the Ferguson plot (section 2.1.4.2.9.1), in that polymers or aggregates of a single, homogeneous monomer would have the same, or nearly the same, isoelectric points, and would focus to a single band.

2.1.4.3 Overall Conclusions of Electrophoresis and Isoelectric Focussing Experiments.

At the beginning of this series of experiments, five questions were asked about the aorta extracts, and the two principal peaks which they produced when fractionated by gel filtration in guanidine, SDS, or urea:

1. Do the guanidine and guanidine/ditheothreitol extracts contain the same or different proteins?

2. Is Peak I composed of discrete proteins, or is it a continuously heterogeneous mixture?

3. Is Peak II a single protein?

4. Are Peak I components aggregates or polymers of the protein making up Peak II?

5. What are the molecular weights of the components in the extracts?

The experiments with polyacrylamide gels did much to answer these questions.

2.1.4.3.1 Comparison of the Whole Extracts.

Electrophoresis in dilute buffer, not containing SDS or urea, confirmed the conclusion from the column chromatographic experiments, that in the absence of denaturing agents the extracts were extensively aggregated.

Electrophoresis in SDS showed the guanidine and guanidine/dithiothreitol extracts from all three aorta extraction experiments to be

complex mixtures of proteins. Many bands were seen, as shown in Figures 12, 13, and 16. Many of these bands were common to both guanidine and guanidine/dithiothreitol extracts, although their relative amounts varied. The final guanidine/dithiothreitol extract (G/DTT-IV) contained no bands except J, K, and several in the area of M-P. More will be said about these bands in section III.

It is worth noting that Figures 13 and 16 show no band at a molecular weight of 68,000 daltons, which would correspond to bovine serum albumin. The absence of this band indicates that, in extraction experiment 3, from which the samples were taken, serum proteins were successfully removed from the tissue by the pre-washes with NaCl, $NaHCO_3$, and EDTA. Anderson (5) has pointed out that serum proteins can be difficult to remove from connective tissue due to the process of entrapment, in which the proteins are caught among the connective tissue fibers (see Historical Setting, section 1).

2.1.4.3.2 Conclusions about Peak I.

As suggested by the results of column chromatography, Peak I, the material excluded from Sepharose 6B in chaotropic medium, was not a single protein. In Figure 12, Peak I was seen to consist of all material migrating with an R_f less than that of Band J. This corresponded to molecular weights in excess of about 100,000 daltons (Table VI). In addition to bands J, K1, K2, M, N, and P, Peak I contained a considerable amount of "background" material not migrating in discrete bands. This is most apparent in the third

column of Figure 12, showing Peak I on 5% gels in SDS on the Ortec slab apparatus. The background material was taken to be degraded fragments of high molecular weight.

Also apparent in this photograph is the heavily stained area at the base of the sample well, corresponding to protein which penetrated the gel very little or not at all. Some of this material was unable to penetrate 3% acrylamide - 0.5% agarose gel in SDS. Peak I therefore contained some very large particles. Centrifugation of the whole guanidine/dithiothreitol extract at a speed sufficient to remove ribosomes and fragments of endoplasmic reticulum did not remove the high-molecular-weight component of Peak I. This ruled out the possibility that it was composed of finely divided tissue residue. Electrophoresis in SDS with 8 M urea, and electrophoresis in SDS after severe treatment of the extract with hot 7 M guanidine or hot dilute alkali, demonstrated that the very large particles were not aggregates of smaller particles.

2.1.4.3.3 Conclusions about Peak II.

Unlike heterogeneous Peak I, Peak II was seen to be composed of protein of a single molecular weight, estimated to be 42,000 - 43,000 daltons. This single band was the most prominent component in the guanidine/dithiothreitol (G/DTT) extracts. It was, however, absent from the final G/DTT extract (G/DTT-IV).

Although Peak II was homogeneous with respect to size as seen on gels in SDS, and in SDS plus urea, it was seen to be heterogeneous with respect to charge when placed on gels in urea alone. The possibility that the multiple bands in urea represented aggregation interrupted by SDS was ruled out by the Ferguson plot (Figure 18), which gave a series of parallel lines, characteristic of a mixture of molecules of the same size but different net charge. The aggregation hypothesis was also disproven by isoelectric focussing in urea, which showed the same bands as did electrophoresis in urea. Aggregates of a single monomer would have focussed to a single band regardless of their particle size.

Some preliminary experiments with standard protein (bovine serum albumin) suggested that the charge heterogeneity of Peak II was not caused by alkylation of thiol groups with impure iodoacetamide containing iodoacetate, or by carbamylation of lysine residues with cyanate, a breakdown product of urea. A preliminary experiment with neuraminidase also suggested that variation of sialic acid content among individual molecules was not responsible for the heterogeneity. None of the preliminary experiments was rigorous, and the nature of the charge heterogeneity of Peak II remained unknown at the conclusion of the electrophoretic studies.

2.1.4.3.4 <u>On the Possibility That Peak I Components Were Made Up of Monomers Represented by Peak II.</u>

As noted earlier, the similarity in amino acid composition

between Peaks I and II (see Table V) suggested that Peak I might be an aggregate or polymer of Peak II. Experiments involving severe treatment of Peak I failed to release any material co-migrating with Peak II (Band E) on subsequent gels in SDS. The severity of one treatment was such that the sample showed evidence of random breakage of covalent bonds, specifically the appearance of stainable material moving with the ion boundary. Nevertheless, no trace of Band E was seen. The small amount of Band E in the Peak I sample shown in Figure 12 was not reproducible, and was taken to be contamination from an adjacent sample on the gel.

These results indicated that Peak I was not a series of aggregates of the subunit seen in Peak II. There remained, however, the possibility that Peak I represented covalent polymers of Peak II.

2.1.4.3.5 Molecular Weights.

In theory, the plot of the logarithms of molecular weight versus relative migration should be a straight line. However, in practice this is often not the case (92), (133). Although the standards shown in Figure 15 did not plot as a straight line, they produced a smooth curve which strongly resembled that obtained by Porzio and Pearson (92). Since their results using the curve were in excellent agreement with the remainder of the literature, curvature of the line does not in itself render the plot useless. This was assumed to be true for Figure 15 also.

Band E, the principal component of Peak II, showed some

variability of molecular weight between preparations. However, it was considered unlikely that these bands represented different proteins. This conclusion was justified as follows. Many bands were common to all the G and G/DTT extracts, indicating that the same protein was found in all of them. The components of one extract were plainly not unique to that one extract alone. Since many other bands were not unique to only one extract, it is reasonable to assume the same would be true of Band E. If there were several components of slightly different molecular weight in Band E, they should therefore have appeared on the gels in SDS as closely-spaced bands, or perhaps as a wide, diffuse band. However, as Figure 12 shows, Band E appeared as a single, sharp band in all preparations. These observations are consistent with the hypothesis that the electrophoretic mobility of Band E material was sensitive to the past history of the preparation. Indeed, it was observed that alkylated Band E migrated just slightly behind reduced, un-alkylated material.

Although the molecular weight of Band E varied within narrow limits, there was no difference in the observed molecular weight of the band between gels of different acrylamide concentrations. Bands F, H, J, K1, and K2, however, appeared to be about 10% larger on 8% gels than on 5% gels. The meaning of this observation is unclear. Segrest and Jackson (121) reported that the apparent molecular weight of glycoproteins decreased with increasing acrylamide concentration. They also mentioned but did not describe other types of anomalous behavior. Furthmayr and Timpl (40) noted that collagen

migrated abnormally slowly on gels in SDS. They did not attempt to determine if the degree of abnormality was a function of the concentration of acrylamide in the gel. More will be said about Bands J and K in Experimental Section III. Bands F and H were minor components of the aorta extracts, and the nature of their anomalous migration on gels in SDS was not investigated further. In view of Segrest and Jackson's observation, their anomalous migration could not be attributed to carbohydrate groups alone.

2.1.4.3.6 Summary of Conclusions from Electrophoresis Experiments.

Electrophoresis and isoelectric focussing established that Peak I as shown in Figure 7 was heterogeneous, being composed of several discrete protein bands and other high molecular weight material which could not be resolved into individual bands. Peak II was apparently homogeneous with respect to size but heterogeneous with respect to charge. The molecular weight of Peak II was 42,000-43,000 daltons. The nature of its charge heterogeneity was not determined.

Both guanidine and guanidine/dithiothreitol extracts were complex mixtures. Band E, the material forming Peak II, was the most prominent band in the guanidine extracts, and a prominent band in the first guanidine/dithiothreitol extract as well. It had been suggested that Peak II might represent a monomer of SGP, and that some or all of the components of Peak I might be aggregates or polymers of this monomer. Electrophoretic experiments showed that Peak I components were not simple aggregates of Peak II subunits.

It was still a possibility that Peak I components were covalent polymers of Peak II monomers.

Molecular weights of the proteins contained in the aorta extracts were calculated and reported in Table VI.

2.1.5 Tryptic Peptides of Peak I and Peak II.

In the last section, involving electrophoresis in polyacrylamide gels, the question arose as to whether Peak I (see Figure 7) could be made up of aggregates or covalent polymers of a subunit represented by Peak II. Subsequent experiments eliminated the possibility of simple aggregation, but left open the possibility of covalent polymerization by crosslinks other than disulfide bonds. As shown in section 2.1.3.3.2, cystine crosslinks in Peak I had been quantitatively reduced to free cysteine, and just as quantitatively alkylated by iodoacetamide, preventing the re-forming of cystine. The experiment to be described in this present section was designed to test the remaining possibility, that covalent crosslinks other than disulfide bonds had bound Peak II subunits together to form the larger species found in Peak I.

The objective of this experiment was to digest Peaks I and II with trypsin, and then examine the digests to see if the same peptides were found in Peak I and Peak II. If Peak I were indeed polymers of Peak II, the tryptic peptides would be expected to be the same from both peaks, except for those peptides involved in the actual covalent crosslinks.

2.1.5.1 Materials and Methods.

2.1.5.1.1 Digestion of Peaks I and II.

Since Peak I was known to contain a small amount of collagen, as indicated by the presence of hydroxyproline (see Table V), it was first

digested with collagenase as described in section 2.1.2.2.1.2. Peak II was also digested with collagenase to eliminate the possibility of an artifactual difference between the two as a result of collagenase digestion of Peak I only. Lyophilized samples, 20 mg of Peak I and 21 mg of Peak II, were dissolved in 5 ml of 7 M guanidinum chloride. This was done because neither solid would dissolve or even disperse in collagenase digestion buffer. The solutions were then dialysed against water, followed by collagenase buffer. Finely-divided precipitates formed, as expected.

The dialysed suspensions were placed in test tubes, to which were added 62 mg of N-ethyl maleimide and 0.2 mg of collagenase. Volumes were made up to 5.0 ml. The suspensions were shaken gently at 37°C for six hours. They were then dialyzed against water overnight with brisk agitation to keep the precipitate in suspension.

Half of each digest, 2.5 ml, was digested with trypsin (TPCK, Worthington) by the method of Brimhall[1]. The suspensions were adjusted to pH 8 (estimated by pH paper) with a few drops of 2% trimethylamine. Next, 0.1 - 0.2 mg of trypsin was added to each sample. The samples were capped and incubated at 37°C for 4 hours with gentle agitation. After 4 hours, all solid material in both samples had dissolved. The solutions were returned to pH 8 with several drops of 2% trimethylamine. An additional 0.1 mg of trypsin was added to each, and incubation was continued for an additional 4 hours.

[1] B. Brimhall, personal communication, 1974.

Digestions were terminated by addition of HCl to pH 3 (by pH paper). The samples were dried at 40°C on a rotary evaporator at 5 mm Hg pressure, and taken up in 1.0 ml of 0.2 M pyridine acetate, pH 3.1. Peak I digest was filtered through glass wool to remove a lumpy precipitate.

2.1.5.1.2 Chromatography of the Digests.

The tryptic digests were chromatographed on a 0.9 x 16 cm column of Bio-Rad Aminex A-5 cation exchange resin. The column was maintained at 50°C. After sample application, the column was eluted at 30 ml/hr, first with 60 ml of 0.2 M pyridine acetate, pH 3.1, and then with a linear gradient over 250 ml, beginning with 0.2 M pyridine acetate, pH 3.1 and ending with 2.0 M pyridine acetate, pH 5.0. Column effluent was continuously monitored at 570 nm for ninhydrin - positive material as described for the automatic amino acid analyzer (section 2.1.1.4).

2.1.5.2 Results and Discussion.

Elution profiles of the tryptic digests of Peak I and Peak II are shown in Figure 19. Only one of the major peaks on these profiles was found at exactly the same point on both chromatograms. Seen to be the highest peak of the Peak I digest, it emerged at the same time as standard ammonia. The dissimilarity of the two chromatograms strongly suggested that the two digests contained entirely different peptides, which meant that Peak I did not contain polypeptide sequences in common with those found in Peak II. This result made it very unlikely that a significant portion of Peak I represented a polymerized form of Peak II monomers.

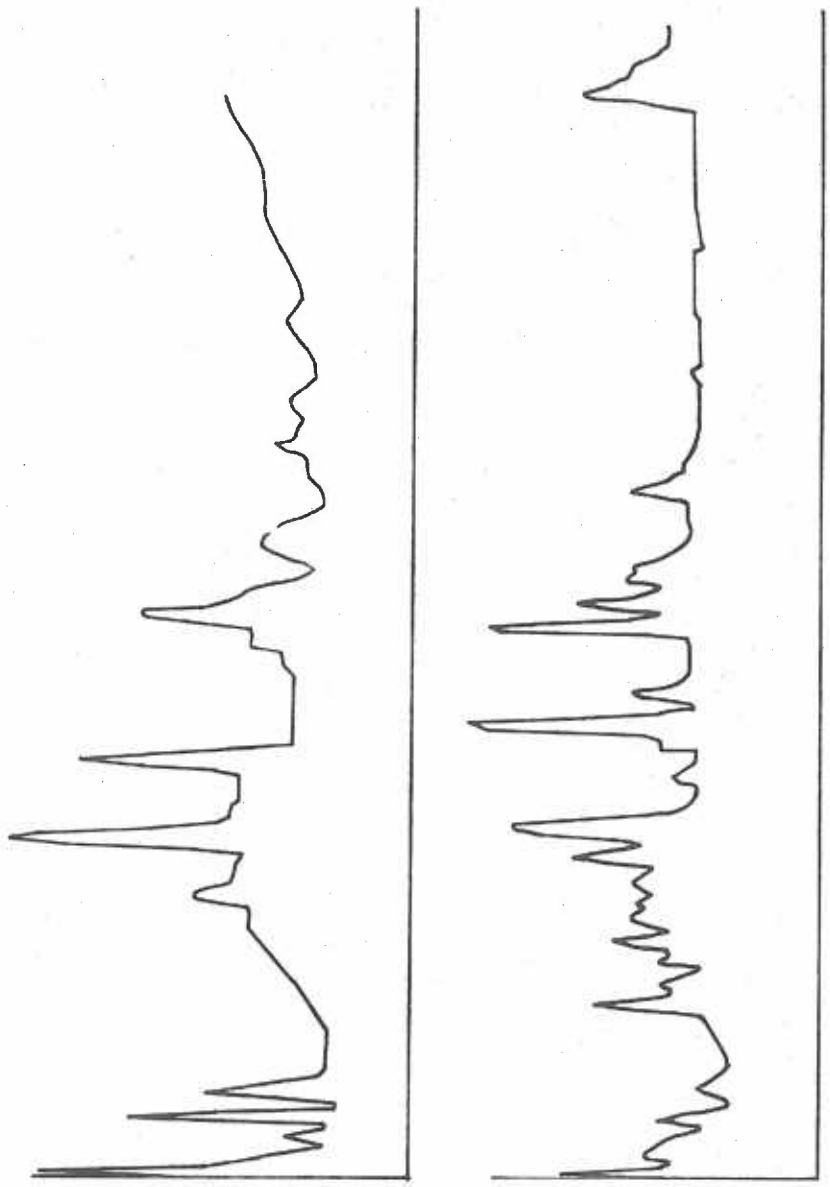
Figure 19. Tryptic Peptide Profiles of Peak I and Peak II from Ion Exchange Column.

Peaks I and II (Figure 7) were digested with collagenase and trypsin, then chromatographed on Bio-Rad Aminex A-5 cation exchange resin. Column effluent was monitored by ninhydrin reaction (section 2.1.1.4.3.5).

Upper trace: Peak I

Lower trace: Peak II

Text section 2.1.5.2, page 166.



One could postulate that, in addition to crosslinking, Peak I had been modified in such a way as to alter the chromatographic properties of virtually every tryptic peptide. However, it was difficult to envision a process which could do this without changing the amino acid composition of the material, since hydroxylated prolines, methylated histidines, and other modifications are immediately apparent on amino acid analysis. Extensive glycosylation of serine, threonine, or asparagine residues was not consistent with the low carbohydrate content of these preparations (section 2.1.3.3.2).

It was concluded that Peak I and Peak II were unrelated.

2.1.6 Summary and Discussion of Experimental Section I: SGP Heterogeneity.

The major findings of Experimental Section I were these. Two putative SGP fractions could be extracted from adult bovine aorta. The first was removed with 5 M guanidine. The second was removed from the tissue residue after the guanidine extractions by extraction with more 5 M guanidine containing 0.05 M dithiothreitol, a mild reducing agent capable of reductive cleavage of disulfide bonds. The fractions had similar but not identical amino acid compositions. Gel filtration of the two fractions in 4 M guanidine on columns of Sepharose 6B gave two peaks which again had similar but not identical amino acid compositions. Polyacrylamide gel electrophoresis in SDS showed both the guanidine-soluble and guanidine/dithiothreitol-soluble fractions to be complex mixtures of proteins. Many of the components appeared to be common to both fractions. The predominent species in both fractions was a protein with an apparent molecular weight of 42,000 to 43,000 daltons. Although homogeneous by size, it showed at least seven components by electrophoresis or isoelectric focussing in 8 M urea. The nature of the charge heterogeneity was not determined. Several other major components were seen on the gels in SDS, having molecular weights in the range of 100,000 to 250,000 daltons. In addition to these discrete bands, the gels showed considerable diffuse staining, especially at molecular weights above 100,000 daltons. This was taken to represent slightly degraded material of high molecular weight. There was considerable evidence which suggested that the

larger species present were not polymers or aggregates of the 42,000 dalton protein, in spite of the similarity in amino acid composition between that protein and the mixture of larger molecules which emerged together at the void volume of the Sepharose column.

These findings could be compared to those in the literature in several ways. The yields were of the same order of magnitude as those reported for SGP from pig aorta, human aorta, chick aorta, and calf aorta (see Table I). Amino acid compositions also matched the rather loose definition of SGP given by Robert and Comte in 1968 (109). As mentioned earlier, solubility and amino acid analysis have long served as the basis for defining SGP in spite of the limited significance of such criteria. Beyond these simple comparisons, SGPs reported in the literature differ widely. Perhaps in keeping with this variability, the discovery of a principle component of molecular weight 42,000 daltons neither agrees nor disagrees with the literature taken as a whole. Although there has been no other SGP molecule of this exact size reported, putative SGP molecules have been reported with molecular weights varying between the extremes of 7,800 daltons (55) and 270,000 daltons (82). A 42,000 dalton species is certainly not unreasonable. Several authors have reported that SGP as extracted from the tissue is a series of aggregates or polymers of a small subunit (26)(138). These conclusions were based on the observation of a number of bands on polyacrylamide gels in SDS, or of a number of overlapping peaks emerging from gel filtration columns. The experiments in Section I revealed similar heterogeneity, but they also showed that the larger species were not aggregates, nor were they polymers of the

predominant smaller species present in the crude tissue extracts. The smaller, 42,000 dalton species compares most closely to proteins recently described by Moczar and Moczar (77) and by Brown, Adamany, and Blumenfeld (19).

The goals of Experimental Section I were to gain an understanding of the heterogeneity of SGP as it is extracted from the tissue, and to isolate and characterize an SGP monomer. The first goal was accomplished, although the understanding gained is not complete. The most significant finding was that the heterogeneity observed by gel filtration or by polyacrylamide gel electrophoresis in SDS was not due to aggregation, but was due instead to the presence of a wide variety of different polypeptides. The second goal, that of isolating and characterizing an SGP subunit, was thought to have been achieved in the description of a protein of molecular weight 42,000 - 43,000 daltons, found in large amounts in the aorta extracts. It met the basic solubility and composition criteria for being SGP. However, it could not be shown to be a subunit of the larger molecules in the extracts. More work would be needed, both in describing this proposed subunit and in determining its relationship to the material of higher molecular weight, before it could be said with certainty that this molecule was in fact the monomeric form of SGP.

At this point in the investigation of SGP, a number of experiments were planned. The several bands observed in the electrophoresis of the proposed subunit in 8 M urea were to be isolated by preparative electrophoresis or isoelectric focussing. These bands were to be

examined separately for amino acid composition, carbohydrate composition, N- or C- terminal sequence, amide nitrogen (a measure of the asparagine and glutamine content), and tryptic or cyanogen bromide peptide maps. An attempt was to be made to obtain antibodies to the proposed monomer, to be used in immunochemical comparisons of this molecule to its proposed polymers. Ultracentrifugation studies were planned to verify the apparent subunit molecular weight of 42,000 - 43,000 daltons. Two-dimensional peptide maps of the proposed monomer and polymers were planned, with isolation and analysis of individual peptides as final confirmation of identity.

But before these experiments could be meaningful, it was necessary to answer one very important question. That question is the subject of the next major section.

2.2 Experimental Section II: Is Peak II Actin?

In Experimental Section I, a protein with a molecular weight of 42,000 - 43,000 daltons was extracted in large amounts from bovine aorta by a procedure comparable to those used by others to obtain a substance presumed to be structural glycoprotein, a poorly characterized component of connective tissues. This protein was designated "Peak II" by virtue of its chromatographic properties on Sepharose 6B in guanidine. It was shown to be homogeneous with respect to molecular weight but heterogeneous with respect to charge. Its solubility and composition of amino acids and carbohydrate were comparable to those of other SGPs in the literature. It was therefore proposed that this protein was a monomer or subunit of SGP.

But before any further experiments were undertaken to characterize the protein, or to confirm or deny its role as a monomer or subunit of SGP, it was necessary to demonstrate conclusively that the protein was not merely the myofibrillar protein actin. This second experimental section was therefore devoted to that end.

2.2.1 Introduction. Arguments Pro and Con.

At this point, it seemed unlikely that Peak II was actin. The solubility of actin is such that it should have been completely removed from the tissue by three extractions with 5 M guanidine. The presence of any measurable amount of actin in the subsequent guanidine/dithiothreitol extracts was viewed as extremely unlikely.

But since the aortic media is about half smooth muscle (50), it was known that actin was present in the fresh tissue in large amounts. Furthermore, Peak II bore a strong resemblance to actin in the literature. The amino acid compositions were very much alike. The molecular weight of actin, a subject of controversy at the time when Peak II was first prepared, had recently been proven to be 42,000 daltons by perhaps the most irrefutable of all arguments. In 1975, Collins, Elzinga, and Jackman (25) published the complete amino acid sequence of rabbit skeletal muscle actin.

Since the consequences of mistaking actin for a subunit of SGP were far-reaching and serious, and since the evidence was not conclusive that Peak II was not actin, a demonstration of the non-identity of the two proteins was seen as an essential part of the characterization of any presumed SGP monomer derived from aorta.

2.2.2 Techniques, Available and Chosen.

Many biochemical techniques were available to distinguish between two proteins, and many of them were considered for use in comparing Peak II to actin. Following is a brief discussion of those techniques, and of the advisability of applying each one to that problem.

Immunochemistry was potentially a powerful technique, but several factors made it less attractive for comparing Peak II to actin. First, actin is a very poor antigen. Pollard (91) suggested this was due to its ubiquity, being conserved throughout virtually all forms of life. He gives the example that amoeba actin or slime mold actin are able to

bind rabbit skeletal muscle myosin and activate the ATP-ase of the contractile process. Even if actin were a good antigen, the insolubility of Peak II except in the presence of chaotropes made immunochemical experiments appear difficult at best.

Finally, there was the possibility that similar carbohydrate groups on dissimilar proteins could give artifactual similarities by immunochemical techniques. For these reasons, immunochemical comparisons between actin and Peak II were not attempted.

Closely related to immunochemical techniques were interactions between actin and myosin, or between subunits of actin itself. Pollard (91) described several such interactions, which could have proven decisive if they had been feasible in the case of Peak II. However, Peak II was denatured and insoluble, and even if it could have been renatured, negative results in an attempt to bind Peak II to myosin and activate the ATP-ase would have proven nothing except that denaturation irreversibly ruined the activity.

Identical N- or C- terminal sequences from Peak II and actin would have been strong evidence of identity. However, the methods for determining these sequences are not without complication, and it was decided not to attempt sequencing unless other, simpler tests proved inconclusive.

An interesting proposal was to culture aortic smooth muscle cells and look for Peak II in the medium. SGP, being an extracellular protein, would presumably have been secreted into the medium, while

intracellular actin would not. Such an experiment also would have involved complex, untried procedures, and it too, was shelved pending results of simpler tests.

Four experiments were chosen for immediate use in comparing Peak II to actin. The criteria of choice were that the methodologies were already worked out in Experimental Section I, and that the experiments would be capable of detecting a major difference between the two proteins quickly and decisively. The objective was to demonstrate a decisive difference quickly rather than to prove that Peak II was actin. These four experiments were:

1. Comparison of Peak II to standard actin by PAGE in SDS. Comparison by PAGE in 8 M urea was not considered a definitive experiment because the nature of the charge heterogeneity of Peak II was not known. The multiplicity of bands could have been only an artifact of the preparation. Therefore, dissimilarity between standard actin and Peak II on a gel in urea would have proven nothing.

2. Comparison of the amino acid analyses of Peak II and standard actin. These were to be obtained from samples hydrolyzed and chromatographed under identical conditions. Even more significant than the ratios of the seventeen amino acids commonly detected in an acid hydrolysate would be the presence of 3-methyl histidine, a modified amino acid found in actin but in very few other proteins (87).

3. Comparison of the tryptic peptides of Peak II and standard actin, by the techniques of section 2.1.5.

4. Extraction with guanidine and dithiothreitol of a tissue known to contain SGP but not muscle. Ligamentum nuchae from fetal calf was chosen for this purpose, after the work of Ross and Bornstein (113) and others (61)(122). The absence of a large amount of Peak II in this extract would imply that Peak II was not SGP.

2.2.3 Materials and Methods.

2.2.3.1 Preparation of Standard Actin was by the method of Carsten and Mommaerts (21), using bovine skeletal muscle as the source tissue.

Bovine skeletal muscle was obtained from adult animals at the time of slaughter and stored in crushed ice until use. All subsequent operations were at 4°C unless otherwise noted. Pieces of tissue totalling 47.8 g wet were frozen in liquid nitrogen and shattered in a pre-cooled stainless steel mortar and pestel. The homogenized tissue was then extracted sequentially with the following solutions by stirring, centrifugation, and decanting of the supernatant:

150 ml of 0.1 M KCl, 15 minutes

75 ml of 0.05 M $NaHCO_3$, 15 minutes

250 ml of 1 mM EDTA, 5 minutes

250 ml of water, two times, 5 minutes each

100 ml of reagent acetone, three times, 5 minutes each.

The residue was then dried at room temperature under a stream of nitrogen. The supernatants were discarded. (Similar supernatants from aorta tissue were examined in Experimental Section III.) The purpose of these pre-extractions was to remove serum proteins and other soluble tissue components.

The dry residue was suspended in 40 ml of freshly-prepared 0.2 mM ATP (Nutritional Biochemicals), 0.2 mM ascorbate, pH 7.5. The suspension was shaken vigorously for 30 minutes, then centrifuged at 47,000 x g for 15 min. The supernatant was decanted and stored during a second extraction of the pellet, with the same solution as before, for 4½ hours. After centrifugation as before, the combined supernatants were centrifuged at 105,000 x g for 30 minutes. Solid KCl and solid $MgCl_2$ were added to the supernatant to final concentrations of 0.1 M and 0.1 mM, respectively. Under these conditions, individual actin monomers (G-actin, or globular actin) form fibrillar aggregates, the native form in muscle (F-actin, or fibrous actin). The solution was incubated for 1 hour at 18°C, then overnight at 4°C. It was centrifuged at 105,000 x g for 3 hours, producing a firm, jellylike pellet. The supernatant was discarded. The pellet was resuspended in 25 ml of fresh ATP/ascorbate solution, in which it slowly dissolved. The solution was centrifuged at 105,000 x g for 1 hour. The supernatant was decanted from a very small pellet. Solid KCl and $MgCl_2$ were added to the supernatant as before, and the solution almost instantly became extremely viscous. It was incubated for 1 hour at 18°C and overnight at 4°C. The viscous solution was then centrifuged at 105,000 x g for 3 hours, producing a clear, firm pellet and a supernatant of low viscosity. The pellet was redissolved in 20 ml of fresh ATP/ascorbate solution, dialyzed against water, and lyophilized.

2.2.3.2 Tests Comparing Peak II to Actin.

The first of the four tests was for co-migration of Peak II and standard actin on PAGE in SDS. This was accomplished using the Ortec slab apparatus and methods as described in section 2.1.4. Prior to electrophoresis, the standard actin was treated with dithiothreitol and iodoacetamide in 5 M guanidine, in an identical manner to the treatment of the aorta extracts prior to chromatography (sections 2.1.2 and 2.1.3).

The second comparison was examination of the 3-methylhistidine content of Peak II. On the single-column amino acid analysis system described in section 2.1.1.4.3.4, standard 3-methylhistidine emerged 3 minutes after histidine (Figure 4). Duplicate hydrolysates of Peak II and standard actin were run successively on the amino acid analyzer. The third comparison of Peak II to actin was by the elution profile of tryptic peptides from Aminex A-5 as described in section 2.1.5 for the comparison of Peak II and Peak I.

For the fourth test, a sample of fetal calf ligamentum nuchae was obtained from a fetus at the slaughterhouse approximately 1 hour after the death of the mother. It was transported to the laboratory in crushed ice. Four grams of tissue were crushed at liquid nitrogen temperature and suspended in 50 ml of 5 M guanidine as described for aorta in section 2.1.2.2.2. All subsequent operations were at 1-4°C. The coarsely homogenized suspension was more completely homogenized with three strokes in a ground glass homogenizer. An additional 30 ml of buffer were needed to rinse the suspension from the homogenizer.

It was then extracted overnight with gentle agitation. The suspension was centrifuged at 12,000 x g for 10 min. The supernatant was decanted and stored. The pellet was resuspended in 80 ml of fresh guanidine. A total of three extractions with guanidine and three more with guanidine and dithiothreitol were carried out in this manner. The extracts were dialyzed free of salt and lyophilized. Samples of the first guanidine (G-I) and first guanidine/dithiothreitol (G/DTT-I) extracts were dissolved to 1 mg/ml in slab gel sample buffer (section 2.1.4.1.3) in SDS and 2-mercaptoethanol. These samples were compared to Peak II on a 5% slab gel in the Ortec apparatus.

2.2.4 Results and Discussion.

2.2.4.1 Actin Preparation.

A total of 52 mg of lyophilized protein were recovered from this preparation. The number does not represent a quantitative estimate of actin in the tissue.

This material was shown to be actin by three independent criteria. First, by amino acid analysis as described in section 2.1.1.4, it contained 2.6 residues/1000 of 3-methyl histidine, a value very close to the 2.7 res/1000 reported by Collins (25) for rabbit actin. This modified amino acid has been found so far only in actin, myosin (traces), histones, and bovine retinal opsin (87). None of these three could have been mistaken for actin in this preparation. Bovine myosin heavy chains have a molecular weight of 200,000 (91), whereas on PAGE in SDS the actin preparation showed the R_f expected for a molecule weighing 42,000 daltons. Histones are small, very basic

proteins (54), having molecular weights of under 20,000 daltons and large numbers of lysine and arginine residues. The amino acid composition of the actin preparation was as expected for actin (Table VII), and not at all similar to that of histones. Opsin is a component of the retina, a tissue which was not present in the bovine skeletal muscle preparation. Furthermore, the amino acid compositions of actin and opsin (123) were not at all alike.

The second indication that the protein obtained from bovine muscle was actin was its behavior during purification. This purification was based on the reversible G to F transition of actin, and any material not able to undergo this transition, under conditions known to cause actin to do so, would have been discarded from the preparation (21)(43).

The third criterion was the appearance of the putative F-actin pellet under the electron microscope. Using a protein prepared as described above, from the same tissue, Gipson[1] observed typical actin filaments, and decoration of those filaments by heavy meromyosin subfragment 1, as described by Nachmias, Huxley, and Kessler (83).

There was no doubt that the protein obtained from this preparation was actin.

2.2.4.2 Co-migration of Peak II With Actin.

Figure 20 shows Peak II, actin, and a mixture of the two on a 5% gel in SDS. Not only did the principal bands migrate together, but the dimers and trimers also co-migrated on this gel. The dimer and

[1] I. Gipson, unpublished observations.

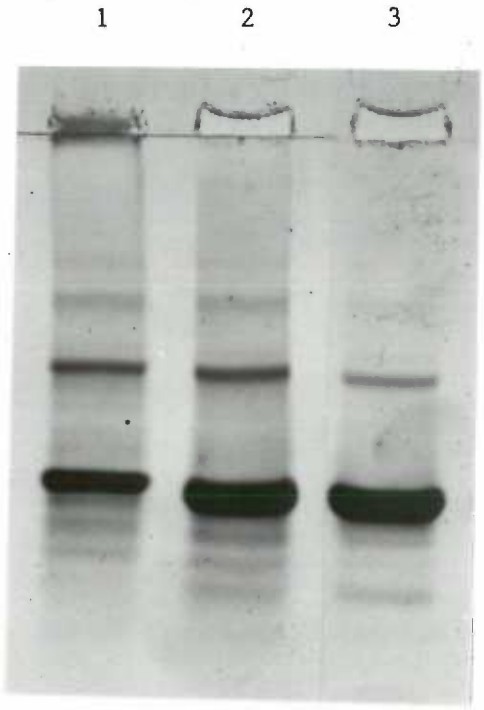


Figure 20. Comparison of Peak II with Actin.

Electrophoresis on 5% polyacrylamide gel in 0.1% sodium dodecyl-sulfate using the Ortec apparatus. All wells were from the same gel.

Well 1: Actin, prepared according to Carsten and Mommaerts (section 2.2.3.1, page 178)

Well 2: Actin plus Peak II.

Well 3: Peak II (see Figure 7)

Text section 2.2.4.2, page 182.

trimer bands of the mixture were as sharp as those of either sample alone. Rather than demonstrating an obvious difference between Peak II and actin, this gel showed that the two preparations were very much alike.

2.2.4.3 Similarity of Amino Acid Composition.

Table VII shows the amino acid compositions of actin from the literature, actin prepared as described above, and Peak II. (The sample of Peak II was a different preparation than the one used to obtain the analysis in Table V, and the amino acid analyzer program was different also. Therefore the results are not identical.) The most significant result in this analysis was the presence of 3-methyl histidine in Peak II, in an amount only slightly less than that reported by Gosselin-Rey et al (43) to be present in bovine carotid actin. Laki (66) has stated that the 3-methyl histidine content in actin is variable between approximately 1.5 and 3 residues per 1000. The conclusion agrees with that of the experiment on the gel in SDS. No significant difference could be found between Peak II and actin. The similar contents of 3-methyl histidine strongly favored the conclusion that Peak II was actin.

2.2.4.4 Similarity of Tryptic Peptide Profiles.

Figure 21 shows the elution profiles of the tryptic digests of standard actin (upper trace) and Peak II (lower trace) when chromatographed as described in section 2.1.5. The column for Peak II was eluted at a slightly slower rate, resulting in a slightly longer time

TABLE VII: AMINO ACID COMPOSITIONS OF PEAK II AND STANDARD ACTIN (RESIDUES PER 1000 RESIDUES).

	Bovine Carotid Actin[1]	Rabbit Skeletal Muscle Actin[2]	Bovine Skeletal Muscle Actin[3]	Peak II
Asp	91	91	88	92
Thr	65	75	68	68
Ser	66	59	67	77
Glu	117	104	125	135
Pro	51	51	48	50
Gly	77	75	79	81
Ala	80	78	85	82
½Cys	12	13	10	13
Val	51	56	54	50
Met	37	43	42	11
Ile	77	78	63	59
Leu	75	70	71	77
Tyr	39	43	43	41
Phe	32	32	32	32
Lys	52	51	50	49
His	21	21	23	21
3M-His	2.4	2.7	2.6	2.1
Arg	50	43	51	52
Other	10	11	--	9

[1]Gosselin-Rey et al (43).
[2]Collins et al (25).
[3]Prepared from bovine skeletal muscle by the procedure of Carsten and Mommaerts (21).
[4]3M-His: 3-methyl histidine.

Text section 2.2.4.3, page 184.

Figure 21. Tryptic Peptide Profiles of Peak II and Actin from Ion Exchange Column.

Peak II (Figure 7) and standard actin (section 2.2.3.1) were digested with trypsin, then chromatographed on Bio-Rad Aminex A-5 cation exchange resin. Column effluent was monitored by the ninhydrin reaction (2.1.1.4.3.5).

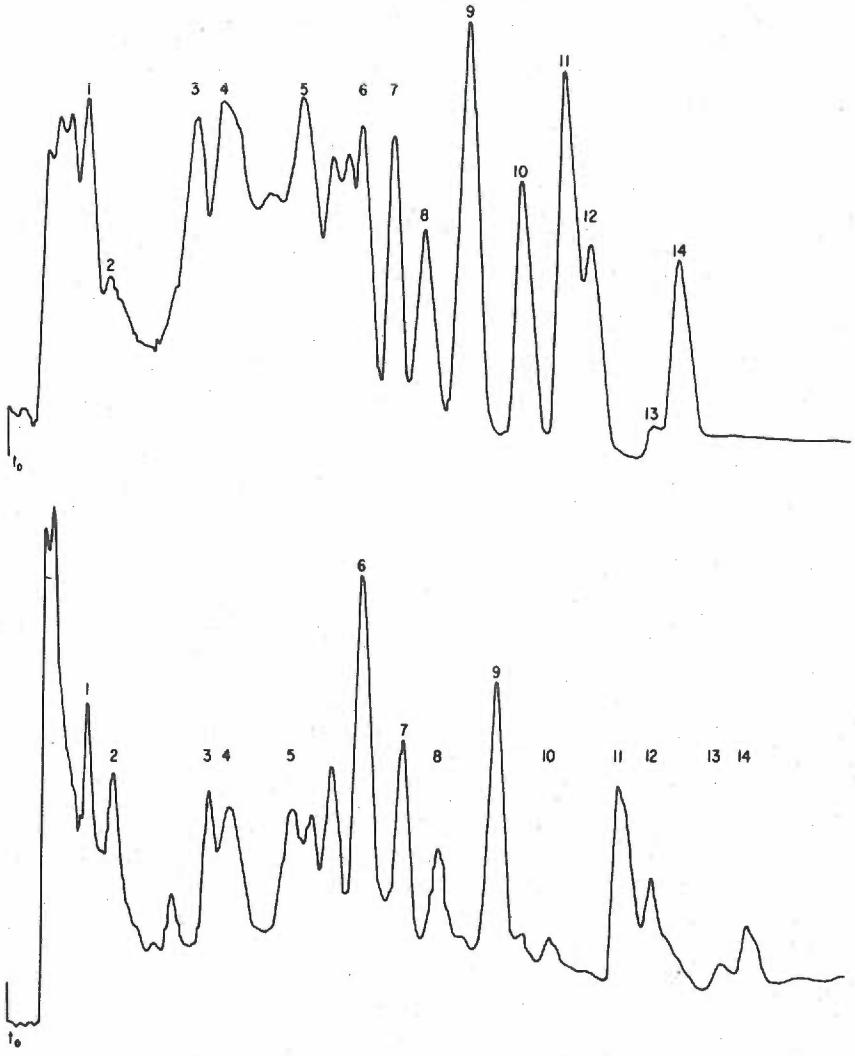
Upper trace: Actin

Lower trace: Peak II

Because of slightly different elution rates, numbered peaks do not appear to correspond between the two profiles as shown on the figure. However, the elution volumes were the same.

Text section 2.2.4.4, page 184.

COMPARISON OF TRYPTIC DIGESTS



scale. At first glance, the two profiles were not at all alike, but if the change in elution rate was taken into account, fourteen separate peaks appeared at corresponding times on the two chromatograms. This could be contrasted with Figure 19, in which only a single peak was common to digests of Peak I and Peak II. By a third independent criterion, Peak II was seen to be very similar to skeletal muscle actin.

2.2.4.5 Absence of Peak II in Ligamentum Nuchae Extracts.

Figure 22 shows the slab gel in SDS comparing Peak II to the first guanidine and first guanidine/dithiothreitol extracts of ligamentum nuchae. The Peak II load was large, and small contaminating bands were quite visible. There was only a minor band in the extracts corresponding to Peak II. If Peak II were the subunit of SGP, it would have been expected to be the dominant band in these extracts of fetal ligament. The small band in the ligament extracts having the same mobility as Peak II could have been fibroblast actin (18), or an unrelated protein of the same molecular weight. The major components of the ligamentum nuchae extracts were thus seen not to correspond to Peak II in mobility in SDS, suggesting that the microfibrillar protein described by Ross and Bornstein (113) and Peak II were not the same protein.

2.2.5 Conclusion and Implications for Future Work on SGP.

It was apparent that denatured actin, in the form of "Peak II", had been mistaken for a possible monomeric form of SGP.

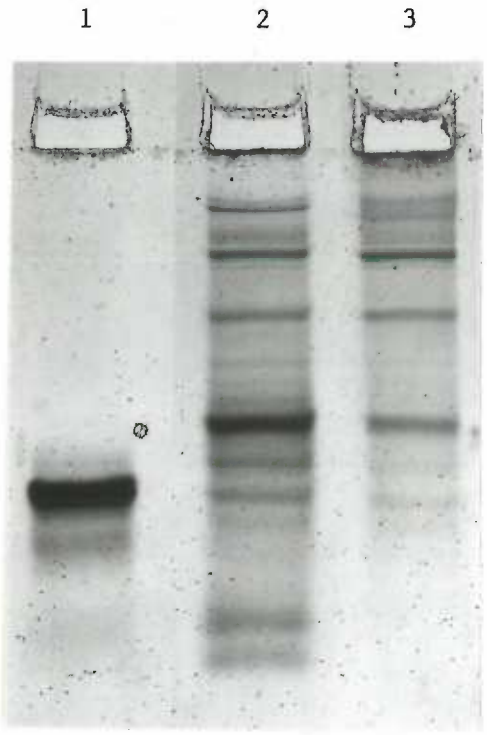


Figure 22. Ligamentum Nuchae Extracts Compared to Peak II.

Electrophoresis in 5% polyacrylamide gel in 0.1% sodium dodecylsulfate. Ortec slab apparatus used. All wells were taken from the same gel.

Well 1: Peak II (Figure 7)
Well 2: Ligamentum nuchae extract G-I
Well 3: Ligamentum nuchae extract G/DTT-I

Text section 2.2.4.5, page 188.

The first result of this discovery was abandonment of all the experiments proposed at the close of Experimental Section I, there being no point in further characterizing an artifact.

The discovery that Peak II was only denatured actin raised a very serious question, with far-reaching implications in the field of connective tissue research. Were the many SGP preparations described in the literature also only actin? Could it be that many investigators had been engaged, for two decades, in the characterization of an artifact?

With the information available from the experiments described so far, it was not possible to answer this question. There were several alternative explanations for the presence of actin in the guanidine and guanidine/dithiothreitol extracts of aorta, explanations which had not been tested. The last major experimental section of this thesis is devoted to additional experiments which were designed to test those explanations and provide data for a firm statement concerning the possibility that aortic SGP is only an artifact.

2.3 Experimental Section III: Survey of Aorta Proteins.

In Experimental Section I, a protein of molecular weight 42,000 - 43,000 daltons was isolated from bovine aorta by a procedure similar to those used by many other investigators to obtain a material thought to be structural glycoprotein, or SGP. It was thought that the protein isolated in Section I might be the monomer of SGP. But in Experimental Section II, this protein was shown to be actin. The question was then asked whether the aortic SGP described by other investigators was also just actin. Experimental Section III is devoted to answering that question.

2.3.1 Specific Objectives of This Section.

At the conclusion of Section II, it was stated that there were a number of postulated reasons why actin had been found in the guanidine extracts, and also in the subsequent guanidine/dithiothreitol extracts of bovine aorta. The overall objective of this section was to confirm or deny those hypotheses.

The first mechanism which was proposed to explain the artifactual presence of actin in "SGP" preparations was that of inadequate tissue homogenization. Peak II, shown to be actin, was isolated from tissue homogenized by freeze-fracturing at liquid nitrogen temperature in a steel mortar and pestel. The resulting homogenate contained some rather large (2-3 mm) pieces of tissue. It was suggested that proteins would be slow to diffuse out of these large pieces, resulting in the presence of actin in tissue residues which had been extracted several times with buffers in which actin was soluble. In recognition of this

possibility, the first specific objective of Section III was to homogenize the tissue more finely and uniformly than had been possible by freeze-fracturing.

It should be mentioned that aorta, being about 50% connective tissue, cannot be homogenized by the more usual techniques. Aorta defies hand-held ground glass homogenizers, porcelain mortars and pestels, and high speed devices such as those manufactured by Virtis or Brinkman. Consequently, it was a considerable problem finding a suitable technique for homogenizing this tough, rubbery tissue.

Another postulated mechanism responsible for the artifactual presence of actin in SGP was the absence of pre-washes. Peak II was isolated from whole aorta tissue which had not previously been washed with water or dilute buffers. As described in the Historical Setting (Section 1) and in Experimental Section I (2.1.2.2.1), these pre-washes were thought to contribute to the heterogeneity of SGP by allowing tissue proteases and other degradative enzymes the opportunity to degrade it. Consequently, these pre-washes were omitted in Section I, where the objective was to isolate undegraded SGP. The second specific objective of Section III, therefore, was to wash the aorta homogenate with a dilute buffer in which actin was known to be soluble, until those pre-washes were shown to be free of protein by the method of Lowry et al (69).

A third postulated mechanism leading to the presence of actin as an artifact in SGP was entrapment by collagen fibrils. Anderson (5) wrote of SGP and microfibrillar glycoprotein as being solubilized

only after the removal of collagen from the tissue. Most authors describing SGP from aorta first removed collagen from the tissue. There were three ways in which this was done. Some investigators used treatments with hot trichloroacetic acid, in which collagen, unlike most other proteins, is soluble. Others used autoclaving to remove the collagen. Still others used collagenase to remove the collagen enzymatically. There are many examples of the three techniques in Table I. Removal of collagen by any of them was thought to degrade SGP, as described in the Historical Section, and for this reason no steps were taken in Section I to remove collagen prior to extraction of the tissue with guanidine. Therefore, the third specific objective of Section III was to solubilize collagen from samples of homogenized aorta by each of the three common techniques of collagen removal, and then examine both the supernatant and the tissue residue for the presence of actin.

Other mechanisms postulated to explain the presence of actin in SGP preparations were entrapment by insoluble elastin, or containment within the tissue by some other mechanism yet to be envisioned. Examination of the relevant literature (see Table I for the large number of references) shows that if actin were held in the tissue by one of these mechanisms, it most certainly would be present in the "SGP" prepared from aorta as described in that literature. Therefore, if incomplete homogenization, lack of pre-washes, and failure to remove collagen were found not to be the cause for the presence of

actin in the guanidine extracts described in Section I above, then most of the aortic "SGP" described in the literature would undoubtedly be only actin.

Another objective of Section III was to find genuine aortic SGP, if indeed it existed in the tissue in the large amounts often reported in the literature. Using the high resolving power of polyacrylamide gel electrophoresis (PAGE) in SDS, all components of all extracts of aorta would be quantitated and compared to standard albumin, actin, myosin, and collagen, the major proteins expected to be found in the aortic media (50).

To achieve these four goals, the following general protocol was designed. Aorta would be homogenized in a Latapie Mill (Thomas). The homogenate would be washed with the sequence of buffers described by Carsten and Mommaerts (21) for the preparation of actin.

The first wash, with 0.15 M NaCl, would contain either 2 mM diisopropyl fluorophosphate (DFP) or 2 mM phenylmethylsulfonyl fluoride (PMSF). These compounds are irreversible inhibitors of proteases with a hydroxyl group at the active site (33), and would be included to inhibit degradation of the tissue proteins by proteases present in the tissue.

EDTA would also be used in the pre-washes to remove the metal ions from any proteases using them as cofactors (82). No inhibitor of sulfhydryl proteases would be used, as was used in the collagenase digestion described above (2.1.2.2.1), since it would presumably also bind to the sulfhydryl groups of actin, a reaction which alters the

protein's solubility and ability to form fibrils (21). Washes with ATP and ascorbate would continue until the washes were free of protein. The tissue would then be extracted with guanidine and with guanidine containing dithiothreitol. Finally, it would be extracted with 0.1 N NaOH. This was termed the "sequential extraction" of aorta. Separate portions of tissue residue immediately prior to the washes with ATP and ascorbate would be autoclaved, extracted with hot TCA, or digested with collagenase. The residues after these treatments, and an untreated control sample of tissue, would be extracted once with guanidine and dithiothreitol. All solutions from all the above steps would be examined by PAGE in SDS and compared to standard proteins. The ATP/ascorbate washes, and the guanidine, guanidine/dithiothreitol, and NaOH extracts from the sequential extraction would be quanitated. The bands shown by these extracts on PAGE in SDS would also be quantitated. Any band totalling more than 5% of any extract would be isolated by preparative PAGE in SDS and subjected to amino acid analysis. Analyses of bands co-migrating with standard proteins would be compared to analyses of those standards.

In this way, it was hoped to demonstrate the presence of actin in aorta extracts previously thought to contain only SGP, and to place an upper limit on the amount of bona fide SGP which could be present in bovine aorta.

2.3.2 Materials and Methods.

2.3.2.1 Sequential Extraction of Tissue.

A flow chart of these extractions is shown in Figure 23.

2.3.2.1.1 Homogenization and Washes With Dilute Salt.

Thoracic aorta from adult cattle were obtained at the slaughter-house and stored frozen until use. They were partially thawed and stripped of adventitia. Histological sections confirmed complete removal of this layer. All subsequent operations were performed with pre-cooled solutions at 4°C. The tissue was cut into 1-2 cm slices and weighed. The sliced tissue, totalling 97.1 grams, was homogenized into 500 ml of 0.15 M NaCl containing 2 mM DFP (Aldrich) or 2 mM PMSF (Sigma), and 1 mM EDTA, pH 7. Homogenization into the saline solution was accomplished by use of a Latapie Mill (Thomas). This mill resembled a small meat grinder, with a set of revolving blades and a piston to force the tissue through them. The exit face of the blades was washed by a flow of buffer which swept the finely-divided tissue into a receiving flask. For the aorta homogenization, the cold saline solution was pumped through the mill at 5 ml/min with a peristaltic pump.

Except for the use of DFP or PMSF as a protease inhibitor, the following pre-washes were described by Carsten and Mommaerts (21) as preliminary treatment of the tissue prior to extraction with ATP and ascorbate to obtain actin. The suspension was capped and stirred magnetically overnight, then centrifuged at 7000 x g for 15 min. The

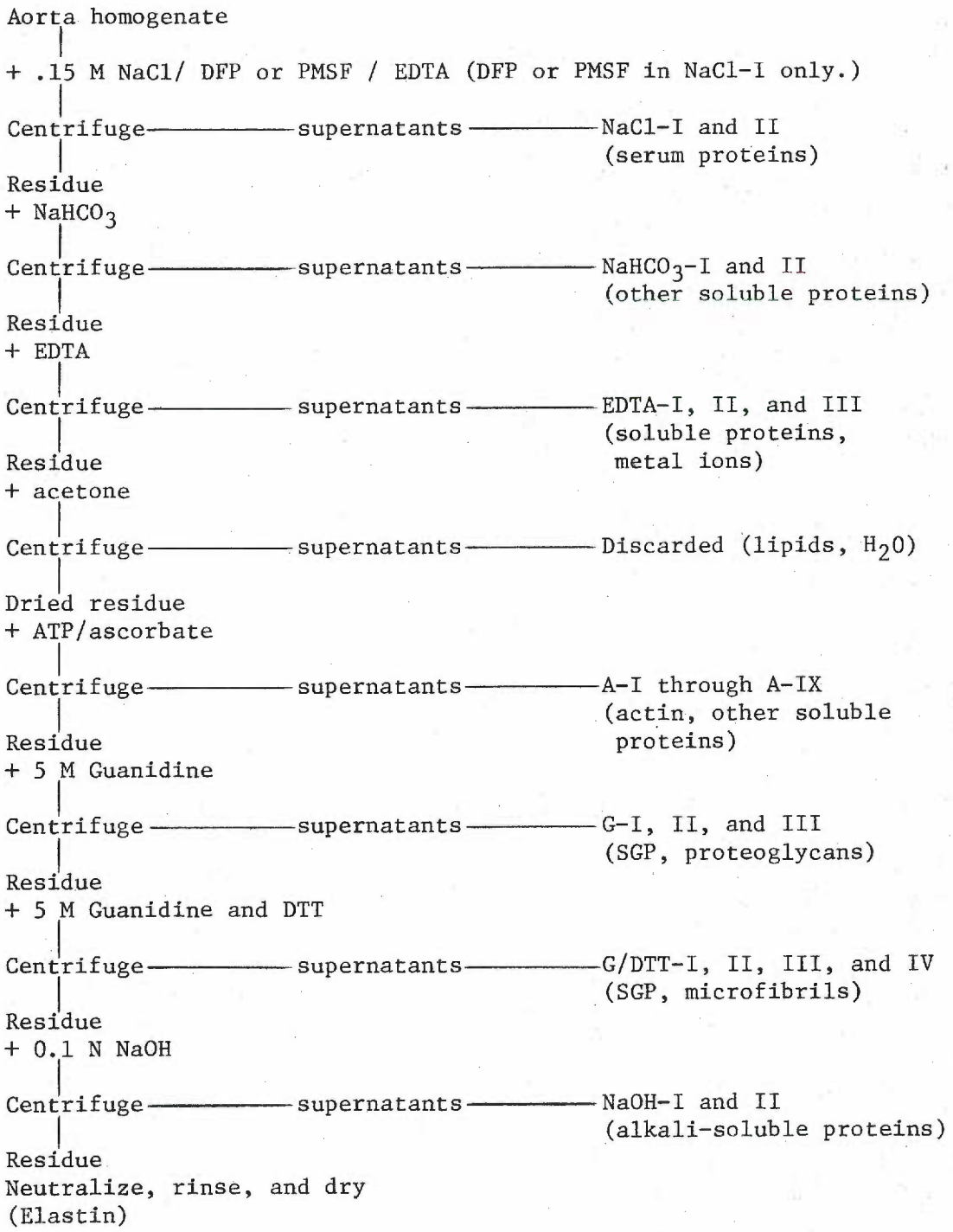


Figure 23. Flow Diagram of Sequential Aorta Extractions.

Items in parenthesis are those species expected to be found in each fraction. DFP: Diisopropylfluorophosphate. PMSF: Phenylmethylsulfonylfluoride.

Text section 2.3.2.1, page 196.

supernatant was decanted, and the residue was resuspended in 500 ml of 0.15 M NaCl, 1 mM EDTA, pH 7. The suspension was stirred for 15 minutes and centrifuged as before. In this same manner, the tissue was washed twice with 500 ml of 0.05 M $NaHCO_3$ containing 1 mM EDTA, three times with 1 liter of 1 mM EDTA alone, and three times with 500 ml of reagent grade acetone. The acetone washes were discarded. The residue was dried under a stream of nitrogen at room temperature and weighed.

2.3.2.1.2 Washes To Remove Actin.

The dried tissue was then extracted by the method of Carsten and Mommaerts (21), which has been shown to be suitable for obtaining actin from arterial smooth muscle (43). The entire preparation was suspended in 500 ml of freshly-prepared 0.2 mM ATP, 0.2 mM ascorbate, pH 7.5, and stirred for 30 min. The tissue was centrifuged at 7000 x g for 15 min, and the supernatant was removed and stored at 4°C. The pellet was resuspended in 500 ml of ATP/ascorbate solution. In this manner, nine sequential ATP/ascorbate washes were collected, designated A-I through A-IX. The pellet was stored overnight at 4°C between washes 4 and 5. Fresh ATP/ascorbate solution was made for washes 5-9. The washes were stored at 4°C.

2.3.2.1.3 Extractions With Guanidine, Dithiothreitol, and NaOH.

The residue after the pre-washes was stirred three times for 24 hours each with 500 ml of 5 M guanidinium chloride, 0.1% EDTA, 0.05 M tris, pH 8.5. After each extraction, the suspension was centrifuged

at 7000 x g for 1 hour. The supernatants, designated G-I through G-III, were stored at -10°C. Next, four extractions with guanidine buffer containing dithiothreitol (DTT) were carried out. The first was begun by adding 3.87 g of DTT (50 mM) and 500 ml of guanidine buffer to the pellet. The bottle was swept with nitrogen, capped, and stirred for 24 hours. The suspension was centrifuged at 7000 x g for 1 hour. The supernatant was stored at -10°C, designated G/DTT-I. Three more G/DTT extractions were performed, but 0.39 g of DTT (5 mM) was used for each, rather than 3.87 g, because of the cost of the reagent. Although Ross and Bornstein (113) had used 50 mM dithioerythritol, Furthmayr and Timpl (39) had shown that concentrations of DTT as low as 1 mM were adequate to extract their "acidic structural protein". 5 mM DTT was not an unusually low concentration.

After the guanidine extractions, the residue was extracted twice for 24 hours with 0.1 N NaOH. Volumes of the first and second extracts were 500 ml and 750 ml, respectively. The residue was centrifuged at 7000 x g for 15 min after each extraction. Supernatants, designated N-I and N-II, were neutralized with HCl and stored at 4°C.

The residue after these extractions was neutralized with HCl, washed once with 1 liter of water, once with 1 liter of acetone: water 1:1, and 3 times with 500 ml of acetone, all by stirring for 10 min and centrifugation at 2500 x g for 5 min. It was then dried under a stream of nitrogen. This solid material was designated the aorta residue.

2.3.2.2 Analysis of the Whole Extracts.

A flow chart of these procedures is shown in Figure 24.

2.3.2.2.1 Preparation of Samples.

After centrifugation at 35,000 x g for 15 min to remove finely-divided tissue not pelletted during low-speed centrifugation of the entire tissue suspension, aliquots of the ATP/ascorbate washes were assayed for protein by the automated Lowry procedure described earlier (section 2.1.1.1). The Lowry-positive washes, I-V, were then filtered free of finely-divided tissue by passage through Whatman #42 filter paper with the help of "Hiflo Super cell", an inorganic filter aid produced by Johns-Manville. Filter aid in the amount of 1 g per 100 ml was suspended in the solution prior to filtration. Washes VI-IX were discarded. A-I and 100 ml of A-V were dialyzed exhaustively versus distilled water and lyophilized. A-II, III, and IV, and the remainder of A-V, were combined and stored at -10°C.

Aliquots of 25 ml of all G, G/DTT, and NaOH extracts except G/DTT-IV were taken for analysis. Extract G/DTT-IV, expected to be very dilute, was concentrated and apportioned as discussed below. The G and G/DTT aliquots were centrifuged at 66,000 x g for 30 minutes to remove finely-divided tissue not pelletted during isolation of the extracts. The supernatants, and the uncentrifuged aliquots of the NaOH extracts, were divided into sub-aliquots for Kjeldahl, uronic acid, and amino acid analysis.

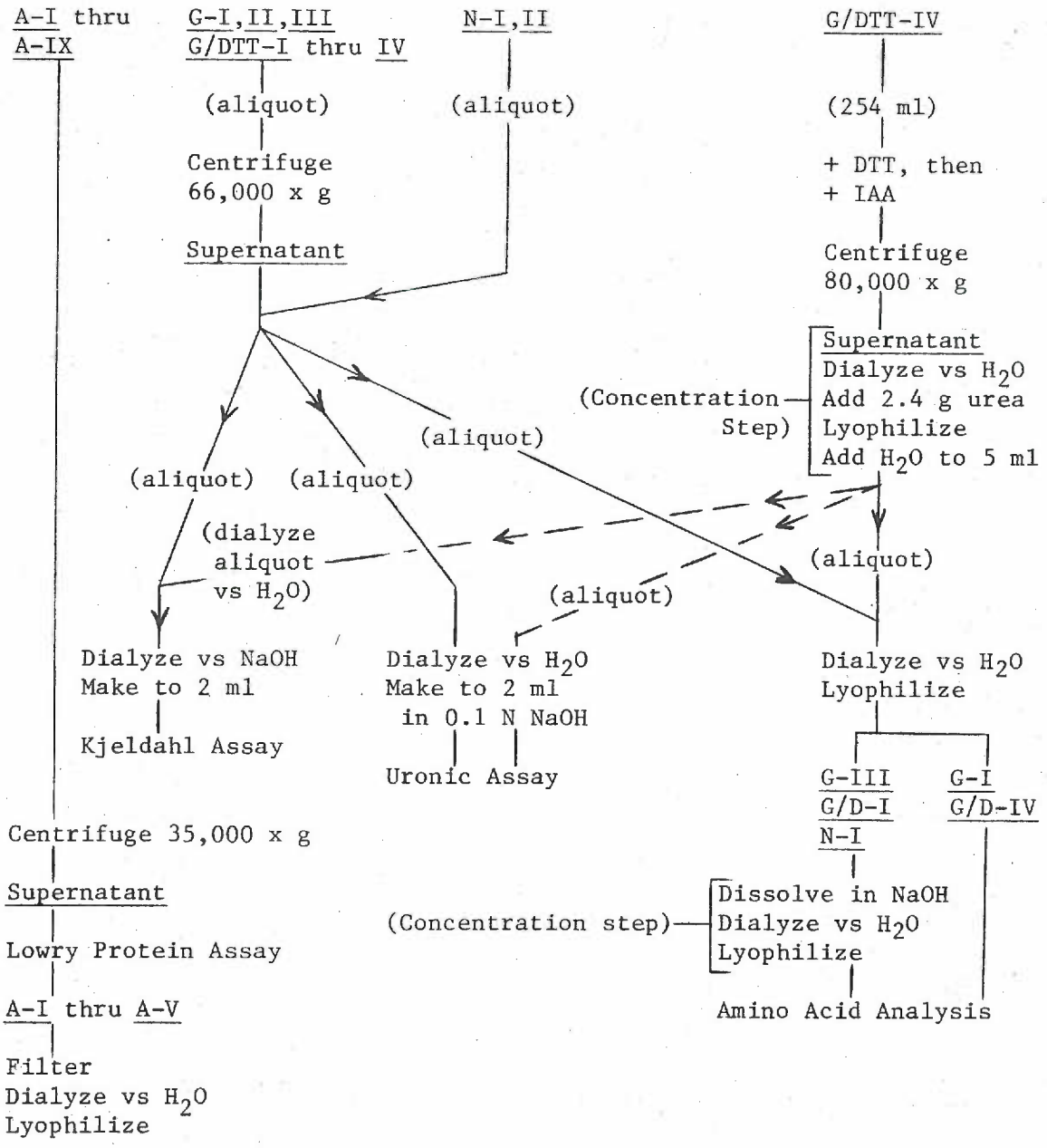


Figure 24. Workup of Sequential Extracts for Analysis.

Extracts shown in Figure 23 were prepared for analysis according to the above flow diagram. Text section 2.3.2.2, page 200.

The entire G-DTT-IV extract, except for 100 ml worked up as described below, was warmed to room temperature. Its volume was 254 ml. To reduce any disulfide bonds which might have formed during storage of the extract, 100 mg of fresh dithiothreitol were added. After 90 min, 1.5 g of iodoacetamide (Aldrich) were added to the solution and stirred to dissolve. The solution was allowed to stand overnight at room temperature. The extract was centrifuged at 80,000 x g for 45 min to remove finely divided tissue. The supernatant was then dialyzed against six changes of water. After addition of 2.4 g of urea, the solution was lyophilized. The solid was taken up in 5 ml of water. This solution in urea was used in the analyses described below.

2.3.2.2.2 Kjeldahl Nitrogen Determination.

For nitrogen determination, samples of 2.0 ml were taken from all the 25 ml aliquots. A 1 ml sample of G/DTT-IV was taken and dialyzed overnight against running tap water. All the samples for Kjeldahl assay were then dialyzed against five changes of 0.1 N NaOH. This was done to keep the protein from precipitating and entrapping traces of guanidine or urea, which would interfere with the Kjeldahl assay. The dialysates were made to 2.0 ml with water. Volumes of 400 µl were digested for nitrogen assay as described in section 2.1.1.2.

2.3.2.2.3 Hexuronic Acid Determination.

For determination of hexuronic acids, samples of 2.0 ml were taken from all the 25 ml aliquots. A 0.5 ml sample of G/DTT-IV was

taken. All samples were dialyzed against four changes of water. To dissolve the precipitate in the dialyzed samples, 0.2 ml of 1 N NaOH was added to each sample. The samples were made to 2.0 ml (0.1 N in NaOH) with water and assayed for hexuronic acid by the m-HDP method (section 2.1.1.5).

2.3.2.2.4 Amino Acid Analysis.

For amino acid analysis, volumes of each sample were taken as follows, to give approximately 5 mg of protein as determined by the Kjeldahl assay:

G-I	1 ml
G-III	10 ml
G/DTT-I	5 ml
G/DTT-IV	1 ml
N-I	10 ml

The samples were dialyzed versus six changes of water. G-I and G/DTT-IV, being of sufficiently small volume, were divided equally between two 5 ml hydrolysis ampoules and lyophilized in the ampoules. The other samples were concentrated by lyophilization in 100 ml flasks. They were taken up in 2 ml of 0.1 N NaOH and dialyzed against two changes of water. These were then divided equally between two 5 ml ampoules and lyophilized. The samples in ampoules were then hydrolyzed and analyzed according to the procedure of section 2.1.1.4. For these analyses, the single-column program was used. The column contained Technicon Chromo-beads type C-2. A Pre-A buffer of pH 3.15 was employed to resolve hydroxyproline from aspartic acid. The pH of A-buffer was 3.30. Changes to B-buffer and to C-buffer were 90 min and 150 min, respectively, after sample application. No temperature change was used.

2.3.2.3 Further Workup of the Extracts.

The remainder of the G/DTT-IV preparation in urea (2.5 ml) was dialyzed against four changes of distilled water and lyophilized. Remainders of the 25 ml aliquots of the other G, G/DTT, and NaOH extracts were stored frozen at -10°C.

Aliquots of 100 ml of the original guanidine extracts (G-I, G-II, and G-III) were reduced with 80 mg of dithiothreitol as described by Ross and Bornstein (113). After overnight incubation at room temperature, 400 mg of iodoacetamide were added to each aliquot. 400 mg of iodoacetamide were also added to 100 ml aliquots of the original G/DTT extracts II, III, and IV. A 3.7 g portion of iodoacetamide was added to a 100 ml aliquot of G/DTT-I because of the larger amount of dithiothreitol used in preparing this extract. Quantities of iodoacetamide represented a 4-fold molar excess over free thiols of the dithiothreitol in each extract. Alkylation of the free thiols with iodoacetamide was allowed to proceed for 12 hours at room temperature. All the reduced, alkylated aliquots of the G and G/DTT extracts, together with 100 ml aliquots of the neutralized NaOH extracts, were centrifuged at 23,000 x g for 50 minutes to remove finely-divided tissue. The supernatants were dialyzed against four changes of water. The resulting suspensions of precipitated material were stored frozen at -10°C. They shall be referred to in subsequent experiments as the reduced, alkylated extracts.

2.3.2.4 Comparison of Techniques for Removal of Collagen.

A portion of acetone-dried tissue, as described in section 2.3.2.1.1, was divided into samples weighing 1 gram each. One of these samples was treated for removal of collagen in each of the following ways.

2.3.2.4.1 Collagenase Digestion.

The tissue was suspended in 30 ml of 0.02 M tris Cl, 0.01 M $CaCl_2$, pH 7.4, containing 20 mg of N-ethyl maleimide (an inhibitor of proteases having sulfhydryl groups at the active site (90) and 0.5 mg of collagenase (Worthington) which had been purified by the method of Peterkofsky and Diegelmann (90). The suspension was shaken for 24 hours at 37°C. It was then centrifuged at 7,700 x g for 10 min. The second supernatant was also decanted and stored at -10°C. The pellet was extracted with guanidine and dithiothreitol as described below. This collagenase digestion was exactly as described by Wolff et al (138) for their preparation of SGP from a variety of tissues, except for the use of purified collagenase and the inclusion of N-ethyl maleimide.

2.3.2.4.2 Autoclaving.

The tissue was placed in 30 ml of water in a tightly-capped tube. It was then heated to 110°C for 24 hours, with frequent agitation to resuspend the settled tissue. While still quite warm, the suspension was centrifuged at 7000 x g for 20 minutes. The supernatant was

decanted and made to 0.5 N with a small amount of glacial acetic acid to prevent gellation. (The process by which heat-denatured collagen gels upon cooling is familiar to connective chemists. The gels can be dispersed, or prevented from forming, by dilute acid (119).) It was then frozen. The pellet was extracted with guanidine and dithiothreitol as described below. This treatment was exactly as described by Keeley and Labella (60) for their preparation of SGP from chick aorta.

2.3.2.4.3 Extraction With Hot Trichloroacetic Acid (TCA).

The tissue was suspended in 30 ml of water and heated to 90°C. TCA (100%, w/v) was added to a final concentration of 2.7% (w/v). After 4 minutes at 90°C, the suspension was cooled in an ice bath and centrifuged at 7,700 x g for 15 min. The supernatant was removed and stored at 4°C. The pellet was resuspended in 30 ml of 2.7% TCA and incubated at 90°C for 25 min. It was then cooled and centrifuged as before. The pellet was extracted with a third portion of TCA at 90°C for 30 min. The tissue was finally washed, by resuspension and centrifugation as before, with two 30 ml portions of water. All five extracts (three TCA extracts and two water washes) were stored at 4°C. The pellet was extracted with guanidine and dithiothreitol as described below. The TCA extraction procedure was as described by Moczar and Robert (76) for the preparation of aortic SGP from several large mammals.

2.3.2.4.4 Guanidine/Dithiothreitol Extraction.

The tissue residues from the three collagen removal treatments, and a fourth 1 gram portion of dry tissue which served as a control, were each extracted once with guanidine and dithiothreitol. The tissue samples were suspended in 30 ml of 5 M guanidine, 0.1% (w/v) Na_4 EDTA, 0.05 M tris Cl, pH 8.5. Dithiothreitol (250 mg) was then added to each sample. The bottles were swept with nitrogen and capped. They were then shaken for 21 hours at 4°C. The suspensions were centrifuged at 17,000 x g for 15 min. The supernatants were decanted and stored at -10°C. Pellets were also stored at -10°C.

2.3.2.5 Polyacrylamide Gel Electrophoresis in SDS.

All PAGE in SDS in this section was performed using the Bio-Rad apparatus and the solutions described in section 2.1.4.1.3. Samples were taken from the reduced, alkylated extracts (section 2.3.2.3) unless otherwise stated.

2.3.2.5.1 Band Quantitation by Gel Scanning.

Slab gels were stained with Coomassie Brilliant Blue dye as described in section 2.1.4. The slabs were then cut between each sample well to produce strips of gel with one sample per strip. These strips were scanned in the manner of a tube gel. The strips were placed in a gel scanning cuvette (Gilford Model 2412) and held against one of the inner faces by the surface tension of the solution (10% HAc). The cuvette was then filled with solution to a point 1-2 mm below the upper edge of the strip. Adding any more solution than this broke the

surface tension holding the strip of gel to the face of the cuvette, and it fell to the bottom of the cuvette. The gel was then scanned with a Gilford Model 2410-S Linear Transport and Model 220 Optical Density Converter mounted to the monochromator of a Beckman Model D.U. spectrophotometer. A plot of absorbance at 580 nm versus distance on the gel was generated by a chart recorder attached to the O.D. Converter.

For quantitation of bands on the gels, the areas of the corresponding peaks on the chart recording were integrated with a planimeter. Three integrations, or five for very small peaks, were averaged for each area. In order to verify that the staining of each band was linear with respect to sample size, samples of from 5 to 40 µg were applied to the slabs. Peak areas from the scans of these gels were then plotted versus micrograms of total sample (Figures 30 and 31). The result was a series of lines, one for each band in the sample. A least-squares linear regression was used to determine the best straight line through the points. The areas of the peaks at an arbitrary loading, usually 20 µg, were then read from these best-fit lines. From these areas, the percentage of each band in the total band pattern was calculated.

The relationship between peak area and micrograms of protein was determined using standard proteins (bovine serum slbumin, standard actin from section 2.2, and standard collagen α and β chains). Each standard was applied to the gel over a broad range of sample loadings. Data from these gels were used to obtain a standard calibration curve of peak area per microgram of protein (Figure 28).

All the plots described above are shown in the Results section to follow (2.3.3.3).

2.3.2.5.2 Preparative PAGE in SDS.

There are two basic techniques for preparative electrophoresis (2). The first involves sectioning the gel after electrophoresis and extracting the desired proteins from the sections. The second involves collecting each protein as it emerges from the lower surface of the gel, usually by washing the surface with a stream of lower electrode buffer and collecting the stream in a series of test tubes using a fraction collector as in column chromatography. The second method normally gives better recoveries of the applied sample. However, the second method was found to be much more difficult technically, much slower, and dangerous in that the stream of buffer entering the fraction collector was at the same voltage potential as the lower electrode of the gel apparatus, typically 500 volts. For many reasons, it was decided to use the simpler, quicker, safer sectioning technique to isolate individual protein bands by preparative electrophoresis.

2.3.2.5.2.1 Apparatus and Procedures.

For preparative work, the Bio-Rad apparatus was able to accommodate two slabs simultaneously. Each slab was 3 mm thick, rather than the 1.5 mm thickness used for analytical experiments. The separating gel, and well and cap gel, recipes given in section 2.1.4 were quadrupled for the preparative experiments. A single sample well 13.7 cm wide was used.

Samples of 5 mg were applied to each gel, in 1 ml of sample buffer. For preparative PAGE of extract G/DTT-I (see Figure 23), 149 mg of the reduced, alkylated extract were fractionated on a 3.1 x 95 cm column of Sepharose 6B in guanidine, by the techniques described in section 2.1.3. All material emerging after Peak I was dialyzed free of guanidine, lyophilized, and made to 5 mg/ml in sample buffer for preparative PAGE. A sample of Peak I was dialyzed free of salt and hydrolyzed for amino acid analysis. Removal of Peak I was necessary because, as seen in section 2.1.4, much of this material did not penetrate the gel during electrophoresis, and because results from quantitative PAGE (below) suggested that the material plugged up the pores of the gel, preventing smaller molecules from passing through. As seen on the strips of preparative gel stained to locate the bands (next section), removal of Peak I reduced the amount of bands with molecular weights over 100,000 d (Figure 16).

Power supply setting for the preparative runs were 1 mfd discharge capacitance, 325 volts. The gels were begun at 200 pulses per second, and increased to 300 per second after 30 minutes. Further increases in power application were within the capability of the power supply, but they were found to produce excessively warm operating temperatures in the electrophoresis cell. Preparative gels were run for a total of 5 hours. It was necessary to interrupt the electrophoresis briefly after 2½ hours in order to remove an accumulation of bubbles from the lower surface of the gels, and to replace the upper electrode buffer. Volumes of this buffer were increased to 400 ml for preparative runs.

Gels of 8% were used in preparing bands from ATP-ascorbate wash A-I while 5% gels were used for guanidine and guanidine/dithiothreitol extracts.

2.3.2.5.2.2 Isolation of Bands.

After electrophoresis, the gels were placed on a glass plate, and strips were cut from each side and from the center of the slab. The strips were stained with Coomassie blue. The remainder of the slabs were stored at 4°C during the staining of the strips. The stained strips were then laid alongside the unstained portions of gel in order to locate the bands on the unstained gel. Bands to be isolated were then sectioned from the unstained portions of gel using the stained strips as guides. The use of a stained strip from the center of the slab was necessary because protein at the center of the gel moved about 5% faster than at the edges, resulting in slightly curved bands across the gel. The center strip allowed accurate compensation for the curvature.

Pieces of gel containing bands to be isolated were homogenized in gel running buffer (see section 2.1.4.1.3) using a Polytron PCU-2 homogenizer (Brinkman) at low speed. The buffer for this procedure also contained 25% isopropyl alcohol, which kept the gel from swelling and allowed smaller extraction volumes to be used. Gel band homogenates were extracted four times for 12 hours each at room temperature with 3-4 volumes of this buffer. Each extract was pipetted from the gel homogenate after a brief, low-speed centrifugation to settle the finely-divided pieces of gel. The extracts for each

band were combined and dialyzed against two changes of 0.15 M NaCl, followed by six changes of distilled water, all at 4°C. A precipitate which formed during dialysis against the NaCl slowly disappeared during the dialyses against water. The gel extracts were assayed for protein by the automated Lowry assay as described in section 2.1.1.1. They were then lyophilized.

The lyophilized band extracts were in the form of small amounts of very finely divided material stuck to the insides of relatively large lyophilization bottles. In order to remove them, it was necessary to redissolve each of them in 2.0 ml of 5 M guanidine, 0.05 M tris Cl, pH 8. A bit of lumpy, jellylike residue in each sample was filtered out by passage of the guanidine solutions through beds of Celite "Hi-Flow Super-Cell" (Johns-Manville) packed in Pasteur pipets. The residue was presumed to be very small particles of polyacrylamide gel not removed earlier by centrifugation. The crystal clear filtrates were then dialyzed free of guanidine.

After dialysis, aliquots of from 16 to 50 µl were removed for examination by analytical PAGE to verify the purity of the isolated bands. Volumes removed were determined by the results of the earlier protein assays. Each volume contained 100 µg of protein. Additional aliquots containing 0.5 mg of protein were taken in duplicate for amino acid analysis. Aliquots for amino acid analysis were placed in 5 ml ampoules, while aliquots for gels were placed in 5 ml test tubes. All aliquots were lyophilized in the ampoules or test tubes. They were

then prepared for PAGE in SDS, and for amino acid analysis, as described in sections 2.1.4.1.3 and 2.1.1.4, respectively.

2.3.2.5.3 Amino Acid Analysis of Bands.

Duplicate hydrolyzates of each band were analyzed for amino acid composition by the single-column method as described in sections 2.1.1.4 and 2.3.2.2.4. The results of the two analyses were averaged to give the values reported below.

2.3.3 Results and Discussion.

2.3.3.1 Sequential Tissue Extraction.

Yields of the ATP/ascorbate (A), guanidine (G), guanidine/dithiothreitol (G/DTT), and NaOH extracts are shown in Table VIII. It should be mentioned that the yields of A-IV and A-V are not reversed on the table. A-V was obtained with a fresh solution of ATP/ascorbate, while the solution for washes A-I through A-IV had been mixed at the beginning of A-I, and had stood for about 3 hours prior to A-IV. This fact may have caused the yield of A-V to exceed that of A-IV. Results of hexuronic acid analysis of these extracts were given in section 2.1.2.3, Table II. Amino acid compositions of many of these whole extracts are given in Table IX.

Some of these results were discussed in section 2.1.2.4, the discussion of the three extraction experiments presented in Experimental Section I. As stated there, the yields of the various extractions were comparable to those in the SGP literature. Many of the extracts contained hexuronic acid or hydroxyproline (Hypro), indicative of acid mucopolysaccharides and collagen, respectively (15)(12).

Examination of Table IX shows several facts relevant to the objectives of Section III. First, extract G-I was seen to contain a small but detectable amount of 3-methylhistidine (3M-His), suggestive of the presence of actin. It is possible that other extracts also contained 3M-His, but the amount was below the detection limits of the analyzer. As seen in Figure 23 and in Table VIII, extract G-I had been preceded by nine "A" washes with ATP and ascorbate, the

TABLE VIII: YIELDS OF SEQUENTIAL EXTRACTIONS.

Extract	Yield (mg)	% of Dry Tissue
A-I	130	0.65
A-II	51	0.26
A-III	29	0.15
A-IV	6	0.03
A-V	30	0.15
A-VI thru IX	0	0
Total A	246	1.24
G-I	1995	9.98
G-II	415	2.08
G-III	120	0.60
Total G	2530	12.66
G/DTT-I	594	2.97
G/DTT-II	122	0.61
G/DTT-III	114	0.57
G/DTT-IV	39	0.20
Total G/DTT	869	4.35
NaOH-I	217	1.08
NaOH-II	294	2.47
Total NaOH	509	2.55
Total of all Extracts	4.2 grams	20.8%

Text section 2.3.3.1, page 214.

TABLE IX: AMINO ACID COMPOSITIONS OF SEQUENTIAL EXTRACTS (RESIDUES PER 1000 RESIDUES).

	G-I	G-III	G/DTT-I	G/DTT-IV	G/DTT-I V_o	NaOH-I	Actin[1]	Collagen[2]
Hypro[3]	3	27	26	66	19	79	0	92
Asp	93	81	91	68	82	63	91	48
Thr	57	45	56	39	52	29	65	17
Ser	66	55	58	48	63	39	66	33
Glu	130	114	117	94	114	86	116	72
Pro	59	74	75	93	76	102	51	129
Gly	93	163	152	245	151	289	77	334
Ala	82	84	73	74	76	91	80	105
½Cys	16	5	7	22	37	tr	12	0
Val	54	47	53	35	56	34	51	19
Met	21	18	16	10	9	8	37	7
Ile	42	34	36	26	38	21	77	11
Leu	82	69	70	46	68	38	75	25
Tyr	30	24	25	15	21	13	39	5
Phe	33	30	32	25	31	21	32	13
Hylys[3]	1	4	6	11	5	7	0	7
Lys	63	49	39	26	44	20	52	25
His	21	20	18	13	14	10	19	5
3M-His[3]	tr	0	0	0	0	0	2	0
Arg	56	57	52	45	47	47	50	48

[1]Bovine carotid (43)
[2]Bovine skin (32)
[3]Hypro: hydroxyproline. Hylys: hydroxylysine. 3M-His: 3-methyl histidine.
Text section 2.3.3.1, page 214.

last five of which contained no detectable protein. From the literature, one would have expected these nine extractions to have removed actin completely.

Also in Table IX it can be seen that all the extracts (G, G/DTT, and N) contained hydroxyproline, indicative of collagen (see the last column of the table). The amount of collagen, as measured by hydroxyproline, was seen to increase from left to right across the table, from earlier to later extracts. Extracts G/D-IV and N-I were largely collagen. Interestingly, the V_O, or Peak I, fraction from G/D-I contained relatively less collagen than did the whole extract, indicating that the large, undefined species making up this peak were principally composed of a protein unlike collagen Finally, it should be noted in Table IX that collagen has a very large proportion of glycine, and that those extracts showing high contents of hydroxyproline also show correspondingly high contents of glycine.

2.3.3.2 Results of Electrophoresis Experiments.

Polyacrylamide gel electrophoresis in SDS was used in this section to accomplish four objectives. First, each of the sequential aorta extracts was resolved into its component proteins and visualized as a series of bands on slabs of polyacrylamide gel. This allowed qualitative comparison of the constituents of the different extracts. Second, the relative amount of protein in each band was determined by densitometry of the stained gels, and the apparent molecular weight of each band was calculated from gels calibrated with standard proteins. Third, the bands were compared to known standard proteins placed in adjacent wells on the slab gels. Several of the most prominent bands were found to co-migrate with these standards. And fourth, the more prominent bands were isolated by preparative PAGE in SDS, and the amino acid compositions of these bands were determined.

2.3.3.2.1 Bands Seen in the Sequential Extracts.

The aorta homogenate was extracted sequentially with a series of solutions, as described in section 2.3.2.1 and as shown in Figure 23. Briefly reviewed, these extracts were:

1. Two pre-washes with NaCl, designated NaCl-I and -II.
2. Two pre-washes with $NaHCO_3$, designated $NaHCO_3$-I and -II.
3. Three pre-washes with EDTA, designated EDTA-I, -II, and -III.
4. Three pre-washes with acetone (discarded).

5. Nine pre-washes with ATP and ascorbate. Only the first five of these contained detectable protein by the method of Lowry et al (69). Designated A-I through A-IX.
6. Three extractions with 5 M guanidine, designated G-I, -II, and -III.
7. Four extractions with guanidine and dithiothreitol, designated G/DTT-I through -IV. These may also appear below as G/D-I etc. where space is limited, as in a table.
8. Two extractions with NaOH, designated NaOH-I and -II, or N-I and -II where space is limited.

Of these, only the last three extracts (G, G/DTT, and NaOH) were expected to contain material defined in the literature as being SGP. As defined in the literature, SGP is not soluble in any of the prewashes.

2.3.3.2.1.1 Bands Seen in Extracts Expected to Contain SGP.

Figure 25 shows a 5% PAGE slab in SDS on which each of the G, G/DTT, and NaOH extracts was fractionated. Especially noteworthy on this gel was the fact that the banding patterns of all the G and G/DTT extracts were strikingly similar. In many cases, the same band could be seen in all the extracts from G-I through G/DTT-IV. There was no marked difference between extracts obtained with guanidine alone and subsequent extracts obtained with dithiothreitol added to the guanidine. This finding strongly supported the conclusion that the same proteins were found, albeit in differing amounts, in all the G and G/DTT extracts.

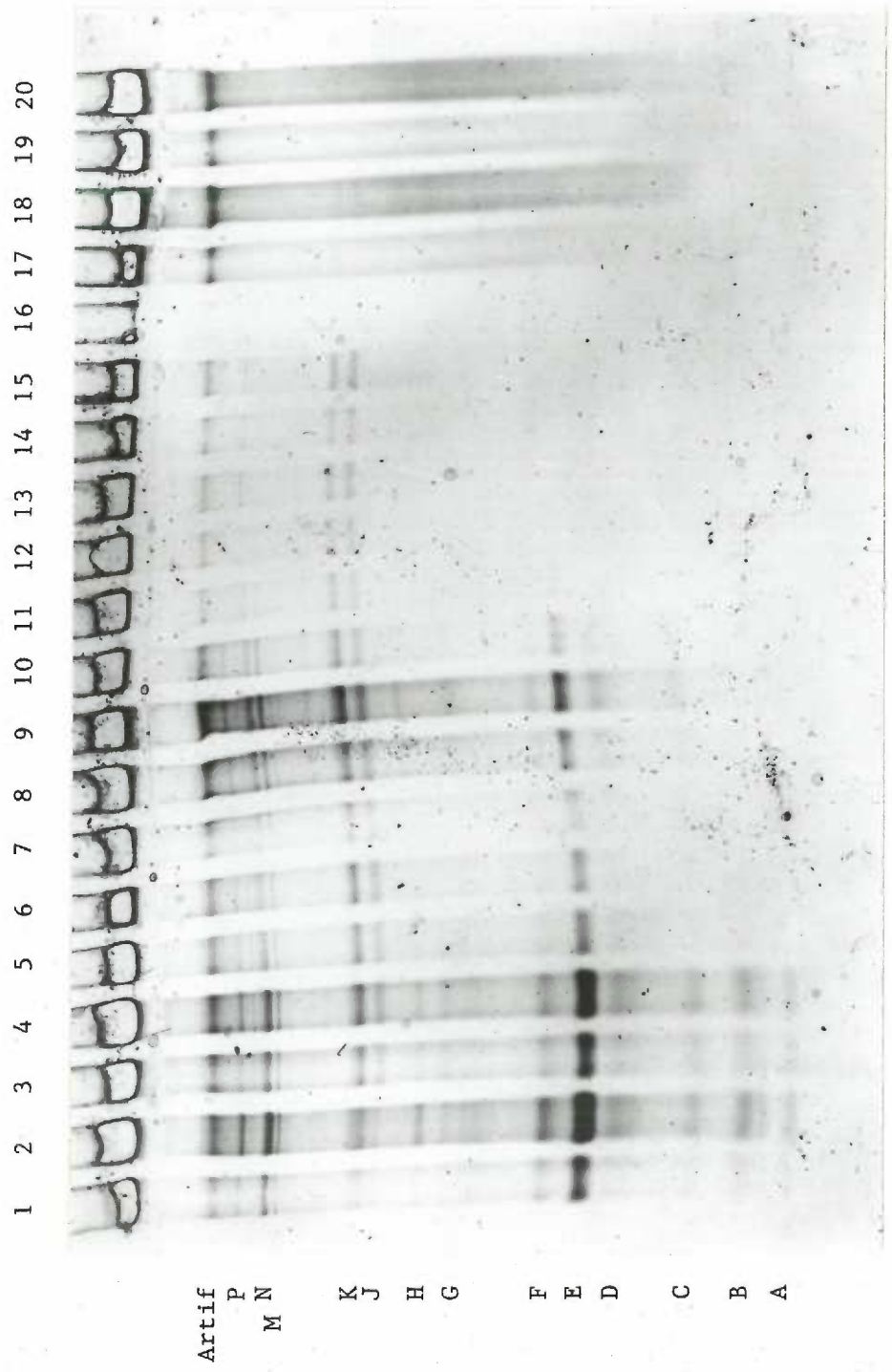
The second noteworthy feature of this gel was the absence of discrete bands in the two NaOH extracts. Identical smeared patterns

Figure 25. Electrophoresis of G, G/DTT, and NaOH Sequential Extracts.

5% polyacrylamide gel in 0.1% sodium dodecylsulfate. Bio-Rad slab apparatus. Samples were from sequential extraction (section 2.3.2.1, page 196, and Figure 23).

Well	Sample
1,2	G-I
3,4	G-II
5,6	G-III
7	Accidental loading
8,9	G/DTT-I
10,11	G/DTT-II
12,13	G/DTT-III
14,15	G/DTT-IV
16	Empty
17,18	NaOH-I
19,20	NaOH-II

Text section 2.3.3.2.1.1, page 219.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Artif
P
M N
K
J
H
G
F
E
D
C
B
A

were seen with or without 2-mercaptoethanol in the sample buffer, and in the presence of 8 M urea in addition to the SDS (gels not shown). In the historical section of this thesis, it was noted that many authors considered material extracted with 0.1 N NaOH to be degraded. These findings demonstrated the truth of their supposition. Since no discrete bands could be seen in the NaOH extracts, no comparison could be made on the gels between the components of these and of previous extracts.

The many bands seen in the various extracts were designated by capital letters as shown in Figure 25. Often, a group of partially-resolved bands was given a single letter to designate the group. Individual bands within the group were then numbered. For example, Band G was a group of closely-spaced bands which were labelled G1, G2, G3, and G4.

Not all bands within a group were present in all extracts. Bands C, D, E, and F were present in extracts G-I through G/DTT-II, while Bands J, K, and N were present in all the G and G/DTT extracts. Band R was an artifact of the slab gel system, and did not represent a discrete protein species (section 2.1.4.2.4).

2.3.3.2.1.2 Bands Seen in Pre-Washes Not Expected to Contain SGP.

Figure 26 is a composite photograph made up of selected sample wells from a single 6½% gel in SDS. This gel was loaded with the NaCl, $NaHCO_3$, and EDTA pre-washes. The last well shown in this figure contained a G/DTT extract for the sake of comparison. In wells 1 and 2, the NaCl washes were seen to contain many bands, as expected. However, few if any of these bands appeared to co-migrate with bands of the G/DTT extract. The $NaHCO_3$ washes, in wells 3 and 4, and the EDTA

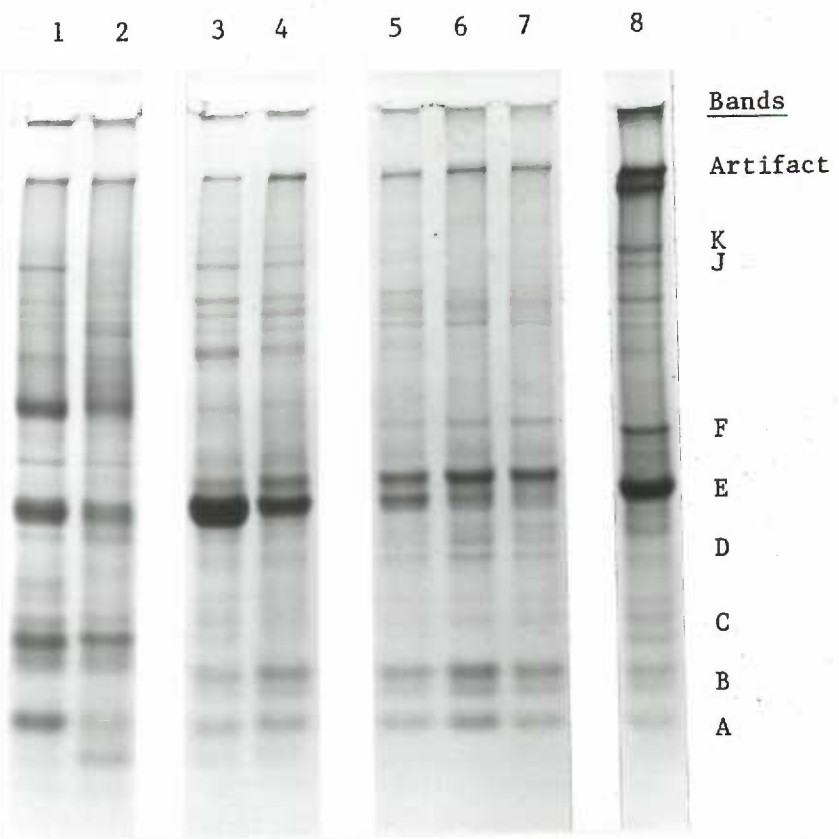


Figure 26. Electrophoresis of NaCl, $NaHCO_3$, and EDTA Pre-Washes.

6½% polyacrylamide gel in 0.1% sodium dodecylsulfate, using Bio-Rad apparatus. Composite photograph made up of wells from one gel. Samples were the various pre-washes as shown in Figure 23. Text section 2.3.3.2.1.2, page 222.

Well	Sample
1	NaCl-I
2	NaCl-II
3	$NaHCO_3$-I
4	$NaHCO_3$-II
5	EDTA-I
6	EDTA-II
7	EDTA-III
8	G-I

washes, in wells 5-7, had quite similar banding patterns. Several bands seen in these washes were also seen in the G/DTT extract (Bands A, B, C, D, and E). However, other bands (Bands F, H, J, and K) did not appear in the washes. These findings suggest that some of the components in the "SGP" extracts were proteins which were incompletely removed from the tissue by the pre-washes. Anderson (5) wrote of the process whereby serum proteins and other soluble proteins become entrapped within the connective tissue matrix, and are thereby inaccessible to mild buffers in which they are normally soluble. Bands A-D may have been examples of this process. However, it should be noted that most of the bands seen in the NaCl washes were not present in the later washes, indicating complete removal of those proteins from the tissue by NaCl.

Figure 27 is a composite photograph made up of three wells from a single 8% gel in SDS. The wells contained pre-washes A-I and A-V, and extract G-I. Bands A, B, C, D, E, and F were present in all three samples, but the banding pattern above Band F was entirely different between the A washes and the G-I extract. As noted just above, Bands A-E were also present in the $NaHCO_3$ and EDTA pre-washes. These observations indicated many components common to the pre-washes and the guanidine extracts. It should be remembered that the A pre-washes were intended to remove actin, according to the procedure of Carsten and Mommaerts (21). The pre-washes clearly removed species in addition to actin, as seen by the complexity of the banding pattern in Figure 27. If, as it was

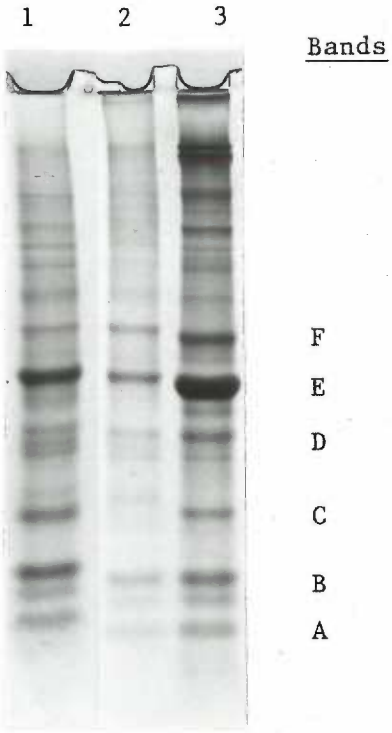


Figure 27. Electrophoresis of ATP/ascorbate Pre-Washes.

8% polyacrylamide gel in 0.1% sodium dodecylsulfate, using Bio-Rad apparatus. Composite photograph made up of wells from one gel. Samples were the various fractions shown in Figure 23. A-I was originally at the right edge of the slab, hence the bands are slightly curved at the right side. Bands A-E of all three samples co-migrated.

Well 1:	A-I
Well 2:	A-V
Well 3:	G-I

Text section 2.3.3.2.1.2, page 224.

suspected, Band E were actin, then the A pre-washes did not remove all of it from the tissue.

2.3.3.2.1.3 Summary of Bands Seen in the Sequential Extracts.

All the pre-washes and extracts contained complex mixtures of proteins. Some of the proteins were found only in the NaCl washes. Some proteins, denoted as Bands A, B, C, D, and E on the gels, were present in all the pre-washes and extracts from $NaHCO_3$-I through G/DTT-I. It was suggested that these bands represented soluble proteins entrapped by the connective tissue matrix. The NaOH extracts were extensively degraded and devoid of discrete bands.

2.3.3.2.2 Quantitation of Bands in the A Pre-Washes and in the G, G/DTT, and NaOH Extracts.

The purpose of these experiments was to determine the relative amount of protein represented by each band in the extracts expected to contain SGP. Since actin was found to be present in similar SGP preparations (section 2.2), it was decided also to quantitate the bands in the first ATP/ascorbate (A-I) pre-wash, which was expected to contain actin (43).

2.3.3.2.2.1 Verification of the Technique.

It is thought by some investigators (34) that bands of protein seen on gels stained with Coomassie Blue cannot be quantitated by scanning the gel in the manner described in section 2.3.2.4.1 above, because, it is asserted, the A_{580} of the dye bound to the protein is not a linear function of the amount of protein in the band. However, Hsieh and

Anderson (56) have shown that good results can be obtained within limited ranges of protein concentration. They stated that the results obtained are very strongly influenced by the techniques of gel staining and scanning, and of peak integration. Because of the many variables, it was necessary to verify that the technique as described in section 2.3.2.5.1 did in fact give reliable estimates of protein quantities before it could be used to determine bands in unknown samples. Data from several standard proteins were therefore plotted on a graph of peak area versus sample load. The results are shown in Figure 28.

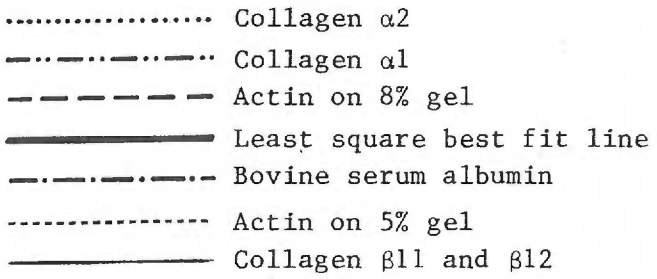
The relationship between sample load and peak area was very clearly linear. Not only was this true for any individual protein, but the different standards used (bovine serum albumin, actin, and collagen α1, α2, and β chains) all produced the same straight line, within experimental error, on either 5% or 8% gels. This was unexpected. It greatly simplified subsequent quantitation of bands on samples from aorta. A linear regression (least-squares) was used to determine the best straight line through all the 49 points. That line had a slope of 150.65 planimeter area units per microgram, a y-intercept of -35.5 area units, and a correlation coefficient (Pearson's r) of 0.9916. (A planimeter area unit was equal to 0.01 square inch.) The technique was entirely feasible. The linear regression line is shown by the heavy solid line on Figure 28. It was used to calculate all unknown bands.

2.3.3.2.2.2 Results of Band Quantitation of Aorta Extracts.

Bands in aorta extracts A-I, A-V, G-I, G-III, G/DTT-I, and G/DTT-IV were quantitated by the scanning technique. Figure 29 shows typical

Figure 28. Standard Curve for Quantitation of Bands on Gels Stained With Coomassie Blue.

Measured amounts of homogeneous standard proteins were applied to 5% and 8% polyacrylamide gels in 0.1% sodium dodecylsulfate. Bio-Rad slab apparatus was used exclusively. Stained gels were scanned as described in section 2.3.2.5.1, page 207. This Figure shows the relationship between the amount of protein applied to a sample well and the area of the peak obtained by scanning the gel. An area unit was 0.01 square inch.



Text section 2.3.3.2.2.1, page 227.

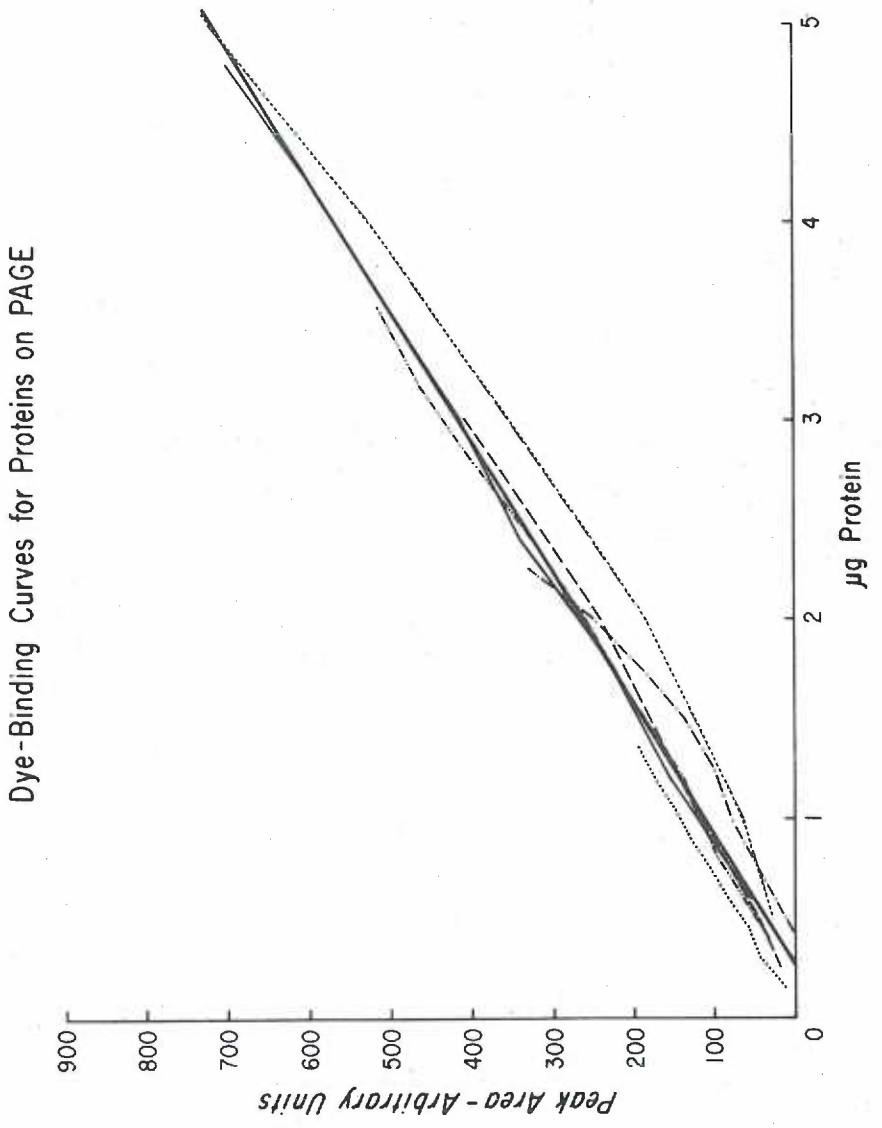

Dye-Binding Curves for Proteins on PAGE
Peak Area - Arbitrary Units
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5
µg Protein

Figure 29. Densitometer Tracings of Electrophoresis Runs From Extracts G-I and G/DTT-I, for Quantitation of Bands.

Representative chart recorder tracings of stained gels scanned as described in section 2.3.2.5.1, page 207.

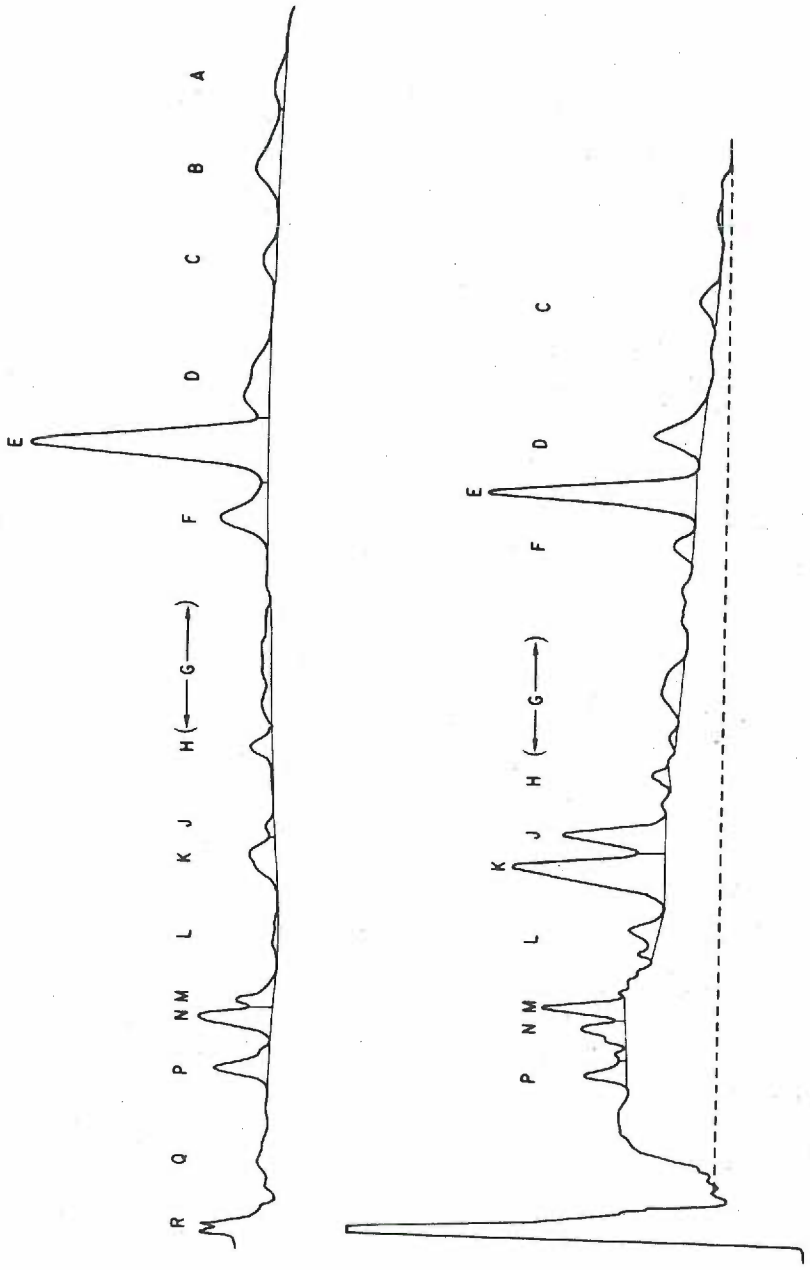
Upper trace: G-I

Lower trace: G/DTT-I

Gels scanned were shown in Figure 16, wells 1 and 2. Peak letters correspond to letters of bands in Figure 16. Solid lines under the peaks are the "local" baseline for determination of peak area. Dashed line is the absorbance of a blank piece of gel.

Band R: Top of Gel

Text section 2.3.3.2.2.2, page 227.


A
B
C
D
E
F
G
H
J
K
L
M
N
P
Q
R
E
D
C
F
G
H
J
K
L
P
N
M

scans used for these determinations. Several features of these scans should be noted. First, many closely-spaced bands were not completely resolved. Bands B, C, D, and G were seen to be multiple bands on the photographs (c.f. Figures 25, 26, and 27). Nevertheless, because the absorbance did not return to baseline between these incompletely-resolved bands, the groups of bands were quantitated as one band.

Second, the baseline used to determine the base of a peak for purposes of integration was the "local" baseline rather than the absorbance of a blank piece of gel. This was of little consequence in scans of A and G extracts, but became very significant in the scans of G/DTT-I and IV. Figure 29 shows local baselines as solid lines under the peaks, and the baseline value of a blank gel as a dashed line. The area between the two lines is a rough estimate of the amount of diffuse "background" staining of the gel. In later extracts, this background made up a major portion of the stainable material. Indeed, in the NaOH extracts, it made up the entire sample. It was taken to represent degraded polypeptide fragments. An attempt was made to estimate the amount of this material, but the data were contradictory and inconclusive. They are not presented in this thesis.

A third feature of the scans is that Band "R" in Figure 29 represents the upper end of the gel. It was not quantitated.

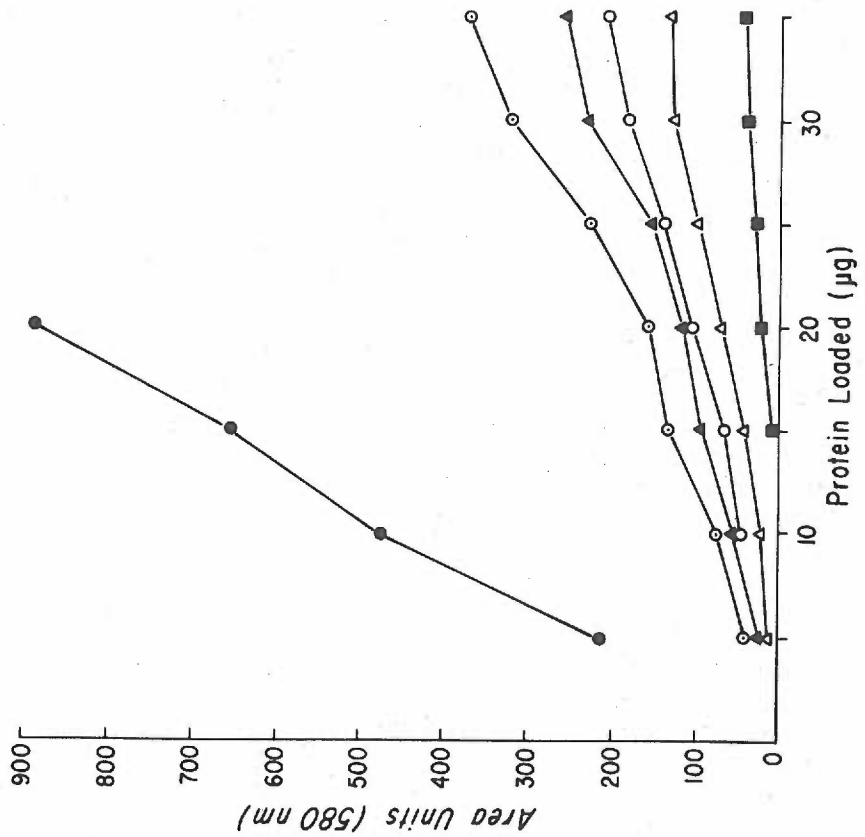
Figure 30 shows plots of peak area versus sample load for several bands in extracts G-I. Plots similar to this were constructed for all bands of all the extracts in which bands were quantitated. The linear relationship between peak area and sample size is apparent. However, it

Figure 30. Plot of Peak Area Versus Total Protein for Sequential Extract G-I.

The area of each peak on scans such as that shown in Figure 29 was measured for a series of samples ranging from 5 to 35 µg of total protein per well on the gel. For each band, the area of the corresponding peak was plotted as a function of sample load. A series of those plots is shown here.

Symbol	Band
Solid circles	E
Open circles with dot	N
Solid triangles	P
Open circles	K
Open triangles	A
Solid squares	J

Text section 2.3.3.2.2.2, page 232.


Area Units (580 nm)
900
800
700
600
500
400
300
200
100
0
10
20
30
Protein Loaded (µg)

was noted that the y-intercepts for individual bands varied. For this reason, it was decided that the slope of the standard curve would be used in conjuction with the y-intercept of each individual unknown curve in determining the quantity of protein in each band. In essence, this meant that the figure expressing the amount of protein in a given band was proportional to the slope of the line given by that band on the area-vs-load plot.

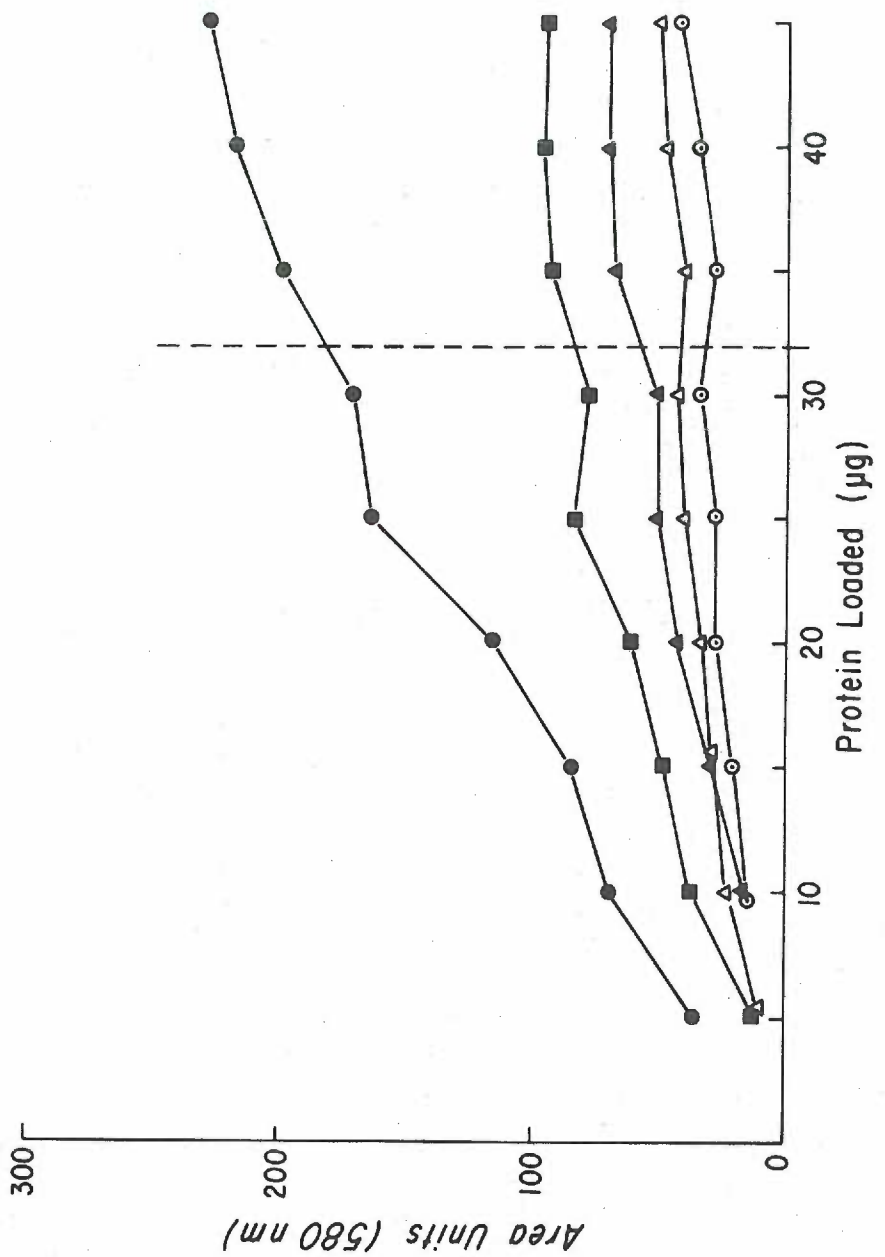
Figure 31 shows peak areas versus sample size for several bands of extract G/DTT-I. When compared to Figure 30, the most striking additional feature of this plot is the decline in the slope of the curves at higher sample loads. Earlier (section 2.1.4) it was seen that this extract contained a large fraction of material which did not penetrate even a 3% gel in SDS. These observations led to the hypothesis that an accumulation of very large molecules on the floor of the sample well made it impossible for smaller molecules to enter the gel. This phenomenon would be more pronounced at higher sample loads in which more and more of the gel pores would be clogged. It would be seen as a decrease in the amount of each band relative to the total amount of protein applied to the gel. With the assumption that this was occurring at the higher loadings of G/DTT-I, resulting in the observed departure from linearity, only data from samples of 30 μg or less were used in quantitating the bands in G/DTT-I. This phenomenon was even more pronounced with extract G/DTT-IV (plot not shown). Consequently, the slope at the lowest sample loading was used to quantitate the bands in this extract only.

Figure 31. Plot of Peak Area Versus Total Protein for Sequential Extract G/DTT-I.

The plot is as described in Figure 30.

Symbol	Band
Solid circles	E
Solid squares	J
Solid triangles	D
Open triangles	M
Open circles with dot	N

Dashed vertical line represents upper limit of data used for calculation of the bands. Note that the plots decrease in slope above this point. Text section 2.3.3.2.2.2, page 235.


Area Units (580 nm)
300
200
100
0
10
20
30
40
Protein Loaded (µg)

Extract A-V contained an extremely small amount of protein (see Table VIII). Consequently, the samples applied to the gel were dilute and the bands from them were weak. This lead to lower correlation coefficients for most of the bands in this extract (Table X).

Table X shows the results of the quantitation of bands in the aorta extracts. The first number of each entry is the percentage of that band of the total bands in that extract. The second entry is the correlation coefficient (Pearson's r) of the line used in computing the value. Since there was a variable amount of background staining on the gels, which was not included in these quantitations (see Figure 29), the values in Table X represent maximum possible percentages of each band in the whole extract. Most correlation coefficients exceeded 0.9 (perfect correlation gives a coefficient of 1.0), and those for the principle bands (B, D, E, J, K, M, and N) exceeded 0.95 except in extracts A-V and G/DTT-IV, for the reasons described just above. From these coefficients it can be seen that most of the band quantitation data were of excellent quality.

Figure 32 shows the quantity of each band in milligrams of protein per gram of dry tissue. These figures were obtained from the data in Tables VIII and X by multiplying the yield of each extract by the percentatage of each band in that extract. From this figure it can be seen that extract G-I, the first guanidine extract, contained the largest amount of protein of any of the extracts. It can also be seen that more Band E, co-migrating with actin, was found in G-I than in all the ATP/ascorbate extracts combined (A-I through A-V). This was in

TABLE X: PERCENTAGE OF EACH BAND IN EACH EXTRACT. SECOND ENTRY IN EACH SPACE IS CORRELATION COEFFICIENT OF LINE USED TO CALCULATE THE PERCENTAGE FIGURE (SEE FIGURES 30 AND 31).

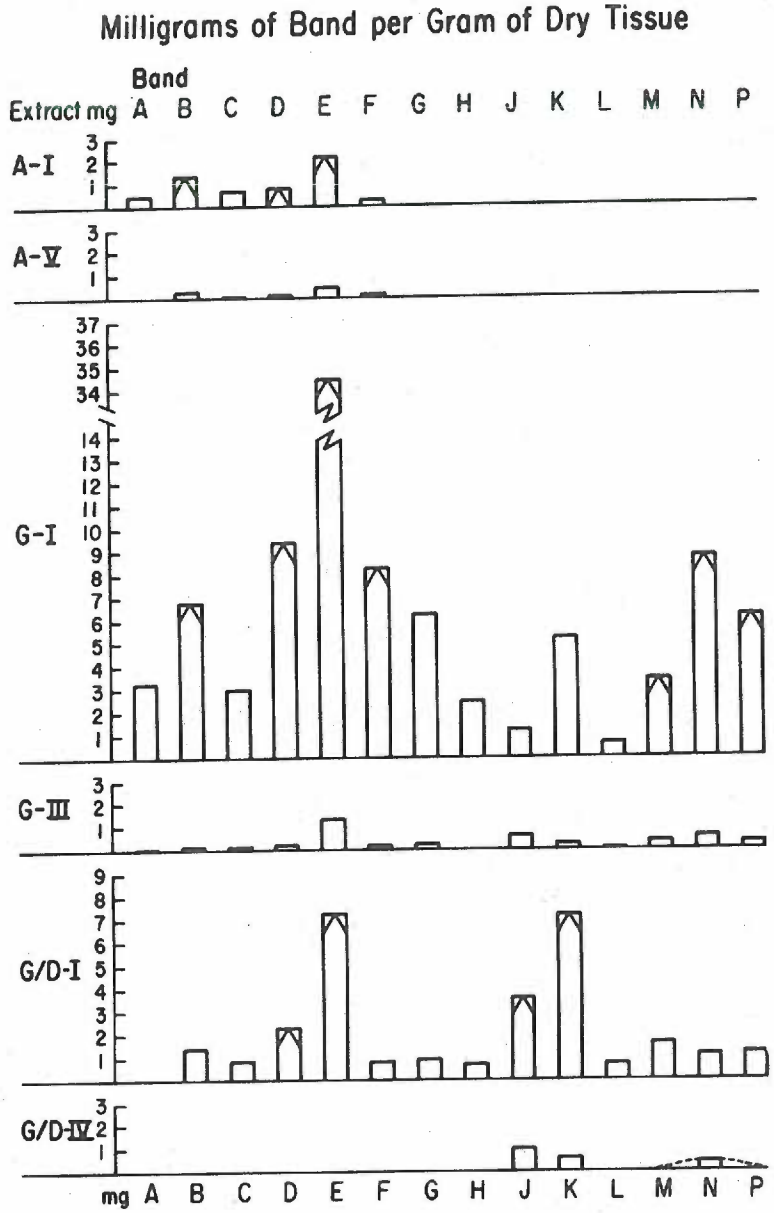
Band	A-I	A-V	G-I	G-III	G/DTT-I	G/DTT-IV
A	6.44 .995	7.24 .880	3.33 .967	.98 .939	0	0
B	21.18 .995	19.26 .815	6.84 .995	3.74 .973	4.82 .995	0
C	11.69 .987	7.32 i.d.[1]	3.06 .974	3.59 .974	3.13 .918	0
D	12.02 .979	14.05 .942	9.44 .991	4.08 .986	7.77 .959	0
E	34.16 .990	30.22 .985	34.58 .998	23.07 .997	24.31 .987	0
F	3.76 .975	13.08 .988	8.36 .991	2.97 .979	2.70 .990	0
G	0	0	6.28 .996	3.94 .834	3.13 .584	3.9 i.d.
H	0	0	2.45 .988	1.46 .992	2.19 .748	0
J	0	0	1.29 .981	11.30 .992	12.05 .963	49.0 i.d.
K	0	0	5.28 .996	18.72 .991	24.10 .987	27.5 i.d.
L	0	0	0.65 .718	2.30 .702	2.24 .900	0
M	0	0	3.47 .990	6.22 .962	5.53 .979	
N	0	0	8.77 .986	9.87 .985	3.85 .972	20.6 i.d.
P	0	0	6.19 .987	5.02 .976	4.18 .985	

[1]insufficient data

Text section 2.3.3.2.2.2, page 238.

Figure 32. Histogram Showing the Amount of Protein Represented by Each Band in the Intact Aorta Tissue.

This chart was derived from Tables VIII and X by multiplying the amount of total protein in each extract by the precentage of each band in the whole extract. The bars represent the amount of each band per gram of dry tissue. Note that the milligram scale for G-I is broken in order to show the large amount of Band E in that extract. Band lettering corresponds to Figure 16. Bands A, B, C, D, and G are not single species (see Figures 25 and 27). Bar for Band N in extract G/DTT-IV represents sum of five bands in the region of Bands M through P (Figure 16). Bars marked at top with diagonal lines represent bands isolated by preparative electrophoresis for amino acid analysis in the next section. Text section 2.3.3.2.2.2, page 238.


Milligrams of Band per Gram of Dry Tissue
Band
Extract mg A B C D E F G H J K L M N P
A-I
A-V
G-I
G-III
G/D-I
G/D-IV
mg A B C D E F G H J K L M N P

spite of the facts that extraction with ATP and ascorbate is a recognized method for removing actin from the arterial wall (43), and that the last four such extracts, prior to the G-I extraction, were devoid of protein as measured by the method of Lowry et at (section) 2.1.1.1). The predominance of Band E in the extracts is even more striking in view of the fact that Bands B, C, D, and G were not single species as illustrated in Figure 32, but were made up of several bands not completely resolved on the gel. Band E in all the extracts was a single band.

2.3.3.2.3 Molecular Weights of Bands.

The apparent molecular weights of the bands in all the sequential extracts were determined as described in section 2.1.4.1.4. Some of these results were given earlier in Table VI. They are all presented together in Table XI. The molecular weight of Band E, the most prominent band in the A, G, and G/DTT extracts, was 42-43,000 d. This is consistent with the conclusion of Experimental Section II that Band E, or Peak II, is actin.

As mentioned earlier (section 2.1.4.2.8), Bands F, H, J, and K migrated anomalously on the gels, leading to different apparent molecular weights on 5% and 8% gels. Although the nature of Bands F and H are still unknown, Bands J and K appeared to be collagen α chains, as described in the next section. It is known (40) that collagen migrates anomalously on gels in SDS. This was confirmed by the gel shown in Figure 34, which gave apparent molecular weights for standard collagen α1 and α2 chains of 116,000 d and 130,000d, respectively.

TABLE XI: MOLECULAR WEIGHT (MWT) OF BANDS FROM SEQUENTIAL EXTRACTS. UNITS ARE DALTONS x 10^{-3}

Band	NaCl	$NaHCO_3$	EDTA	ATP/asc	G-I	G/D-I	G/D-IV	Mean
Pre-A[6]	(14)[1]	14						14
A	(16)	16	16	16	16			16
B1		17	17	17	17			17
B2	many	18	18	18	18			18
C1		21						21
C2		23	22		24	24		23
C3		25		25				25
D1		29	29	30	30			30
D2	(32)	33	32	33	33	34		33
E1		37[4]			37			37
E2			38					38
E3				42	42			42
E4			43	43		43		43
E5		44						44
F(5%)[5]				53	50	50		51
F(8%)[5]			61	59	57	57		59
G1[3]	(66)				75			75
G2					89			89
G3		(96)			95			95
G4		(107)	(105)		102			102
H(5%)[5]					94	95		94
H(8%)[5]					115	117		116
I[2]					128			128
J		(116)	(112)			115	116	116
K1		(126)	(129)		124	125		124
K2					128	131	130	130
M					188	189		188
N					200	201		200
P[6]					225	230		228

[1]Bands in parenthesis did not co-migrate with bands in other extracts.
[2]Band I seen only in 8% gels.
[3]Bovine serum albumin in NaCl extract only.
[4]Underline denotes predominant E band. Others very faint.
[5]Only one band. Apparent MWT varied between 5% and 8 % gels.
[6]Pre-A and P were outside calibration limits of the gels.
Text section 2.3.3.2.3, page 242.

The actual molecular weight of both is 95,000 d (40)(41). The molecular weights of bands migrating ahead of hemoglobin (16,000d) or behind myosin (200,000 d) were outside of the calibration range of the gels. Therefore, the weights of Bands Pre-A and P were estimates based on extrapolations only.

2.3.3.2.4 Identification of Bands by Co-Migration with Standards.

Several of the bands seen in the various pre-washes and extracts were found to co-migrate with standards of known proteins. This section describes the tentative identification of those bands as indicated by their co-migration with the standards.

Bovine serum albumin was from Sigma. Human hemoglobin was a gift of R. T. Jones. Actin was prepared from bovine skeletal muscle by the method of Carsten and Mommaerts (21) as described in section 2.2.3.1. Myosin was prepared from rabbit skeletal muscle by I. Gipson and R. Anderson according to the method of Perry (89). As described in section 2.2.4.1, this preparation was used by Gipson and Anderson to prepare heavy meromyosin subfragment 1, which in turn was used to "decorate" actin filaments as described by Nachmias et al (83). Pollard (91) has stated that this test is perhaps the most specific of all known criteria for the identification of myosin or actin. There was no doubt that this preparation was indeed myosin. Acid-soluble collagen was prepared from rat skin by B. Rokosova. It contained hydroxyproline in the amount expected for pure collagen (32), and displayed the characteristic pattern of alpha, beta, and gamma

chains described by Furthmayr and Timpl (40) when fractionated by PAGE in SDS.

Figure 33 shows the NaCl-I pre-wash on an 8% PAGE slab in SDS. Neighboring wells contained hemoglobin, actin, serum albumin, and whole blood. Band A, which did not co-migrate with the Band A of later washes and extracts (Figure 26), was found to co-migrate with hemoglobin alpha and beta chains. The same band was prominent in whole blood. Band E in the NaCl pre-washes did not co-migrate with actin, but instead migrated with an apparent molecular weight of 37,000 d. Band G, which did not co-migrate with the Band G of later washes and extracts (Figure 26), co-migrated with bovine serum albumin and also with a prominent band in bovine whole blood. Tentatively, Bands A and G in the NaCl pre-washes were hemoglobin and serum albumin, respectively. Since the NaCl pre-washes did not contain SGP as SGP is defined in the literature, nothing more was done to identify the bands in these washes.

Figure 34 shows standard collagen, myosin, and actin on a 5% gel slab in SDS, adjacent to extracts A-I, G-I, G/DTT-I, G/DTT-IV, and N-I. As expected from Experimental Section II, Band E co-migrated with actin. The small variations of mobility seen for Band E in this figure were not considered significant, since they were not seen on other gels (see Figures 25 and 27, for example). Also on Figure 34, Band J co-migrated with collagen $\alpha 2$ chains, Band K co-migrated with collagen $\alpha 1$ chains, and Band N co-migrated with either collagen $\beta 12$ chains or myosin. (This myosin band represents the heavy chain rather than the

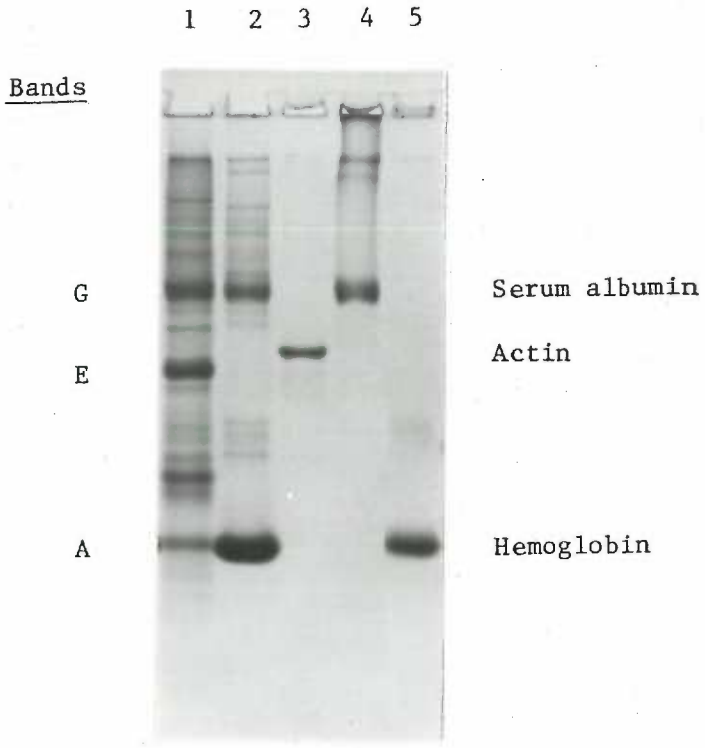


Figure 33. Identification of Bands in NaCl Pre-Washes.

Electrophoresis in 8% polyacrylamide gel in 0.1% sodium dodecylsulfate using Bio-Rad apparatus. Composite photograph made up of selected wells from a single gel.

Well	Sample
1	NaCl-I (See Figure 23)
2	Whole bovine blood
3	Actin standard
4	Bovine serum albumin standard
5	Human hemoglobin (subunits) standard

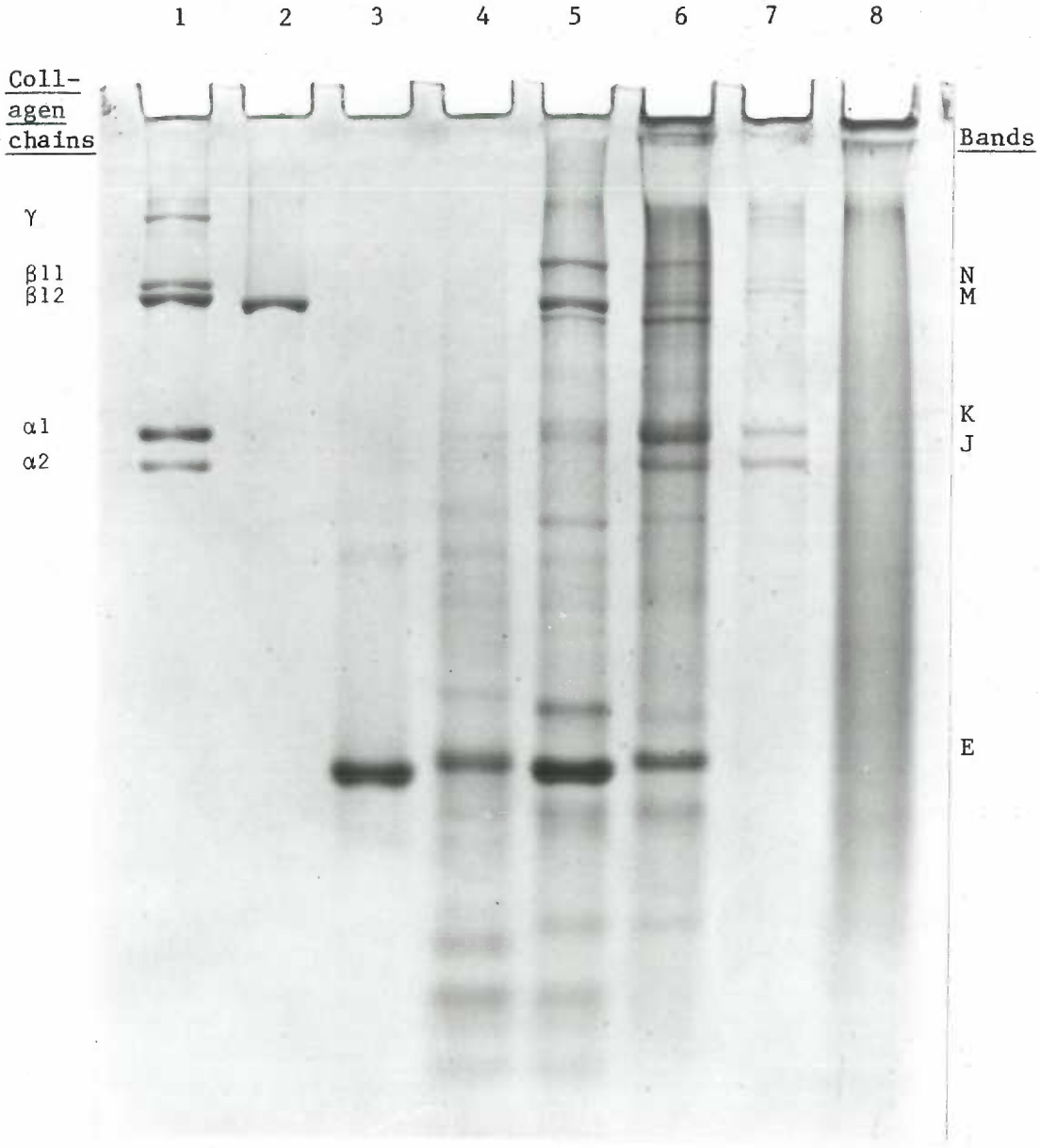
Text section 2.3.3.2.4, page 245.

Figure 34. Identification of Bands in A, G, and G/DTT Extracts.

Electrophoresis in 5% polyacrylamide gel in 0.1% sodium dodecylsulfate using Bio-Rad apparatus, showing co-migration of bands in A, G, and G/DTT extracts with known standard proteins. Extracts were obtained as shown in Figure 23. Photo from a single gel.

Well	Sample
1	Collagen α, β, and γ chains
2	Myosin heavy chain
3	Actin
4	A-I
5	G-I
6	G/DTT-I
7	G/DTT-IV
8	NaOH-I

Text section 2.3.3.2.4, page 245.


1
2
3
4
5
6
7
8
Coll-
agen
chains
Bands
γ
β11
β12
α1
α2
N
M
K
J
E

entire myosin molecule, which dissociates in SDS and 2-mercaptoethanol (91)). Band M, migrating just ahead of collagen β12, was also thought to be collagen, migrating slightly ahead of rat skin collagen due to species and tissue differences (41).

Two observations from gels not photographed tended to confirm the identity of Bands J, K, M, and N as collagen and/or myosin. Samples of extracts G-I and G/DTT-I were digested with collagenase as described in section 2.1.2.2.1. The digested samples were dialyzed extensively against water, lyophilized, and compared to undigested samples, and to a sample of the enzyme itself, on a 5% gel. Bands J, K, and M (migrating near collagen α1, α2, and β12 chains, respectively) had disappeared, while the remaining bands were unchanged. However, several new bands had appeared in the region between Bands J and M. They did not correspond to the bands in the collagenase itself. Their nature was not determined. These observations suggested that Bands J, K, and M were collagen, in keeping with their co-migration with known collagen. A second indication that these bands were collagen came from an attempt to fix the protein bands in the 3% polyacrylamide - 0.5% agarose composite gel described in section 2.1.4.2.6. The gel was soaked in 5% trichoroacetic acid (TCA) prior to staining and destaining. The destained gel showed all bands except the collagen standard and Bands J, K, and M of the extracts. Band N, corresponding to standard myosin or to β12, was unaltered. In section 2.3.1 it was noted that, unlike most other proteins, collagen is soluble in TCA, and that extraction with TCA has been used by several investigators to remove

collagen from connective tissue prior to extraction of SGP. Therefore it is reasonable that collagen would be removed from polyacrylamide gels, rather than fixed to them, by 5% TCA. Band N, migrating with both collagen β12 and myosin, had not been removed from the gels after collagenase or TCA. This suggested that at least some of it was not collagen and therefore presumably was myosin. The identity of Band N was unknown, pending amino acid analysis.

To summarize, the tentative identification of bands was:

Band E	Actin
Band J	Collagen α
Band K	Collagen α
Band M	Collagen β
Band N	Myosin, with some collagen β

From Table X, these bands made up a total 52% of the G-I extract and 70% of the G/DTT-I extract.

2.3.3.2.5 Results of Preparative Gels and Amino Acid Analysis of Isolated Bands.

Although the major components of the aorta extracts were seen in the previous section to co-migrate with known standard proteins, it was necessary to show by another method that the bands in the extracts were the proteins they appeared to be. The other method chosen for this comparison was amino acid analysis. This is not to suggest that the combination of co-migration on gels in SDS and a similar amino acid composition is sufficient evidence to prove conclusively that two proteins are identical. *Such proof was not the purpose of Experimental Section III. The purpose of these experiments was to provide strong evidence against the conclusion in the literature that certain fractions of mammalian aorta, obtained by extracting the tissue with denaturing and reducing agents, represented SGP, a new component of connective tissue.* If the major components of those extracts could be shown to co-migrate with standard proteins, and to have amino acid compositions similar to the compositions of the same standard proteins, then the evidence would heavily favor the conclusion that these proteins were not SGP, but rather were collagen or actin or myosin. If the amino acid compositions agreed with the data from the analytical gels, then the objective of this section would be fulfilled.

2.3.3.2.5.1 Developing the Technique for Recovery of Bands From the Gel.

Initially, it was attempted to recover a band from a stained gel. However, trials with bovine serum albumin (BSA) showed this to be difficult, if not impossible. The dye and the protein were insepar-

able except by extended dialyses against 6 M urea in 25% pyridine. Recovery of protein after this treatment was essentially zero. The technique was discarded.

In the pursuit of a quick method of obtaining amino acid analyses from proteins in polyacrylamide gel, a section of gel containing BSA was placed directly in HCl and hydrolyzed as described in section 2.1.1.4. The chromatogram showed extensive, sometimes total, destruction of many amino acids. This technique was also discarded.

A section of unstained gel containing BSA was extracted as described in section 2.3.2.5.2.2, except that the isopropanol was not used and only three extractions of the gel were done. Analysis of the extracts by the method of Lowry et al (section 2.1.1.1) showed a 66% recovery of the starting load. This was considered adequate.

Since unstained portions of preparative gel were stored at $4^{o}C$ for about fifteen hours during the staining and destaining of the marker strips, it was necessary to determine how far a protein band diffused during storage of the unfixed, unstained gel. Sections of gel containing Band C from an actual preparative run were stained at the time the gel was sectioned according to the stained marker strips. The test section showed very little band broadening. Diffusion was therefore found not to be a problem with the technique as it was employed in these experiments.

A minor problem was caused by the swelling of the stained strips during staining and destaining in 10% HAC, 5% isopropanol. The destained strips were longer than the unstained gel. A change to 14% isopropanol in 10% HAC solved this problem. However, the bands lost

all stain if left in this solution longer than ten hours.

As described under Materials and Methods, the preparative gel technique reflects the findings of this section.

2.3.3.2.5.2 Choice of Bands to Be Isolated.

Ideally, the goal was to isolate every band making up more than 5% of any extract which, according to the literature, should contain SGP. However, this ideal was modified by the fact that some extracts, such as G/DTT-IV, contained very small amounts of material (see Figure 32). A second goal was to verify that the bands in the ATP/ascorbate extracts which also appeared in the guanidine extracts had the same composition from both extracts. Finally the high "background" in extract G/DTT-I, especially at higher molecular weights, made isolation of Bands M and N from this extract unrealistic. Considering all these factors, it was decided that the following bands would be isolated:

From A-I, Bands B, D, and E.

From G-I, Bands B, D, E, F, M, N, and P.

From G/DTT-I, Bands D, E, J, and K.

These bands are indicated in Figure 32 by small triangular figures at the tops of the appropriate bars.

2.3.3.2.5.3 Recovery of Bands.

Bands from A-I were isolated from 8% gels. Bands from G-I and G/DTT-I were isolated from 5% gels. All bands were extracted from the gels as described in section 2.3.2.5.2.2. Assay of the gel extracts

for protein by the method of Lowry showed recoveries ranging from 50% to 172% of the expected recovery, based on the amount of each band in the aorta extract and the earlier recovery of BSA from trial gels. During slicing of the preparative gels it was found to be impossible to section the slabs accurately enough to separate Bands M and N. They were therefore isolated together.

2.3.3.2.5.4 Purity of Isolated Bands.

In order to verify that the bands had been isolated free from adjacent bands, portions of each recovered band were placed on an analytical slab. This slab is shown in Figure 35. Most of the isolated bands were pure. Bands E showed dimers and trimers on this gel, which was done without 2-mercaptoethanol. Bands D and F from G-I contained an appreciable amount of E. Band P from G-I also contained an appreciable amount of N. Bands J and K from G/DTT-I were not completely separated from each other. It was not as clean a separation as desired, but the results were usable. Band E, the most important band in this study, was free of neighboring bands in all three preparations.

2.3.3.2.5.5 Amino Acid Analysis of Isolated Bands.

Results of amino acid analysis of each isolated band are shown in Table XII.

2.3.3.2.5.5.1 Comparison of Co-migrating Unknown Bands to One Another.

Before the discussion of individual compositions, one must consider for a moment the validity of comparing amino acid compositions.

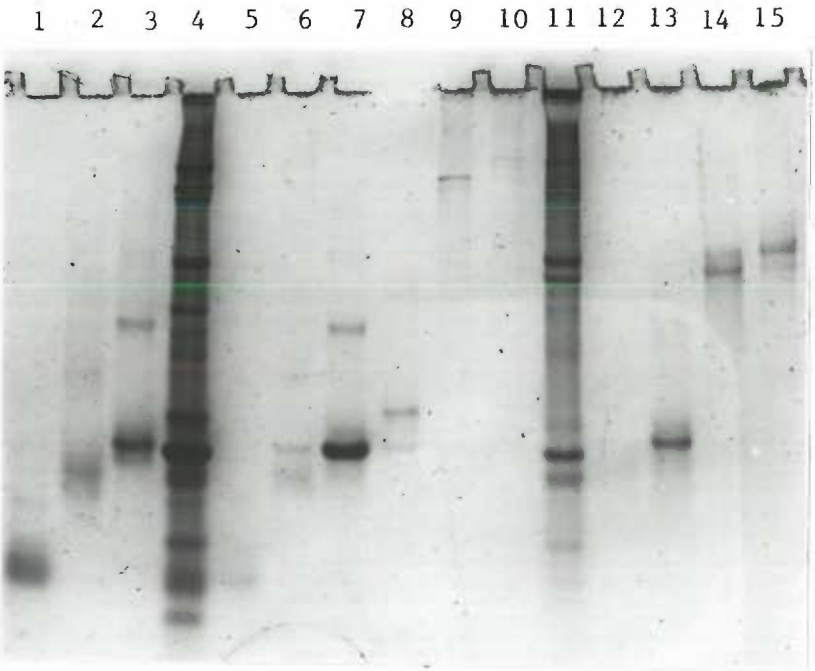


Figure 35. Bands Isolated by Preparative Polyacrylamide Gel Electrophoresis.

Bands isolated by preparative electrophoresis were returned to an analytical slab to verify purity. 5% polyacrylamide gel in 0.1% sodium dodecylsulfate. Bio-Rad apparatus.

Well	Sample
1	Extract A-I, Band B
2	A-I D
3	A-I E
4	Extract G-I before fractionation
5	Extract G-I, Band B
6	G-I D
7	G-I E
8	G-I F
9	G-I M+N
10	G-I P
11	Extract G/DTT-I before fractionation
12	Extract G/DTT-I, Band D
13	G/DTT-I E
14	G/DTT-I J
15	G/DTT-I K

Text section 2.3.3.2.5.4, page 254.

TABLE XII: AMINO ACID COMPOSITION OF ISOLATED BANDS (RESIDUES PER 1000 RESIDUES).

	Extract A-I			Extract G-I						Standards	
	Band B	D	E	B	D	E	F	MN	P	Actin[1]	Myosin[3]
Hypro[4]	--[5]	--	--	--	--	--	--	tr	tr	--	--
Asp	93	102	100	88	96	95	99	94	92	91	103
Thr	62	52	64	59	55	64	55	55	61	65	46
Ser	62	79	70	72	77	73	98	67	69	66	44
Glu	128	127	139	126	120	120	150	158	127	116	213
Pro	53	49	52	46	61	60	51	58	76	51	17
Gly	74	81	71	83	90	76	86	92	112	77	39
Ala	93	95	84	93	81	80	80	82	79	80	86
½Cys	tr	7	4	11	16	15	5	9	16	12	10
Val	52	45	48	56	54	46	50	55	70	51	45
Met	20	17	30	15	22	36	18	14	11	37	27
Ile	39	35	53	41	41	56	38	33	37	77	38
Leu	80	95	88	79	80	79	83	87	72	75	108
Tyr	21	35	33	23	34	39	29	20	25	39	16
Phe	40	42	32	34	39	34	30	28	28	32	33
Hylys[4]	--	--	--	--	--	--	tr	1	2	0	0
Lys	88	72	56	83	60	55	41	67	55	52	99
His	25	18	20	23	22	21	28	22	22	19	14
3M-His[4]	--	tr	1.4	--	--	2.2	--	--	--	2.4	tr
Arg	71	50	57	68	51	52	60	58	48	50	57

TABLE XII: AMINO ACID COMPOSITION OF ISOLATED BANDS (RESIDUES PER 1000 RESIDUES)(Continued).

	Extract G/DTT-I				Standards	
	D	E	J	K	Actin[1]	Collagen[2]
Hypro[4]	--[5]	tr	52	44	--	92
Asp	104	99	81	76	91	48
Thr	57	57	42	38	65	17
Ser	71	71	54	65	66	33
Glu	120	124	106	107	116	72
Pro	54	65	79	86	51	129
Gly	100	110	208	189	77	334
Ala	73	76	76	77	80	105
½Cys	32	34	23	23	12	0
Val	51	52	38	43	51	19
Met	18	10	10	11	37	7
Ile	42	45	30	31	77	11
Leu	84	76	55	59	75	25
Tyr	35	31	16	18	39	5
Phe	43	36	24	28	32	13
Hylys[4]	4	4	8	9	--	7
Lys	46	44	33	30	52	25
His	15	14	11	13	19	5
3M-His[4]	--	tr	--	--	2.4	--
Arg	53	54	53	55	50	48

[1]Bovine carotid artery (43)
[2]Bovine skin (32)
[3]Bovine carotid artery (66)
[4]Hypro: hydroxyproline. Hylys: hydroxylysine. 3M-His: 3-methylhistidine.
[5]Dash indicates value not determined
Text section 2.3.3.2.5.5, page 254

Anderson (5) has written a concise summary of the problem. He describes a test of homology between two proteins which involved summing the squared differences between residues when the compositions were expressed as residues per 100 residues. A sum of less than 100 was said to indicate homology. This test has been used to indicate homology between SGP preparations. However, as pointed out by Anderson, the test also indicated homology between calf tendon SGP and bovine serum albumin (BSA). It is reasonable to conclude that statistical comparisons of overall amino acid compositions lead to results of questionable significance. Similar amino acid compositions, in themselves, do not necessarily imply homology. However, widely differing compositions are reliable evidence of the lack of homology. Anderson also points out that amino acid compositions are a valuable criterion of purity for collagen or elastin because of the highly atypical amino acid profiles of these proteins. Because of the presence of 3-methylhistidine in actin, and because of the very great number of glutamic acid residues in myosin, these two proteins can also be validly compared to others by amino acid composition.

Turning now to Table XII, it can be seen that Bands B from A-I and G-I are superficially similar. As shown above, this does not prove homology, but neither does it rule it out. Similar conclusions can be drawn about Bands D from A-I, G-I, and G/DTT-I. Band E from these same three extracts shows similar composition, except that from G/DTT-I it contains a relative excess of glycine. As stated earlier, this extract contained a large quantity of "background" material,

some of which extended into the region of Band E on the gel (see Figure 29). If this material were fragments of collagen or elastin, its presence along with Band E would be expected to increase the glycine content. A trace of hydroxyproline was present in Band E from G/DTT-I, also suggestive of collagen.

2.3.3.2.5.5.2 Comparison of Band E to Actin.

As measured by the presence of 3-methylhistidine, actin was present in Band E from all three extracts. In the case of the G/DTT-I band, the exact amount of it was difficult to determine because of the presence of hydroxylysine, which emerged from the column just after 3-methylhistidine (see Figure 4). The lesser amount of 3-methylhistidine in Band E from A-I was a reproducible finding. Laki (66) has stated that the 3-methylhistidine content of actin preparations is variable. Perhaps the ATP/ascorbate washes preferentially removed a fraction of actin having less of this modified residue. It was interesting to note that Band D from A-I contained a trace of 3-methylhistidine. It is not known whether this represented contamination from Band E not visible in Figure 35, or whether Band D was a degradation product of actin.

At the beginning of Section III, it was theorized that the presence of actin in the guanidine and guanidine/dithiothreitol extracts was due to incomplete tissue homogenization or to the omission of pre-washes which would have removed it. The findings of the present section do not support this. The presence of actin must be due to another process.

2.3.3.2.5.5.3 Comparison of Bands J and K to Collagen.

Although several of the bands were seen to contain hydroxyproline, only J and K contained large amounts of it. This would be expected if these bands were collagen α chains, as suggested by their co-migration with standard collagen. Bands J and K also contained large amounts of glycine, again suggestive of collagen. However, these bands did not have enough hydroxyproline or glycine to be pure collagen. As seen in Figure 29, about one-third of the total absorbance of these stained bands on the gels was "background". The fraction of "background" would be larger in the isolated bands, which were taken from preparative gels which could not be sectioned precisely at the edge of the bands because of the difficulty of exactly locating them on an unstained gel. The nature of this "background" remains unknown. Its composition may resemble that of Peak I, the V_o peak from G/DTT-I fractionated on Sepharose 6B in guanidine (Table IX). Peak I had much glycine but only a small amount of hydroxyproline. If this material were contaminating Band E of G/DTT-I, the result would be elevated glycine but only traces of hydroxyproline. In spite of these equivocations, it is safe to say that Bands J and K were largely collagen, in confirmation of the observations from analytical gels.

2.3.3.2.5.5.4 Comparison of Bands M and N to Collagen and Myosin.

Bands M and N, isolated from extract G-I, were thought to be collagen and/or myosin because of their co-migration with those standards. The amino acid composition of the sum of the two bands,

which were not resolved on the preparative gels, was that of neither pure collagen nor pure myosin. It contained more glutamic acid than did any other band or fraction analyzed, and far less glycine than it would be expected to contain if it were largely collagen. It also contained small but detectable amounts of hydroxyproline and hydroxylysine.

As seen in Table XII, myosin has a high content of glutamic acid (213 residues per 1000) and a low content of glycine (39 residues). By contrast, collagen contains 72 residues of glutamic acid and 334 of glycine. Myosin is devoid of hydroxyproline, while collagen has 92 residues per 1000 residues. The amounts of aspartic acid, leucine, and lysine are also vastly different between collagen and myosin. In view of these widely divergent compositions, an attempt was made to determine what if any combination of collagen and myosin would give an overall composition resembling that of "Band" MN. Compositions of various hypothetical mixtures were calculated and compared to that of the unknown bands. A mixture of 28% collagen and 72% myosin gave a reasonable facsimile of the composition of the mixture isolated from the gels. The two are compared in Table XIII.

One could continue this exercise by adding mathematically a portion of "background material" as well. But having done this successfully, one would have proven only that mathematics, like Scripture, can be taken out of context and used to "prove" almost anything. The mathematical mixing of collagen and myosin only demon-

TABLE XIII: AMINO ACID COMPOSITION OF MIXTURE OF BANDS M AND N, COMPARED WITH CALCULATED MIXTURE OF 28% COLLAGEN/72% MYOSIN. (RES PER 1000 BASED ON DATA IN TABLE XII)

	M + N	28/72
Hypro[1]	(30)[2]	(26)
Asp	94	88
Thr	55	38
Ser	67	41
Glu	158	174
Pro	58	48
Gly	92	122
Ala	82	91
½Cys	9	7
Val	55	38
Met	14	21
Ile	33	30
Leu	87	85
Tyr	20	13
Phe	28	27
Hylys[1]	1	2
Lys	67	78
His	22	11
Arg	58	54

[1]Hypro: hydroxyproline. Hylys: hydroxylysine.
[2]Estimate from very small peak on chromatograms.
Text section 2.3.3.2.5.5.4, page 261.

strated that the composition of "Band" MN was not inconsistent with the suggestion that the bands were a mixture of myosin and collagen.

2.3.3.2.5.5.5 Mathematical Reconstitution of G-I.

At the suggestion of Black[1], one further mathematical exercise was carried out. Table X shows the percentages of each band in the total G-I extract. Table XII shows the amino acid compositions of many of those bands. From these two sets of data, a mathematical reconstitution of G-I was performed. More specifically, each amino acid composition in Table XII was expressed in terms of weights rather than moles of amino acids. The weight of each residue from each band was then multiplied by the weight percentage of that band in the total extract as seen in Table X. These weights were summed for each amino acid residue, to give the weights of each residue in the mixture. Finally, the weight composition of the mixture was expressed in terms of residues per 1000 residues and compared to the actual composition of G-I from Table IX. If the data in Tables IX, X, and XII were accurate, it would be expected that the actual and "reconstituted" compositions of G-I would agree closely.

Bands B, D, E, F, J, K, M, N, and P were used for the calculations. Since J and K were not isolated from G-I, their compositions in G/DTT-I were used. Taken together, these bands added up to 84.1% of G-I (Table X). The results of the calculations are shown in Table XIV. The actual and reconstituted compositions were in remarkable agreement. Only two residues differed by more than three residues per

[1]J. Black, personal communication, 1976.

TABLE XIV: MATHEMATICAL RECONSTITUTION OF EXTRACT G-I FROM THE AMINO ACID COMPOSITION OF ITS CONSTITUENT BANDS (RES PER 1000).

	Reconstituted G-I	Actual G-I
Hypro*	4	3
Asp	93	93
Thr	58	57
Ser	74	66
Glu	129	130
Pro	61	59
Gly	94	93
Ala	81	82
½Cys	14	16
Val	51	54
Met	24	21
Ile	45	42
Leu	78	82
Tyr	31	30
Phe	32	33
Hylys*	1	1
Lys	56	63
His	21	21
3M-His*	1	tr
Arg	55	56

* Hypro: hydroxyproline. Hylys: hydroxylysine. 3M-His: 3-methyl histidine.

Text section 2.3.3.2.5.5.5, page 263.

1000. Serine differed by 8, while lysine differed by 7. These differences might be attributable to the incomplete resolution of serine from threonine by the amino acid analyzer, and to the fact that lysine sometimes appeared at the edge of the ammonia plateau, making it difficult to establish a realistic baseline (see Figure 4). These results were a good indication that the analyses of the individual bands were accurate.

2.3.3.2.6 Summary of Conclusions from PAGE About the Components of the G and G/DTT Extracts.

The G and G/DTT extracts were those extracts expected to contain the bulk of all SGP in the tissue, according to the literature (Table I). Nevertheless, the principal components of these extracts co-migrated with actin, collagen, or myosin on PAGE in SDS. The amino acid compositions of those bands were in agreement with those of the standard proteins with which they co-migrated. The agreement was especially good in the case of Band E and standard actin. Band E was the principal component of extracts G-I through G/DTT-I. Table XV summarizes the findings of this section.

TABLE XV: SUMMARY OF CONCLUSIONS FROM POLYACRYLAMIDE GEL ELECTROPHORESIS IN SODIUM DODECYLSULFATE.

Extract	Band	Percent of Extract	Co-migrates with	Amino Acid Composition Similar to
G-I	E	34.6	Actin	Actin
	D	9.4		
	N	8.8	β11 or Myosin	Collagen + Myosin
	F	8.4		
	B	6.8		
	P	6.2		
	K	5.3	Collagen α1	Collagen, impure
	M	3.5	Collagen β12	Not separated from N
	A	3.3		
	C	3.1		
G/DTT-I	E	24.3	Actin	Actin
	K	24.1	Collagen α1	Collagen, impure
	J	12.1	Collagen α2	Collagen, impure
	D	7.8		
	M	5.5	Collagen β12	Not separated from N
	B	4.8		
	P	4.2		
	N	3.9	β11 or Myosin	Collagen + Myosin

Text section 2.3.3.2.6, page 265.

2.3.3.3 Comparison of Aorta Extracts Following Attempts to Remove Collagen From the Tissue.

The objective of these experiments was to determine if autoclaving, extraction with hot trichloroacetic acid (TCA), or digestion with collagenase would also remove actin from aortic tissue. Other investigators almost always used one of these three methods for removal of collagen prior to extractions with urea, guanidine, or NaOH to obtain aortic SGP described in the literature.

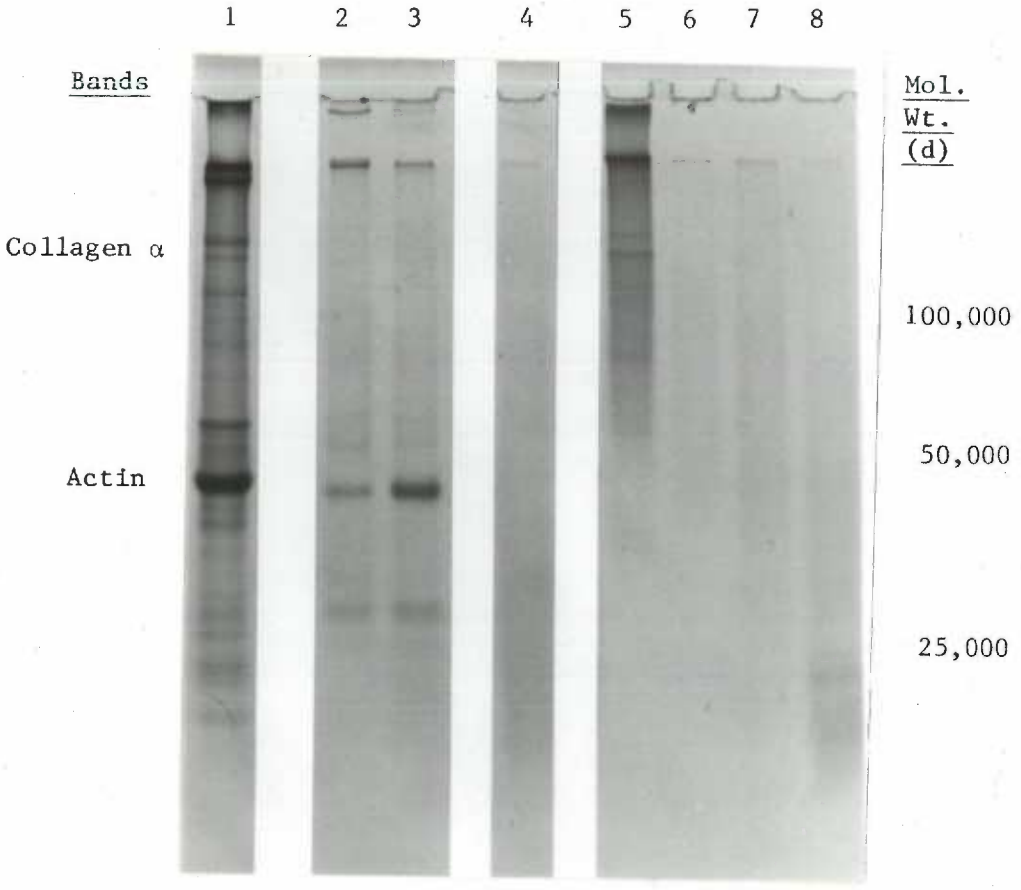
Figure 36 shows the supernatants from the collagenase digestions, from the autoclave procedure, and from the TCA extractions, all on a 6½% gel in SDS. After dialysis, the collagenase extract contained primarily a band migrating slightly ahead of the standard actin. Also seen were bands having molecular weights of about 23,000 d, 48,000 d, and 55,000 d. This gel was not calibrated. The molecular weights were estimated by comparison with bands on an adjacent guanidine extract used as a control. Collagenase appeared to liberate a small amount of something resembling actin. The supernatant after autoclaving showed only a broad smear of stained material, mostly of low molecular weight. No bands were seen whatsoever. The first TCA supernatant, after five minutes at 90°C, showed faint bands corresponding to collagen α and β chains, and a far greater amount of heterogeneous material of high molecular weight. No actin band was seen. The second and third TCA extracts were entirely heterogeneous, showing smears but no discrete bands. The two water washes of the residue after TCA showed some suggestion of bands smaller than 20,000 d. But

Figure 36. Aorta Components Solubilized by Collagenase, Autoclaving, or Hot Trichloroacetic Acid (TCA).

Electrophoresis on 6½% polyacrylamide gel in 0.1% sodium dodecylsulfate using Bio-Rad apparatus. Composite photograph, all wells taken from a single gel. Samples for this gel were obtained as described in section 2.3.2.4.

Well	Sample
1	G/DTT extract of untreated tissue (section 2.3.2.4.4)
2	1st Collagenase supernatant
3	2nd Collagenase supernatant
4	Autoclave supernatant
5	1st TCA supernatant
6	2nd TCA supernatant
7	3rd TCA supernatant
8	Combined H_2O washes after TCA

Text section 2.3.3.3, page 267.


1
2
3
4
5
6
7
8
Bands
Collagen α
Actin
Mol. Wt. (d)
100,000
50,000
25,000

the material in these washes was also largely heterogeneous and degraded. These findings confirmed the conclusions in the Historical Setting section, that autoclaving or hot TCA would degrade the protein extracted from the tissue.

Figure 37 shows the guanidine/dithiothreitol extracts of the tissue after the three treatments to remove collagen. Collagenase removed most of the collagen, as seen by the near absence of collagen α chains in the extract. Collagen β chains moved with the artifact band on 8% gels such as this one. Collagenase very plainly did not remove a large portion of actin from the tissue, since actin was the most prominent band in the extract. As expected, the G/DTT extract of autoclaved tissue was extensively degraded. Most of it appeared as a smear on the gel. The one sharp band in the entire extract co-migrated with standard actin. Several other broad, diffuse bands were also seen. Also as expected, the G/DTT extract of the tissue after TCA treatments was extensively degraded. More bands were seen here than after autoclaving; nevertheless, the majority of this extract was also a smear of degraded polypeptides. A faint band co-migrated with standard actin.

As a control, standard actin was subjected to autoclaving or TCA treatment as described for the tissue. It was insoluble in both treatments. The residues after these treatments were dissolved in sample diluent for PAGE in SDS (autoclaved actin gave a very viscous solution, for undetermined reasons). These samples, together with the G/DTT extract of tissue treated the corresponding way, are shown on

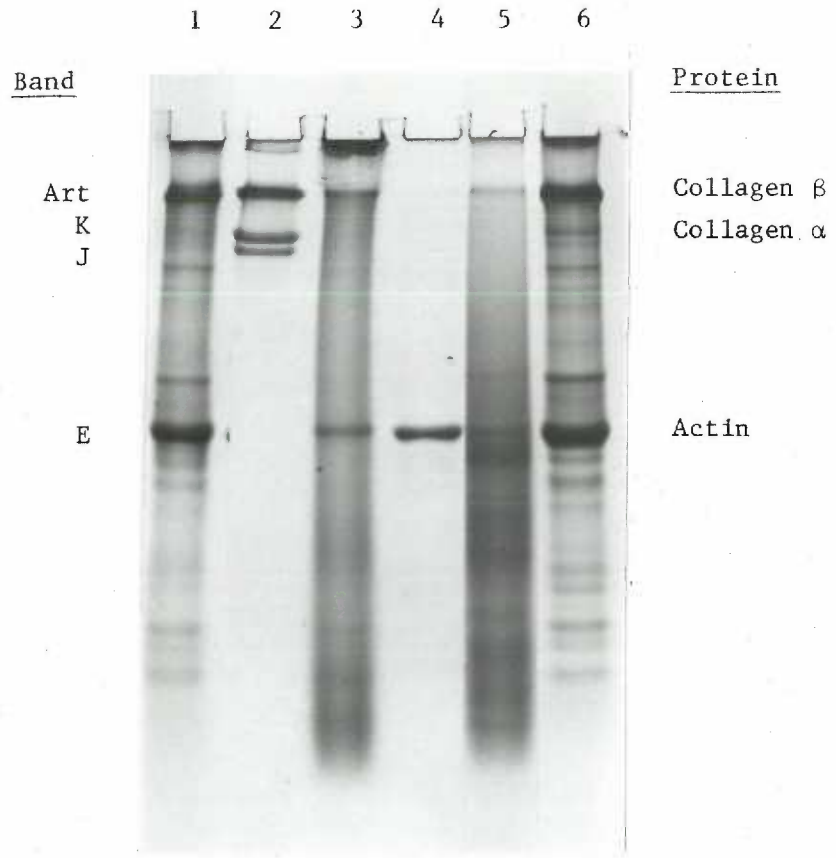


Figure 37. Aorta Components Soluble in Guanidine and DTT After Treatment of Tissue with Collagenase, Autoclave, or Hot TCA.

Electrophoresis on 8% polyacrylamide gel in 0.1% sodium dodecylsulfate using Bio-Rad apparatus. Composite photograph, all wells from the same gel. All samples obtained as described in section 2.3.2.4.4.

Well	Sample
1	G/DTT extract after collagenase
2	Collagen standard
3	G/DTT extract after autoclave
4	Actin standard
5	G/DTT extract after hot TCA
6	G/DTT extract of untreated aorta

Text section 2.3.3.3, page 270.

the 8% gel pictured in Figure 38. Autoclaved actin was an amorphous smear, devoid of discrete bands. Actin after TCA showed a series of sharp bands at lower molecular weights, apparently products of hydrolysis of specific, labile peptide bonds. Most of these bands co-migrated with the bands seen in the G/DTT extract of tissue after hot TCA. There was also a sharp band corresponding to undegraded actin.

Table XVI shows the molecular weights of all bands seen in the various extracts of this series of experiments.

The conclusion from these experiments was that none of the most popular methods for removing collagen from aorta succeeded in removing actin as well. It was clear that actin was retained in the tissue by a mechanism other than entrapment by collagen fibrils. These experiments also showed that, following autoclaving of the tissue, or extraction of the tissue with hot TCA, the material extractable from the residue with guanidine and dithiothreitol was extensively, almost totally, degraded and unrecognizable. This finding invalidates the characterizations of SGP in the literature which followed autoclaving or extracting with hot TCA as part of the tissue fractionation procedure. These characterizations are summarized in Table I.

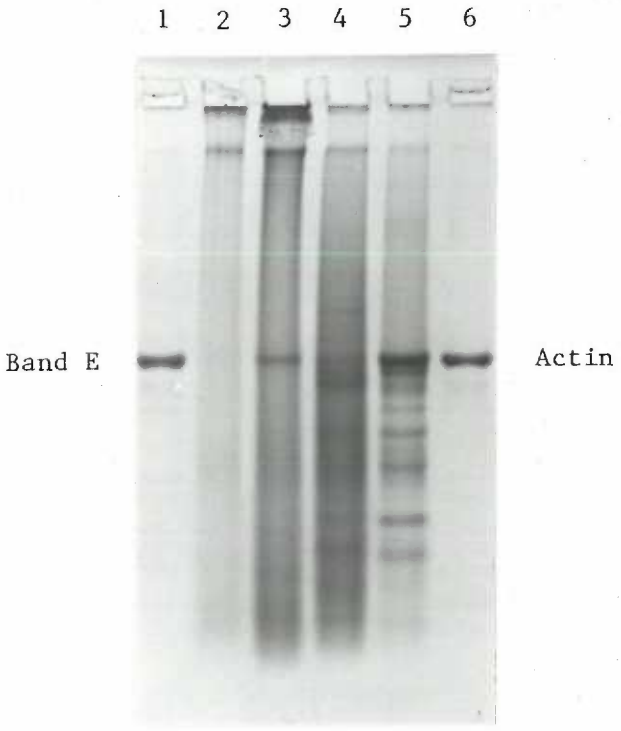


Figure 38. Actin After Treatment With Autoclave or Hot TCA.

Electrophoresis on 8% polyacrylamide gel in 0.1% sodium dodecylsulfate using Bio-Rad apparatus. Standard actin was autoclaved or heated in TCA as described in section 2.3.2.4. Composite photograph, all wells from the same gel.

Well	Sample
1	Untreated actin
2	Actin after autoclave
3	G/DTT extract of tissue after autoclave, as in Figure 37, well 3
4	G/DTT extract of tissue after hot TCA, as in Figure 37, well 5
5	Actin after hot TCA
6	Untreated actin

Text section 2.3.3.3, page 272.

TABLE XVI: MOLECULAR WEIGHT (MWT) OF BANDS FROM COLLAGENASE, AUTOCLAVE, AND HOT TRICHLOROACETIC ACID (TCA) EXTRACTS, AND FROM THE G/DTT EXTRACTS OF THE TISSUE AFTER THESE TREATMENTS. UNITS ARE DALTONS X 10^{-3}.

Band	Collagenase Extract	G/D After Collagenase	G/D After Autoclave	TCA Extract	G/D After TCA	Actin After TCA
Pre-A[1]			14		14	
A		16	16		15	
B1		17			17	17
B2		18	18		19	19
C2	22					
C3			25		24	24
D1					29	29
D2		33				(33)[2]
E1		36			37	(35, 36)
E2		38				39
E3	42[3]		42		42	42
E4		43				
F(8%)		57				
H(8%)		124				
J				142		
K2				167		

[1]Band Pre-A was beyond the calibration limits of the gels.
[2]Bands in parenthesis did not co-migrate with bands in other extracts.
[3]Underline denotes predominant E band. Others very faint.
Text section 2.3.3.3, page 272.

2.3.4 Summary and Discussion of Experimental Section III: Survey of Aorta Proteins.

At the beginning of this experimental section, two objectives were described. The first was to confirm or deny several postulated mechanisms by which actin was retained in homogenized aortic media after repeated extractions of the tissue with 5 M guanidinium chloride. The second objective was to isolate genuine SGP from the tissue, if indeed SGP existed in the large amounts reported in the literature, and if indeed it could be extracted as a discrete polypeptide in undegraded form. These two objectives were established in order to verify the hypothesis from Experimental Section II, that much if not all of the aortic "SGP" described in the literature was actually not SGP but only denatured actin.

The findings in Section III met these objectives. The first proposed explanation for actin retention in the tissue, namely incomplete homogenization of the tissue, was discounted. The Latapie Mill produced a much finer homogenate than had freeze-fracturing, so fine, in fact, that it was difficult to separate it from the supernatant after each extraction. Yet the guanidine and guanidine/dithiothreitol extracts contained no less actin than had corresponding extracts from more coarsely homogenized tissue.

The second proposed explanation for actin retention in the tissue was also discounted. Pre-extractions of the homogenized tissue with NaCl, $NaHCO_3$, and EDTA, followed by exhaustive extraction of the residue with ATP and ascorbate -- a procedure shown to extract

actin from cardiovascular tissue (43) -- failed to removed actin quantitatively from the tissue. In fact, the first extraction with 5 M guanidine, following these pre-washes, removed more actin than had all the pre-washes combined.

The possibility of entrapment by collagen fibrils as an explanation for actin retention in the tissue was more difficult to eliminate. Prolonged digestion of the tissue homogenate with collagenase removed most but not all of the collagen. It removed almost none of the actin, leaving open the rather remote possibility that the remaining collagen entrapped all the remaining actin. Autoclaving of the tissue also failed to release all of the actin. It did, however, degrade the tissue to the extent that the subsequent extraction with guanidine and dithiothreitol obtained material which produced little more than a smear of unresolved species on polyacrylamide gels in SDS. The only recognizable band in this extract was actin. Similarly, extraction of the tissue with hot TCA led to extensive degradation of the residue. The only recognizable bands in the subsequent guanidine/dithiothreitol extract appeared to be actin and fragments of it. None of these treatments guaranteed the removal of actin from the tissue. Two of them degraded the residue severely.

With these proposed explanations eliminated for the retention of actin in the tissue, one is left with the choices that either actin is entrapped by the elastin fibers, or it is rendered very insoluble by some mechanism yet to be envisioned.

The second overall objective of Section III was to find SGP. In all the sequential extracts of aorta, beginning with the saline washes and ending with two extractions with 0.1 N NaOH, no single polypeptide could be found in large amounts which met the solubility and amino acid composition criteria for the definition of SGP. The major components of these extracts were actin, collagen, and in all probability, myosin. The saline, bicarbonate, and EDTA washes contained prominent, unidentified components not found in later extracts, but, by virtue of their extractability in these mild salt solutions, they were too soluble to be SGP as it is described in the literature. The NaOH extracts resembled the extracts after autoclaving or hot TCA, in that they were extensively degraded and heterogeneous.

In the Historical Setting section, three reports of a monomeric form of SGP with a molecular weight of 12,000 to 16,000 d were described (section 1.7). These molecules were as small as, or smaller than, any band seen on any gel described in this thesis. It is probable that molecules smaller than Band A (Figure 16 or Table XI) were able to pass through the pores of the dialysis tubing used in removing salt from the various aorta washes and extracts. Therefore, the findings of this thesis do not rule out the existence of an SGP molecule with a molecular weight of under 15,000 d. However, those papers describing large amounts of aortic SGP usually showed that the extract had been dialyzed (72)(76), and the most recent papers describing aortic SGP report molecular weights much larger than 15,000 d (19)(77).

One is left with the conclusion that the only protein resembling SGP which is found in bovine aorta in amounts sufficient to account for the high yields of aortic "SGP" reported by many investigators (19)(26)(60)(72)(76)(80)(85) is actin. Fragments of myosin and other myofibrillar proteins are probably also present in these preparations.

3 Conclusions of the Thesis. Implications for Future Research.

Many investigators have been studying SGP metabolism, SGP correlations with atherosclerosis, the function of SGP, and other matters relating to SGP as a major component of connective tissues. The Historical Setting outlines many of these studies. Yet in spite of these studies, SGP has not been defined any more rigorously than its extractability and amino acid composition. Until SGP has been defined as a molecule, with a known molecular weight, a known amino acid sequence, and the like, metabolic, pathologic, and immunologic studies are virtually meaningless.

On this basis, the work described in this thesis was begun, with the objective of isolating and defining a monomer of SGP. Procedures were chosen to avoid what appeared to be the shortcomings of previous SGP isolation schemes in the literature. These shortcomings had led to apparent degradation of the polypeptide chain by any or all of a number of mechanisms, including hydrolysis of peptide bonds by strong acid or base, digestion by nonspecific proteolytic enzymes in commercial collagenase preparations, and digestion by endogenous tissue proteases.

The attempts to isolate and characterize an SGP monomer from bovine aorta led to a startling conclusion. Actin, myosin, and collagen were the only major, definable components of the extracts which, according to the literature, should have contained large amounts of SGP. Subsequent to this discovery, a search was made throughout all fractions which could be solubilized from the tissue with

denaturing agents, reducing agents, or dilute NaOH. These were the solutions used by other investigators to extract SGP from aorta. No large quantity of any unidentified protein was found which could have been SGP. The major components of all the extracts were actin, collagen, and myosin. Pre-treatment of the tissue by hot 2.7% TCA or by autoclaving to remove collagen led to extensive degradation of the extractable proteins remaining in the tissue. Likewise, extraction of the tissue with cold 0.1 N NaOH produced extensive degradation.

Although they strongly suggest the conclusion, these findings do not necessarily mean that SGP does not exist, or that there is no such thing as SGP in bovine aorta. They do, however, mean that if SGP exists as a discrete, definable molecular species, of specific molecular weight and composition, it cannot be more than a few tenths of one percent of the dry weight of the tissue. This is in contradiction to the many reports of large amounts of SGP in the aortas of many species, amounts as high as 44% of the dry weight of human aorta (see Table I).

This work has demonstrated the existence of actin and myosin in large amounts in "SGP" preparations, and the absence of any single unknown, presumably SGP, in large amounts. Because of the many conclusions being drawn by other investigators about the involvement of SGP in atherosclerosis, morphogenesis, and aging, this revelation of a major artifact in SGP preparations from arterial wall is an important contribution to the connective tissue literature.

This work, however, was not as rigorous as the chemistry done to define a new protein. It was asserted that certain components of the aorta extracts were actin on the basis of co-migration on polyacrylamide gels in SDS, of similar amino acid compositions, and of the presence of 3-methylhistidine, a modified amino acid found in very few other proteins, none of which could easily be mistaken for actin (87). Similarly, it was asserted that other components were collagen or myosin on the basis of co-migration on gels in SDS, of amino acid composition, of resistance or susceptibility to collagenase, and of solubility in cold dilute TCA.

For none of these proteins were these arguments irrefutable proof of identity. If an irrefutable argument were required, at least two more independent criteria would be needed. Such criteria could be two-dimensional peptide maps, partial sequencing of the polypeptide chain, immunologic identity by double diffusion or immunoelectrophoresis, interaction of putative actin with standard myosin to give normal ATPase activity, similar interaction of putative myosin with standard actin, or decoration of putative actin filaments with known heavy meromyosin subfragment one. These, and other tests as well, might be done, although, as described earlier in the thesis, none of them would be straightforward. Peptide mapping would be complicated and confused by the demonstrated charge heterogeneity of the actin from the guanidine extracts of aorta. Immunological tests would be difficult since actin is a very poor antigen, and since the proteins are

denatured by extracting with guanidine. Denatured actin or myosin would not show normal solubility, ATPase activity, or interaction with standards or with each other.

Whether or not these tests are feasible, they would be of little value to the purpose of this paper or to science. Actin, myosin, and collagen are known proteins, well-characterized by other investigators who obtained them in undenatured form. It is of little value to the study of these proteins that they can be extracted from aorta, in denatured, somewhat degraded form, using 5M guanidine. The experiments that were done showed that much if not all of the aortic SGP described in the literature was probably denatured or degraded actin or myosin. It is now the duty of each investigator in the field to prove that his or her putative SGP is not actin, myosin, or a degraded fragment of one of them.

4. A Closing Speculation.

Actin is known to be present in many non-muscle cells (91)(118). Recently, Mannherz, Kabsch, and Lehermann were able to crystallize a complex of skeletal muscle actin and pancreatic DNase I (70). They described how actin had earlier been found to have the same molecular weight, amino acid composition, and tryptic peptide map as bona fide bovine pancreatic DNase I inhibitor, and furthermore that actin possessed the same ability to inhibit the enzyme. They offered no explanation for the remarkable similarity between actin and the inhibitor.

It has also been found that the gamma subunit of muscle glycogen phosphorylase kinase appears to be an isozyme of actin (35). In lower vertebrates, such as the dogfish, the two polypeptides have the same molecular weight and amino acid composition. There are minor differences between the two in higher vertebrates such as the rabbit. The gamma subunits also form filaments similar to those of F-actin, and the filaments decorate with heavy meromyosin subfragment 1 as described by Nachmias et al (83).

In view of these unusual findings, and in view of the findings of this thesis, it is not unrealistic to ask if actin, or a protein almost identical to it, might not exist as an extracellular component of connective tissue, closely associated with collagen or elastin. If such a protein were as similar to actin as are the DNase inhibitor or the gamma subunit described above, it would be indistinguishable from actin by the tests applied in this thesis. Therefore, this

thesis has not ruled out the possibility that SGP is an extracellular protein very closely related to actin, indistinguishable from actin by even the most definitive comparisons. This hypothesis could be tested by culturing smooth muscle cells or fibroblasts and assaying the medium for a protein similar or identical to actin. Such an experiment would be an interesting sequel to the work of this thesis.

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