AN ABSTRACT OF THE THESIS OF

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Abstract approved Redacted for Privacy

Wilbert Gamble

The vascular system transports components essential to the survival of the individual and acts as a barrier to substances that may injure the organism. Atherosclerosis is a dynamic, lesion producing disease of the arterial system that compromises the functioning of the organ by occlusive and thrombogenic processes. This investigation was undertaken to elucidate some of the normal biochemical processes related to the development of atherosclerosis. A significant part of the investigation was directed toward developing and combining methods and protocols to obtain the data in a concerted manner.

A postmitochondrial supernatant of bovine aorta, using mevalonate-2-^{14}C as the substrate, was employed in the investigation. Methods included paper, thin layer, and silica gel chromatography; gel filtration, high performance liquid chromatography (HPLC), and mass spectrometry.

This current research demonstrated direct incorporation of mevalonate-2-^{14}C into the *trans*-methylglutaconic shunt intermediates. The aorta also contains alcohol dehydrogenase activity, which converts dimethylallyl alcohol and isopentenol to dimethylacrylic acid, a constituent of the *trans*-methylglutaconate

Small, radioactive peptides, named Nketewa as a group, were biosynthesized using mevalonate-2-^{14}C as the substrate. They were shown to pass through a 1000 D membrane. Acid hydrolysis and dabsyl-HPLC analysis defined the composition of the Nketewa peptides. One such peptide, Nketewa 1, had a molecular weight of 1038 and a sequence of his-gly-val-cys-phe-ala-ser-met (HGVCFASM), with a farnesyl group linked via thioether linkage to the cysteine residue.

Methods were developed for the concerted investigation of the *trans*-methylglutaconate shunt, the isolation of mevalonate-2-^{14}C labeled peptides, and characteristics of neutral and acidic metabolites of mevalonate. The question as to whether or not mevalonate was the direct precursor was answered in the affirmative. These results contribute to the understanding of the biochemistry of the vessel wall and the associated atherogenic processes. Mevalonate-derived volatile and acidic compounds may represent an alternate metabolic pathway. The prenylated Nketewa peptide may be, as are other prenylated peptides, participants in the intracellular signaling process, release of cytokines, expansion of extracellular matrix, and calcium release.

Protocols, Pathways, Peptides and the Aorta: Relationship to Atherosclerosis

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Marilyn L. Walsh

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Marilyn L. Walsh, Author

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Dedication

This work is dedicated to my parents, two truly good people; to my husband, whose patience and love through this long process have been tried, but have survived intact; and to Wil, who has taught me the importance of angels in life.

PROTOCOLS, PATHWAYS, PEPTIDES AND THE AORTA: RELATIONSHIP TO ATHEROSCLEROSIS

INTRODUCTION

Atherosclerosis

The term arteriosclerosis entered the scientific lexicon when J.F. Lobstein (1) first used the term to describe the brittle and fragile appearance of portions of the vascular system observed during post mortem examinations. In 1904, Felix Marchand used the term atherosclerosis to describe the pathology of this complex, multi-faceted disease (for a historical review, see 2,3, and references therein). Other researchers provided descriptions of the disease as early as 1700 (2). The reservoir of information accumulated by the amount of cardiovascular research reflects the elaborate, multi-step description required to define atherosclerosis. This research has encompassed both genetic and environmental factors in an attempt to define the etiology and the course of the disease. The intense interest in atherosclerosis, quite obviously, comes from its widespread occurrence: atherosclerosis remains the principal cause of myocardial and cerebral infarctions and their related conditions, giving it the dubious first place as cause of death in the United States, Europe, and Japan (4). This standing has been in place over some time, and is reflected in the emphasis on research directed to both defining the causes of atherosclerosis and to finding treatments to ameliorate or cure the disease.

Structure of the Vessel Wall

The arterial wall is a complex organ, consisting of layers of specialized cells and extracellular structures suited for the function of transporting blood to smaller vessels and, ultimately, cells. The ultrastructure of arteries will vary according to size of the vessel itself (5). Those vessels with large diameters—such as the aorta and the carotid artery are elastic arteries; smaller vessels are designated muscular arteries.

The arterial wall consists of three defined layers or tunica: intima, media and adventitia. The intima is formed by a single layer of endothial cells supported by thin layer of elastic tissue, the basal lamina. In some species the endothelium and basal lamina are accompanied by a third component of the intima, a subendothial layer composed of collagen, elastic fibers, and smooth muscle cells. The bovine aorta, the tissue used in this study, lacks such subendothial layer. Endothelial cells in the intimal layer are metabolically active and form a non-thrombogenic barrier between circulating blood and underlying tissue. The endothelium is also a source of components necessary for the normal functioning of the cardiovascular system (a review of endothial cell function can be found in 6, 7).

Below the intima layer is the tunica media—a middle layer composed of smooth muscle cells, elastic sheets (lamina) and networks of elastic fibrils. The highly structured arrangement of the elastic sheets (40-60 layers) gives the large vessels (aorta) their great resilience. Within this framework are found smooth muscle cells, oriented at angles to the elastic sheets (8). The principal function of the smooth muscle cells is contraction (9), but such cells will proliferate during angiogenesis and synthesize the matrix components (collagen, proteoglycans) of the vessel wall.

Atherosclerosis Development

Atherosclerosis is better viewed as a progressive condition rather than a discrete state [among the many comprehensive reviews available, see (3, 10-14)]. Changes in the functioning of the components of the arterial wall as well as changes in the circulatory blood elements result in the development of lesions within the vessel wall. The etiology of the disease is not known, even though an extraordinary amount of research has been directed toward treating and understanding the condition. The events that participate in atheromatous lesion development have been well characterized and a brief overview of those events is given below.

Lesion development in the arterial wall begins with the appearance of a fatty streak which can occur in childhood or adolescence. These early deposits of lipid contain free cholesterol, cholesterol esters, and triglycerides. Foam cells, which are lipid-laden macrophages, are also observed in this early lesion and are characteristic components of the atherosclerotic lesion. Plasma lipids are engulfed by monocyte/macrophages which then become the foam cells; monocytes from the blood enter the subendothelial layer and there differentiate into macrophages. Such recruitment of monocytes to the intimal layer is a pivotal point in disease progression, since monocytes may be induced to infiltrate the vessel wall in response to several postulated circumstances (see below). Subsequent factors which stimulate monocyte infiltration are the appearance of adhesion molecules on the surface of endothelial cells, the expression of cytokines, growth factors, and leucocyte attachment to the endothelial layer.

Smooth muscle cells, in response to intercellular signals generated by the developing lesion, proliferate in the next stage of atherogenesis and in turn, generate

the fibrous components of the extracellular matrix. Growth factors and cytokines expressed and released by foam cells, smooth muscle cells, and the endothelium itself promote lesion development to a more advanced state, the atheroma. Foam cells and lipids continue to accumulate in the core of the atheroma. Cell death within the lesion releases more lipids and thrombogenic factors.

The development of the plaque progresses through the formation of a fibrotic cap, composed of smooth muscle cells and collagen. Overlaying the lipid core, the cap varies in thickness and composition while the core is characterized by a predominance of extracellular lipids. Other mechanisms now intervene including thrombosis, plaque rupture, calcification, and occlusion of the vessel wall, along with continuing necrosis of the plaque itself. These events lead to the clinical signs of the disease: heart attack, gangrene, stroke and other disorders associated with diminished blood flow.

The etiology of atherosclerosis is not known, but at least two general hypotheses exist. The injury hypothesis (10) postulates that a destructive event or state (stress or turbulence, disease, xenobiotics, risk and genetic factors) may compromise the endothelial lining of the vessel wall with accompanying inflammation. The response to the injury then leads to recruitment of platelets and monocytes to the arterial wall site with subsequent lesion development. The lipid hypothesis (15) postulates that elevated levels of plasma lipids [low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chlylomicrons] increase the rate of lipid uptake by cells found in the vessel wall: endothelial cells, smooth muscle cells, macrophages. The LDL receptor protein on the surface of macrophages and other cells regulates the uptake of cholesterol into the cell. Excess cholesterol and lipids accumulate outside the cell when intracellular levels are

adequate. This excess of lipid is viewed as an inflammatory event and subsequent recruitment of monocytes takes place.

While the etiology of atherosclerosis is not known, the explanation for the initiation of the disease has been integrated into a single, multifactorial approach (11). An excess of circulating lipids, leading to their deposition, especially oxidized low-density lipoprotein (OX-LDL), and subsequent organization into the arterial wall may be viewed as either an injury or a response to excess lipids Once injured, the endothelium will recruit platelets and monocytes to the arterial wall as well as lipid deposits; either or both events will result in the formation of the initial lesion (12, 13). Injury to the endothelium also results in thrombosis (15) which, in atherosclerosis, may lead to occlusion in vessels. Platelets involved in the process are also a source of growth factors which promote smooth muscle proliferation (16,17).

The uptake of modified LDL (OX-LDL) is a part of the atherogenic process [reviewed in (15)]. The production of a second receptor protein, the scavenger LDL receptor, is not regulated and thus cells with this receptor will take up uncontrolled amounts of OX-LDL. Oxidative modification to LDL is induced by smooth muscle cells, endothelial cells, and macrophages as well as extracellular components such as xenobiotics or disease states. OX-LDL also stimulates the release of the monocyte chemoattractant protein-1 from endothelial cells (19).

Research in this laboratory has focused on selected aspects of the normal metabolism of the aortic wall to gain knowledge of its functioning within the cardiovascular system, with the possibility that this information will contribute to the understanding of the atherogenic process. The present research is an investigation of the metabolic fate of mevalonic acid in the aorta.

Mevalonic Acid

The Mevalonate Pathway

Mevalonic acid (MVA) biosynthesis (Figure 1B) begins with the condensation of three molecules of acetyl coenzyme A (acetyl CoA) to yield decarboxylation and reduction, a branched 5-carbon hydroxy acid. This compound was first isolated by Lemuel Wright and others as a carbon source for Lactobacillus acidophilus (23). Members of this research group tested mevalonic acid as a precursor to cholesterol (24) and found it to be quantitatively converted to cholesterol. Earlier studies (25) had shown that acetate was the carbon source for cholesterol. A second report (26) describing the conversion of the C30 polyisoprenoid compound, squalene, to cholesterol suggested that mevalonate was the source of the biological isoprene unit (reviewed in 27).

The structure of squalene was known and followed the so called "isoprene rule"—a concept over a century old (reviewed in 27). The isoprene rule states that terpenes were constructed by the assembly of branched, five carbon units related to isoprene (Figure 1A). Given these relationships, the search for a small, branched precursor began.

Since cholesterol biosynthesis required a branched chain intermediate, investigators looked for compounds that were composed of 2-carbon units that could be assembled into mevalonic acid. Studies with acetoacetyl coenzyme A and acetyl coenzyme A were shown to be intermediates in the formation of HMG-CoA (28). The conversion of HMG-CoA to mevalonic acid required NADPH and a microsomal fraction containing the enzyme, HMG-CoA reductase. This step is a regulatory point in isoprene biosynthesis (29, 30).

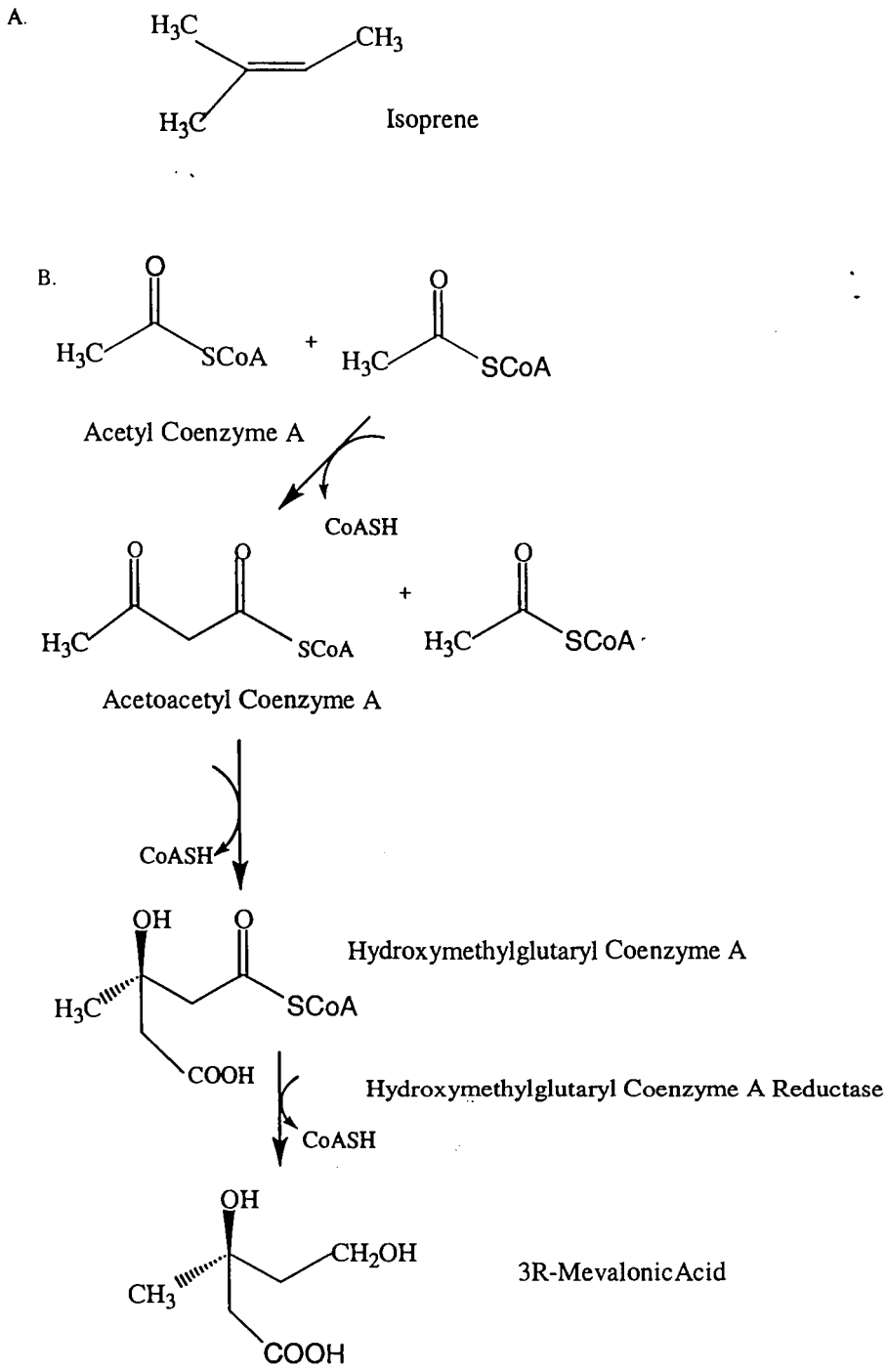

Figure 1. A. Isoprene. B. The pathway for the biosynthesis of mevalonic acid.

The reduction of HMG-CoA to form mevalonate is irreversible in biosynthetic pathways. The catalytic domain (carboxy terminus) of HMG-CoA reductase (30) is located in the cytosol and is highly conserved across species. The amino terminal region varies with different sources and resides in the endoplastic reticulum. It has also been shown that the catalytic domain can function without membrane attachment (31, 32), but the membrane-bound domain is required for enzyme degradation (30). In archaebacteria, HMG-CoA reductase functions without membrane attachment (32)

The mevalonate pathway has been shown to function in archebacteria (31) and plants (34), as well eukaryotes. A review of sterol biosynthesis in various kingdoms is available (35). In eukaryotes, the enzymes for the pathway converting acetyl CoA to HMG-CoA are found in the cytoplasm, and as noted, reduction of HMG-CoA to mevalonate occurs on the endoplastic reticulum. A HMG-CoA reductase has also been isolated from peroxisomes (36).

Isoprenoid Biosynthesis

The steps leading to the synthesis of farnesyl pyrophosphate and squalene from mevalonate are shown in Figure 2. ATP and Mg^{++} participate in the first step, the formation of mevalonate-5-phosphate, which is catalyzed by mevalonate kinase. A second phosphorylation step, catalyzed by phosphomevalonate kinase, produces mevalonate-5-pyrophosphate. The simultaneous dehydration and decarboxylation of mevalonate-5 pyrophosphate produces isopentenyl pyrophosphate, a reaction carried out by the enzyme phoshosphomevalonate decarboxylase; ATP and a divalent cation are required. Isopentenyl pyrophosphate isomerase, in a reversible manner,

catalyzes the isomerization of the substrate, isopentenyl pyrophosphate to dimethylallyl pyrophosphate and requires a divalent cation for activity.

The last step, leading to the formation of farnesyl pyrophosphate, is catalyzed by farnesol synthase. The enzyme catalyzes sequential head-to-tail condensations of isopentenyl pyrophosphate and dimethylallyl diphosphate to produce first, a 10-carbon terpenoid pyrophosphate, geranyl pyrophosphate. Addition of a second molecule of isopentenyl pyrophosphate forms farnesyl pyrophosphate.
Other enzymes of the mevalonate pathway leading to the synthesis of farnesyl pyrophosphate are located in the cytosol. Evidence leading to the synthesis of farnesol pyrophosphate in peroxisomes has been reviewed (37). Utilization of this molecule, via a number of different pathways, results in the synthesis of cholesterol, prenylated proteins, ubiquinone (coenzyme Q), and dolichols. Isopentenyl pyrophosphate is also used in the synthesis of a modified purine base, isopentenyl adenine, found in some transfer ribonucleic acids. A 20-carbon isoprenoid, geranylgeranyl pyrophosphate is also used to modify proteins. Enzymes catalyzing the conversion of farnesol pyrophosphate to squalene are located in the endoplastic reticulum. Two molecules of farnesol pyrophosphate are condensed, head to head, to form this 30-carbon intermediate. Squalene synthase facilitates this reaction, and the cyclization of squalene in the presence of oxygen forms the first sterol intermediate, lanosterol. Nineteen further reactions are required to produce cholesterol, briefly summarized in Figure 3.

The capacity of isopentenyl pyrophosphate and dimethylallyl pyrophosphate to condense into repeated 5-carbon branched chain molecules open to further modification and cyclization leads to the large number of "isoprenoid" compounds. Some of these compounds act as signaling molecules—either in an intracellular mode to transmit information within the cell or to control the activity of enzymes

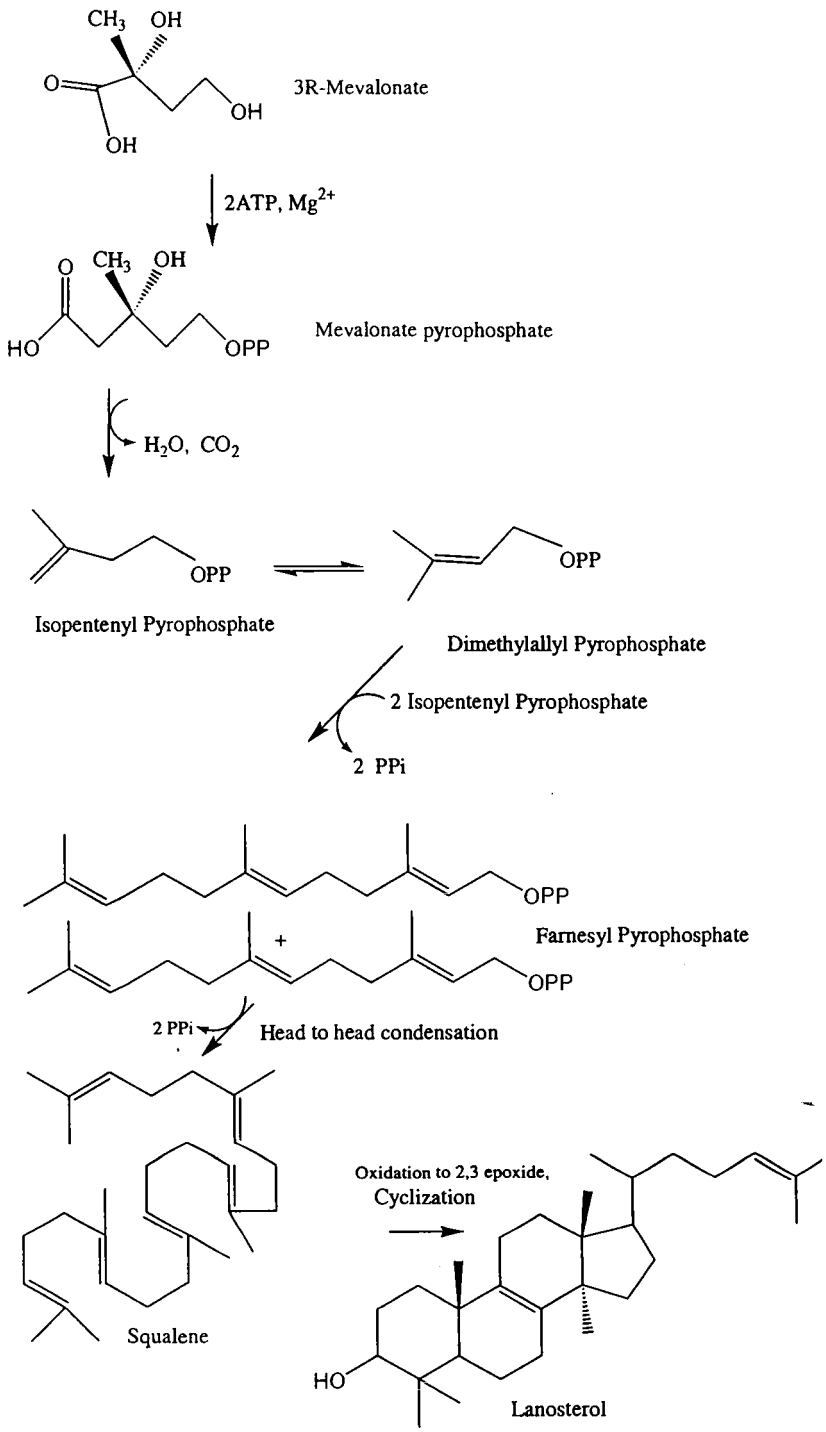

Figure 2. Biosynthesis of farnesyl pyrophosphate, squalene, and lanosterol from mevalonic acid

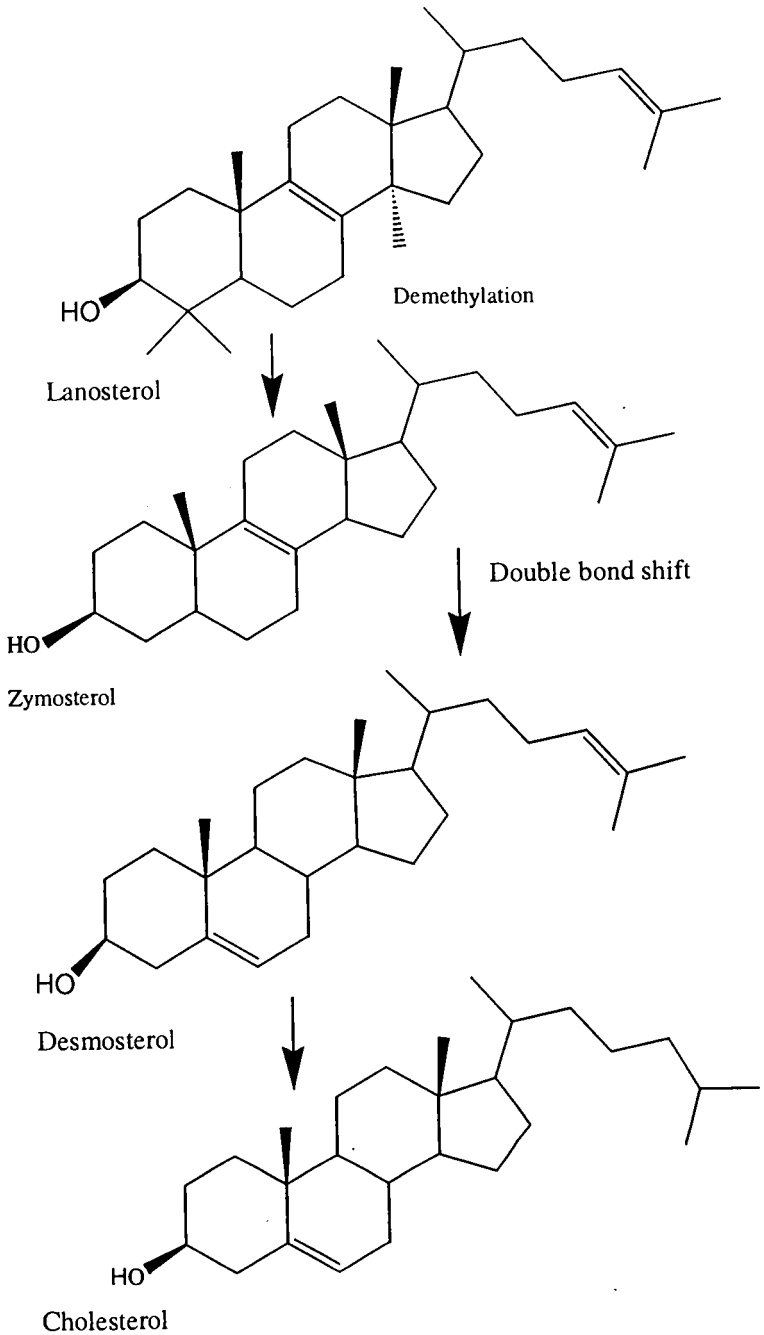

Figure 3. Biosynthesis of cholesterol from lanosterol.

along the mevalonate pathway itself. The varied functions of isoprenoid compounds and modifications are of particular relevance to the processes observed in the development of atherosclerosis: lipid biosynthesis, cell proliferation, cell cycle progression, lipoprotein uptake, DNA replication and cell cycle progression.

Cellular functions of mevalonate derived products

Cholesterol, beyond its historical importance in the elucidation of the mevalonate pathway, is also the most studied and most abundant of the mevalonate derived lipids in animals (38, 39). Dietary intake also supplies the cellular needs for cholesterol. Maintaining the appropriate balance between synthetic and dietary sources to achieve healthy cholesterol levels inside and outside the cell is a subject of extensive research, since cholesterol deposition is characteristic in atherosclerotic lesions (40-42).

Prior research in this laboratory (43-45) has shown that little synthesis of cholesterol takes place by the arterial wall, yet cholesterol accumulates in atherosclerotic lesions both within cells and in the extracellular space. The presence of cholesterol (free and esterified) in the core of advanced lesions arises from the death of foam cells within the core, but cholesterol (and cholesterol esters) may also be deposited independently outside of the lipid accumulating cells (46). First observed by Windaus (47) in 1910 as a significant component of atherosclerotic plaques, the presence of cholesterol in the vessel wall as well as the dynamics of deposited cholesterol has also been the subject of extensive research (48). The amount of total cholesterol in the lesion appears to arise from sources other than biosynthesis by the vessel wall.

Other biosynthetic products formed from mevalonate include dolichols, ubiquinone (coenzyme Q) and heme A. Dolichols are long chain isoprenoid alcohols that are also synthesized (49, 50) from mevalonate. These molecules may contain up to 24 isoprene units, the length depending on the biosynthetic source. Dolichol, as dolichyl phosphate, functions in the endoplasmic reticulum 's biosynthesis of glycoproteins in eukaryotes. Bacteria synthesize dolichols in conjunction with the synthesis of cell wall polysaccharides. Dolichol, in contrast to dolichyl phosphate, is present in a variety of tissues, while the amount of dolichyl phosphate is rate limiting in glycosylation reactions (52) where the polyprenol phosphate functions as a glycosyl carrier. The role of free dolichol itself is not known but may be involved with regulation of membrane stability, fluidity and permeability (52).

Ubiquinones contain a long isoprenoid-derived side chain and participate in a wide variety of metabolic functions; in eukaryotes, the ubiquinone coenzyme Q acts as a diffusable carrier in electron transport. Ubiquinone is required for normal functioning in the inner mitochondrial membrane, where respiration takes place. Ubiquinol is an antioxidant in biological membranes (32, 53), protecting lipids, proteins, and DNA from oxidative damage induced by free radicals. Ubiquinone may be significant in protecting lipoproteins against oxidative damage; oxidized lipoproteins are known to be atherogenic(54, 55). Ubiquinol may be more efficient in inhibiting LDL oxidation, in particular, than other endogenous antioxidants such as α-tocopherol (56).

There are yet other molecules derived from mevalonate: heme A is a component of fetal hemoglobin and contains a farnesyl side chain (57), and isopentenyl adenine, a modified nucleoside, contains an N-6 isoprenoid moiety. This substituted base is found in some forms of transfer RNA (ribonucleic acid) (58) and evidence for an isopentenyladenine-modified protein has been reported (59).

This nucleoside also acts as a plant growth regulator (60). Lining the inner nuclear membrane is a polymeric protein structure called the nuclear lamina, composed of three major proteins, lamins A, B, C. Prelamin A, precursor to the mature lamin A, contains a carboxy terminal farnesyl group; this group is required for the proteolytic removal of an 18-amino acid peptide, along with the isoprenoid moiety, to form the mature protein (61, 62).

Protein Prenylation

The nuclear lamins comprise a small sector of a large group of proteins that are post-translationally modified by a prenyl group after protein synthesis (63-65). These modifications affect the function of the proteins as well as their localization. Three enzymes that transfer a prenyl group (15 or 20 carbons) to a target protein have been identified: farnesyl transferase, geranylgeranyl transferase I and geranylgeranyl transferase II. Target proteins have a distinct C-terminal sequence, CAAX, where C is cysteine, A is any aliphatic amino acid, and X is the signal amino acid for the specific prenyl group added. For farnesyl transferase, X is methionine, serine, or alanine. For geranylgeranyl transferase I, X is leucine or phenylalanine. Geranylgeranyl transferase II will add one or two geranylgeranyl groups to proteins that terminate in a sequence CXCX or XXCC, where C represents cysteine and X, any amino acid. Farnesyl transferase and geranylgeranyl transferase I will modify substrates as short as four amino acids, while the third transferase requires other recognition signal yet to be identified.

Prenylation of "CAAX proteins" is followed by further modifications to the target protein. These include the proteolytic removal of the terminal AAX residues and subsequent methylation of the new carboxy terminal cysteine. Methylation

requires S-adenosyl methione; and substrate recognition requires that the cysteine residue be modified with a farnesyl or geranylgeranyl group. The addition of the methyl group to the prenylated protein, removing the carboxy terminal negative charge, enhances the membrane association of such proteins. Proteins with two geranylgeranyl groups, as in the case of CC or CXC terminal substrates, are also modified in some instances by a fatty acyl group. A stretch of basic residues upstream of the CAAX sequence also positively influences interaction with the lipid bilayer. Proteins modified by geranylgeranyl transferase II that exhibit the C-terminal CXC sequence are methylated after prenylation; those with the CC motif are not methylated.

The significance of prenylation of proteins comes partially from the observation that Ras and Ras-related GTP binding proteins are prenylated. Subsequent research has demonstrated that subunits of trimeric G proteins are also prenylated. These proteins are important participants in signal transduction. Such signaling events may be important in atherogenesis where cell proliferation, lipid deposition, and synthesis and secretion of extracellular matrix material are influenced by such signals (66). It has been estimated (67) that 0.2 to 4% of intracellular proteins may be prenylated.

Mevalonate products are also critically involved with cell growth and proliferation (68), a connection observed when mevalonate-deprived cells did not enter the S-phase (DNA synthesis) of the cell cycle (for reviews, see 68-69). The relationship between cell division and mevalonate availability was observed using highly specific inhibitors of HMG CoA reductase (68), since prior research noted that increased HMG CoA reductase activity correlated closely with DNA synthesis. The effect of HMG-CoA reductase inhibition on S-phase DNA synthesis, moreover, could be relieved by the addition of mevalonate to inhibitor-treated cells. This

observation has stimulated research to identify the mevalonate-related component(s) responsible for cell cycle entry into S-phase. Addition of cholesterol was ineffective in removing the block to DNA synthesis in the S-phase cycle, and inhibition of mevalonate production did not affect DNA synthesis in the G_2, M, and G_1 phases. Mevalonate thus appears to have a dual role in the cell cycle: (1) providing adequate amounts of cholesterol for membrane integrity, and facilitating—either directly or via a mevalonate derived product—activation of S-phase DNA synthesis. Subsequent research investigating this relationship has to date not identified the mevalonate derived product(s). Furthermore, the H-ras protein, which requires farnesylation for activity, is transcribed from a protooncogene (71); thus, the mevalonate pathway could be a potential target for drugs which are designed to limit cell proliferation (72-74).

Mevalonic acid is utilized in the *trans*-methylglutaconate shunt, in which mevalonate is converted in a series of reactions to acetoacetyl coenzyme A(75-82). This product is then available for fatty acid synthesis. The proposed pathway is illustrated in Figure 4, with mevalonic acid first converted to dimethylallyl alcohol and then to dimethylacrylic acid. In steps common with the pathway for leucine catabolism, dimethylacrylic acid is converted to HMG-CoA, which is then cleaved to acetyl CoA and acetoacetate by HMG-CoA lyase. Studies with ^{14}C labeled mevalonate in rats (83) demonstrated a labeling pattern consistent with the proposed shunt. However, direct evidence for the intermediates has not been reported. Investigations in this laboratory (78) demonstrated that mevalonate-2-^{14}C was incorporated into diacylglycerols by bovine aorta, with the greatest amount of label present in medium-chain n-fatty acids.

An alternate pathway for mevalonate-independent biosynthesis of isoprenoids exists in bacteria, algae, and plants (84-88). These organisms synthesize 1-deoxy-D-

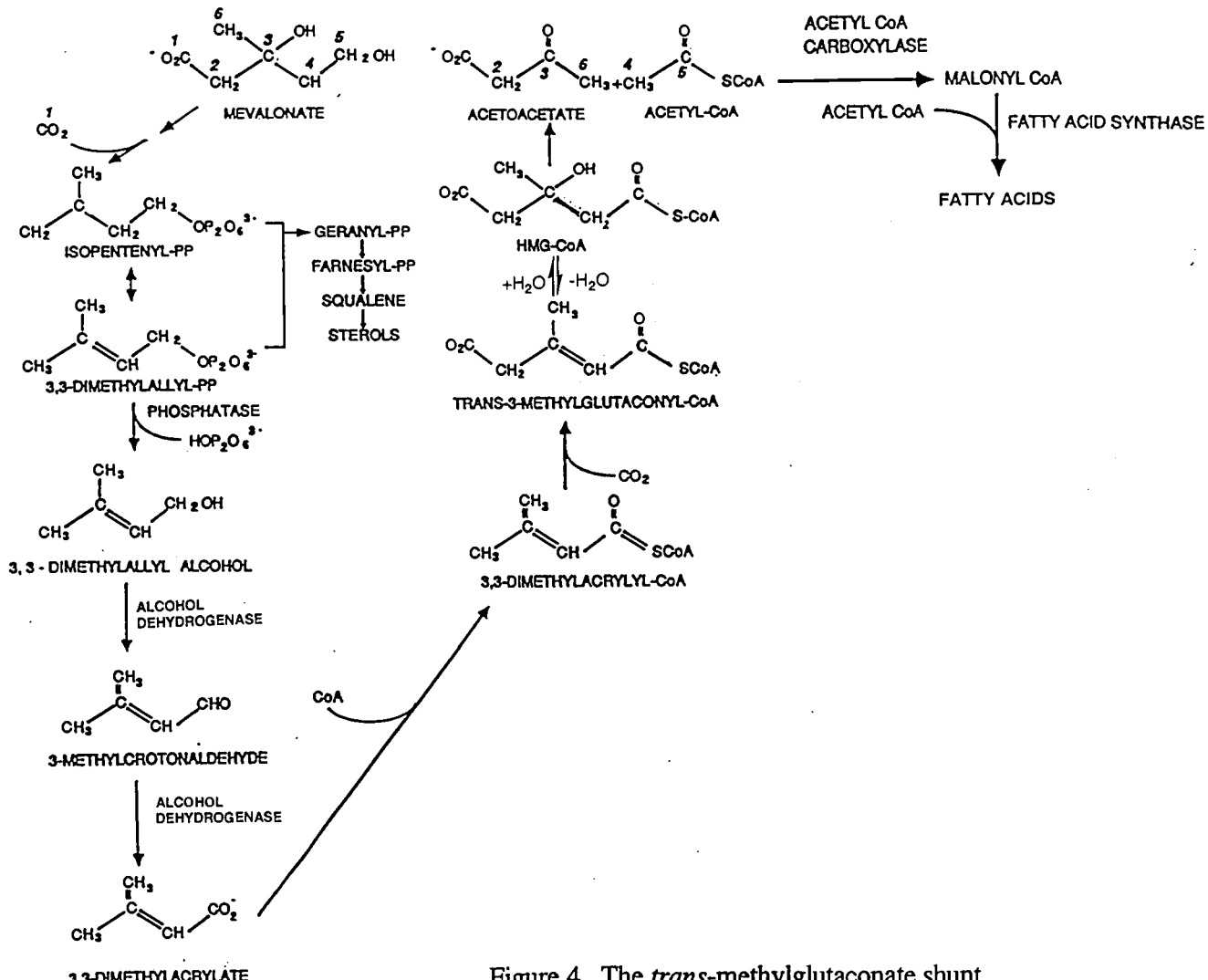

Figure 4. The *trans*-methylglutaconate shunt.

xylulose-5-phosphate from glyceraldehyde-3-phosphate and pyruvate. This intermediate is converted to isopentenyl pyrophosphate for incorporation into isoprenoids. A recent report (89) describes the incorporation of the intact skeleton of the amino acid leucine into sterols by trypanosomatid protozoa. In this study the carbons from leucine were observed in the sterol backbone in a manner consistent with a pathway which utilized dimethylacrylic acid as a precursor to dimethylallyl alcohol.

Cholesterol itself is utilized for the stabilization of cell membranes and is further modified for synthesis of steroid hormones. Recent research in developmental biology has revealed a new role for cholesterol and thus mevalonate. The correct morphogenesis of multicellular organisms depends on the careful and timely signaling so that the organism will develop and grow properly. Signaling often depends on proteins with post translational modifications. One such group of signaling protein molecules are those encoded by the hedgehog gene family. The Hedgehog family of tissue patterning factors are protein(s) covalently modified with cholesterol (90, 91). These signals function in the development in a diverse group of organisms, from embryonic segmentation in insects to neural tube development in vertebrates. The cholesterol-Hedgehog connection arises when the immature Hedgehog protein cleaves itself, acting as an autoprotease. Cholesterol is then covalently linked to the carboxy terminus of the new amino terminal fragment (92-94). The new amino terminal fragment (signaling domain) remains associated with the cell surface, and the Hedgehog-carboxy terminal fragment (the processing domain) then diffuses from the originating cell. This latter domain has no known additional function.

Mevalonate and vascular biochemistry

Mevalonate, because of the biosynthetic products for which it is a precursor, is important in a variety of cellular functions. Research directed toward understanding the utilization of mevalonate in vascular tissue is important, not just because we want to learn more about mevalonate biochemistry in the aorta, but vascular pathobiology enlists those processes in which mevalonate is a critical component: cholesterol biosynthesis, cellular proliferation, signal transduction, and cell metabolism.

Since a clear connection exists between plaque development and accumulation of cholesterol in the vascular wall, a number of attempts have been made to demonstrate the biosynthesis of cholesterol by arterial tissue (reviewed in 45). However, vascular synthesis of cholesterol is low compared to hepatic tissue. Prior studies in this laboratory have demonstrated the low cholesterol-synthesizing ability of the arterial wall (43) from acetate and mevalonate. The bovine aorta has been shown to synthesize mevalonate-5-phosphate and mevalonate-5-pyrophosphate (43) from mevalonate. A cell-free preparation of bovine aorta was used in these studies, as well as in a more recent study in which labeled mevalonate was incorporated into medium (C_8 and C_{10}) n-chain diacylglycerols; this result verified *trans*-methylglutaconate shunt activity in the artery.

Other evidence for mevalonate utilization in arterial tissue has come from the use of inhibitors of HMG-CoA reductase, collectively called statins (96, 97). The inhibition of this enzyme by these compounds reduce the availability of mevalonate to the cell. In clinical settings, statins have been generally shown to lower LDL cholesterol and to have antithrombogenic and anti-inflammatory effects. However, their effects on vascular health are not entirely explained by lowered LDL. Statins appear to exert direct inhibitory effects on cells primarily involved in atherogenesis.

Treatment of cells in culture with these inhibitors (98-100) has shown the importance of mevalonate in normal cell functioning as well as in atheroslerosis. In *in vitro* investigations (101-102), statins inhibited cell cycle proliferation . Further research with vascular smooth muscle cells treated with statins (103-104) showed that muscle cell migration and proliferation were diminished in the presence of these compounds. Plaque stability was increased with the compounds, and macrophages accumulated less cholesterol in the presence of statins.

Inhibitors of the mevalonate pathway have been tested to investigate their effect on cultured and proliferating arterial smooth muscle cells (103). Both compactin (a HMG-CoA reductase inhibitor) and 6-fluoromevalonate, an inhibitor of mevalonic acid pyrophosphate decarboxylase, prevented vascular smooth muscle cell proliferation. Inhibition of squalene synthase and squalene epoxidase blocked cholesterol synthesis but not smooth muscle proliferation. The results of this study also pointed to protein isoprenylation as a probable controlling event in cell proliferation. Similar effects on human and porcine vascular cells (104) were all observed using fluvastatin. Collectively, inhibitors of the mevalonate pathway have generated much interest directed toward both direct atherogenic processes (cholesterol accumulation, cell proliferation), and the development of potential therapeutics in the treatment of proliferative diseases (102).

When vascular smooth muscle cells are deprived of mevalonate using HMG-CoA reductase inhibitors, an impairment of insulin growth factor-insulin signaling is observed, with the secondary impairment of cell proliferation pathways (105). Roullet et al. (106) studied mevalonate availability in isolated whole aortas and observed that exposure to mevalonate decreased blood vessel resistance functions; in a similar study (107) this group tested the level of mevalonate availability on the effects of vasoconstrictors. It was concluded from their results that deprivation of

mevalonate directly contributed to increased vessel resistance function caused by vasoconstrictors. This effect may be related to those observed by Finder et al. (108) relating the expression of nitric oxide synthase-2 (NOS-2) in vascular smooth muscle cells. Treatment with inhibitors of either geranylgeranyl transferase or HMG-CoA reductase caused a 5-10 fold induction of NOS-2 stimulated by the cytokine interleukin-1ß, while inhibitors of protein farnesylation blocked this induction of NOS-2 by interleukin-1ß. These results suggested a tight control of interleukin-1B induction, and hence production of nitrous oxide, a molecule with has extensive physiological effects. These effects include vasodilation and antithrombogenic action (109), and suggest that the NOS-2 synthase protein participates in the control of several atherogenic processes. These results indicated that induction of the NOS-2 synthase depends upon protein isoprenylation.

The effect of cholesterol enrichment in arterial smooth muscle cells was investigated by Pomerantz et al. (111). Cultured smooth muscle cells were induced to take up cholesterol by exposure to modified LDL. This cholesterol enrichment reduced the cell membrane content of proteins known to be modified by isoprenylation: low molecular weight G-proteins and heterotrimeric G-protein complexes. This suggested that high cellular cholesterol reduced both G-protein expression and therefore signaling; these effects occurred by inhibition of protein prenylation and consequently, membrane targeting of signaling proteins in vascular cells.

Resarch Goals

Use of the bovine model

The bovine has been shown to be an excellent model for the study of human atherogenesis (112-114). Prior research in this laboratory has established the ability of post-mitochondrial supernatant prepared from bovine aorta to convert mevalonate to cholesterol (43, 95) and medium chain fatty acids (78).

Research Focus

This research aims to obtain information about vascular wall metabolism using mevalonic acid as a means to gain the information. Mevalonate is a key intermediate in several metabolic processes: biosynthesis of isoprenoids and sterols, prenylation of proteins, the *trans*-methylglutaconate shunt, and control of the cell cycle. A review of metabolic processes related to mevalonate utilization has shown a clear connection between mevalonate metabolism and processes that are directly or indirectly involved in atherogenesis: cholesterol synthesis, cell proliferation, cell migration, and monocyte adhesion. Since it has been shown that mevalonate is utilized at low levels for cholesterol biosynthesis by the aorta, other pathways may be of significance in normal arterial metabolism and in atherosclerotic lesion development. For these reasons, it is appropriate that mevalonate metabolism by vascular tissue be studied. In particular, the postulated intermediates of the *trans*-methylglutaconate shunt have not been directly demonstrated in this or any other tissue. The fate of mevalonate with respect to protein and peptide modification has also not been directly studied in vascular tissue.

Research goals

In this research three questions were to be addressed, relative to the metabolic fate of mevalonate in arterial tissue. (1) Can one demonstrate the direct conversion of mevalonate to the constituents of the *trans*-methylglutaconate shunt? (2) Can one characterize the previously observed radioactive compounds , neutral and acid lipids, which were biosynthesized using mevalonate-2-^{14}C as a substrate? (3) Does the aorta utilize mevalonate to covalently modify peptides and proteins? To answer these questions the first objective was to develop a series of protocols which would permit the investigations in a concerted manner. The combination of the protocols will allow for the identification of mevalonate metabolites. The research will use a previously employed postmitochondrial supernatant of bovine aorta.

MATERIALS AND METHODS

Introduction

This investigation required the use of defined methods as well as the development of procedures needed for the unique requirements of the research. Protocols existed for preparation of bovine aorta and for end analysis of amino acids, among others. However no protocols existed for isolation of the *trans*-methylglutoconate shunt intermediates. Therefore, method development was involved in a significant portion of this project. A combination of techniques (described in this section) was involved to analyze for the intermediate shunt acids, including silica gel column chromatography, thin layer chromatography, and mass spectrometry.

Likewise, a series of methods, was used to isolate and characterize the small peptides, which are too small to be detected by the usual electrophoretic techniques. The peptides also were not significantly bound by ion exchange columns. Therefore, gel exclusion chromatography, membrane filtration, thin layer chromatography, and paper chromatography were used to separate the peptides found in this work. Some protocols, however, remained essentially as described in earlier research from this laboratory (78).

Chemicals, unless specified otherwise, were of reagent grade. Vendors of specific reagents and supplies are noted in the text, or were purchased from Fisher Scientific Company, Santa Clara, CA.

Preparation of the crude homogenate of bovine aorta

Abdominal aortas, from cattle two to three years old, were obtained immediately after sacrifice and stored on ice. Animals brought to the Carlton Packing Company in Carlton, Oregon were the source of this tissue. During the preparation of the crude homogenate the aortas were kept cold and protective gloves were worn to prevent contamination of the tissue. The vessel length was typically 12-14 inches, measured from just above the point where the aorta bifurcates into the femoral arteries.

Exterior fat and adventitia were removed with scissors and the trimmed vessels were then cut into small pieces approximately 0.25 cm^2. The minced tissue was stored in large beakers on ice prior to the homogenization step. A Waring blender at high speed was used to grind the tissue: 100 grams of tissue were combined with 100 ml cold 100 mM potassium phosphate buffer, pH 7.0, containing 6 mM $MgCl_2$. The homogenized tissue was filtered through two layers of cheesecloth; the combined filtrate was stored, at -20°C , in approximately 150 ml aliquots. This filtrate, referred to as the crude homogenate, could be stored frozen for three to four months without loss of activity (95).

Preparation of the postmitochondrial fraction of the crude homogenate

The crude homogenate was centrifuged in a Beckman Model L-65 ultracentrifuge using a 30S rotor at 60,000 x g for 120 minutes. The resulting clear red supernatant was pooled and stored on ice until further use. This cell-free preparation is referred to as the postmitochondrial supernatant, and was used in experiments within several hours after the centrifugation step.

Preparation of mevalonic acid-2-^{14}C

DL-mevalonic acid-2-^{14}C (MVA-2-^{14}C) was obtained as a solution of the N-N'-dibenzylethylenediamine salt, from Dupont New England Nuclear Laboratories (Boston, MA). It was necessary to remove the amine and to obtain mevalonate as the free acid for use in the incubation experiments with the postmitochondrial supernatant. An aliquot of the ethanol stock solution (5-10 μCi, 50.4 mCi/mmole) was evaporated under nitrogen gas and then redissolved in three mls of water. The aqueous solution was adjusted to pH 11 with 1 N KOH and allowed to stand at room temperature overnight, then extracted four times with one ml of diethyl ether each time to remove the liberated amine. The aqueous layer was adjusted to pH 7 with 1 N HCl, then diluted with water to a final activity of 0.5-1.0 μCi per ml, and stored -20 ^{o}C. The purity of this preparation was monitored throughout this study by thin layer or paper chromatographic techniques.

Methods of detecting radioactivity in metabolites

Radioactivity in dissolved samples was detected by scintillation counting; for thin layer and paper chromatograms, Geiger counter technology (Bioscan System 200 Imaging Scanner, Bioscan Inc., Washington, DC) was employed. Scintillation fluid for toluene-soluble samples contained four grams of 2,5-diphenyloxazole) (PPO) and 30 mg of *p-bis*-[2-(5-phenyloxazolyl)-benzene (POPOP)]per liter of scintillation grade toluene. Aqueous samples were counted in Aquasol, a commercially available aqueous fluor, obtained from New England Nuclear (Boston, MA). Samples were mixed with ten ml scintillation fluid in glass vials for counting.

The concerted protocol

The sections below and Figures 5, 6 and 7 diagram the concerted protocols Throughout this project both incubated (for 2 hours, 55 minutes) and initial time(0 to 15 minutes) incubation mixtures were separated into various fractions to investigate the incorporation of mevalonate-2-^{14}C into separate fractions. Figure 5 illustrates the first steps in obtaining the two main fractions, the soluble peptides and the aqueous fraction.

Figure 6 shows how the aqueous fraction and extracted lipids were obtained and analyzed. Figure 7 diagrams the protocols used to characterize the peptide components isolated in both the soluble peptide fraction and the aqueous extract. The use of the concerted protocol allowed for observation of components of a fraction by using different systems and comparison of that behavior with known standards. This allowed for deduction of the identity of the component. This strategy was particularly useful in the identification of the *trans*--methlylglutaconate shunt intermediates. The concerted protocols shown in Figures 5, 6, and 7 give a general approach to the research and do no necessarily reflect the absolute order of protocols used in all the experiments.

Method of incubation of the postmitochondrial supernatant with mevalonic acid-2-^{14}C

The postmitochondrial supernatant was kept on ice until the incubation period was initiated (Figure 5). Incubations were carried out in Erlenmeyer flasks and contained, per five ml of supernatant, 16.9 μmoles of adenosine triphosphate (ATP), 0.05-0.1 μCi mevalonate-2-^{14}C, and additional phosphate buffer to obtain a final

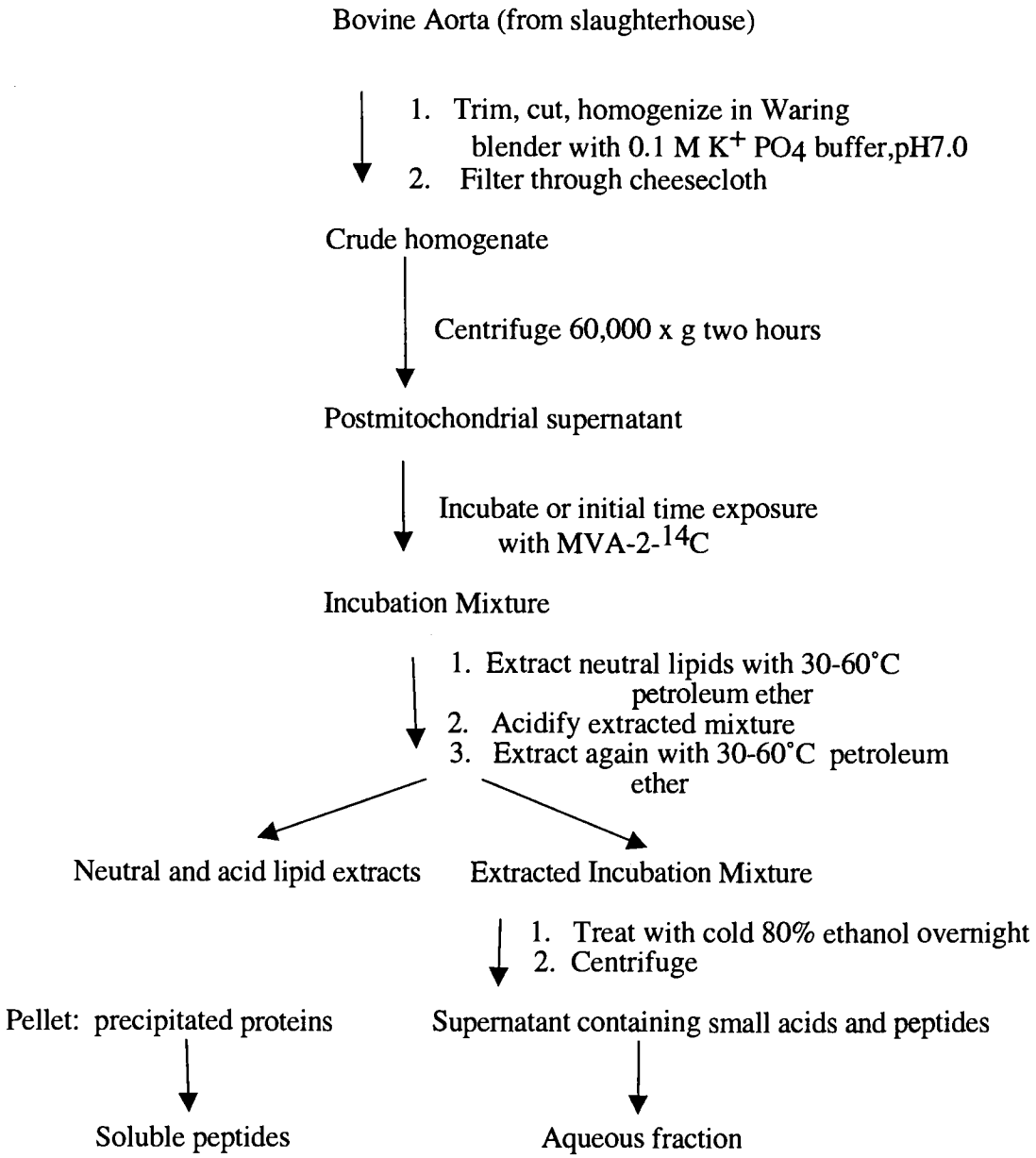

Figure 5. Isolation of various fractions from postmitochondrial supernatant. The figure diagrams the steps leading to the isolation of the supernatant (aqueous fraction) and protein pellet remaining after the precipitation of proteins. The pellet was the source of the soluble peptide fraction.

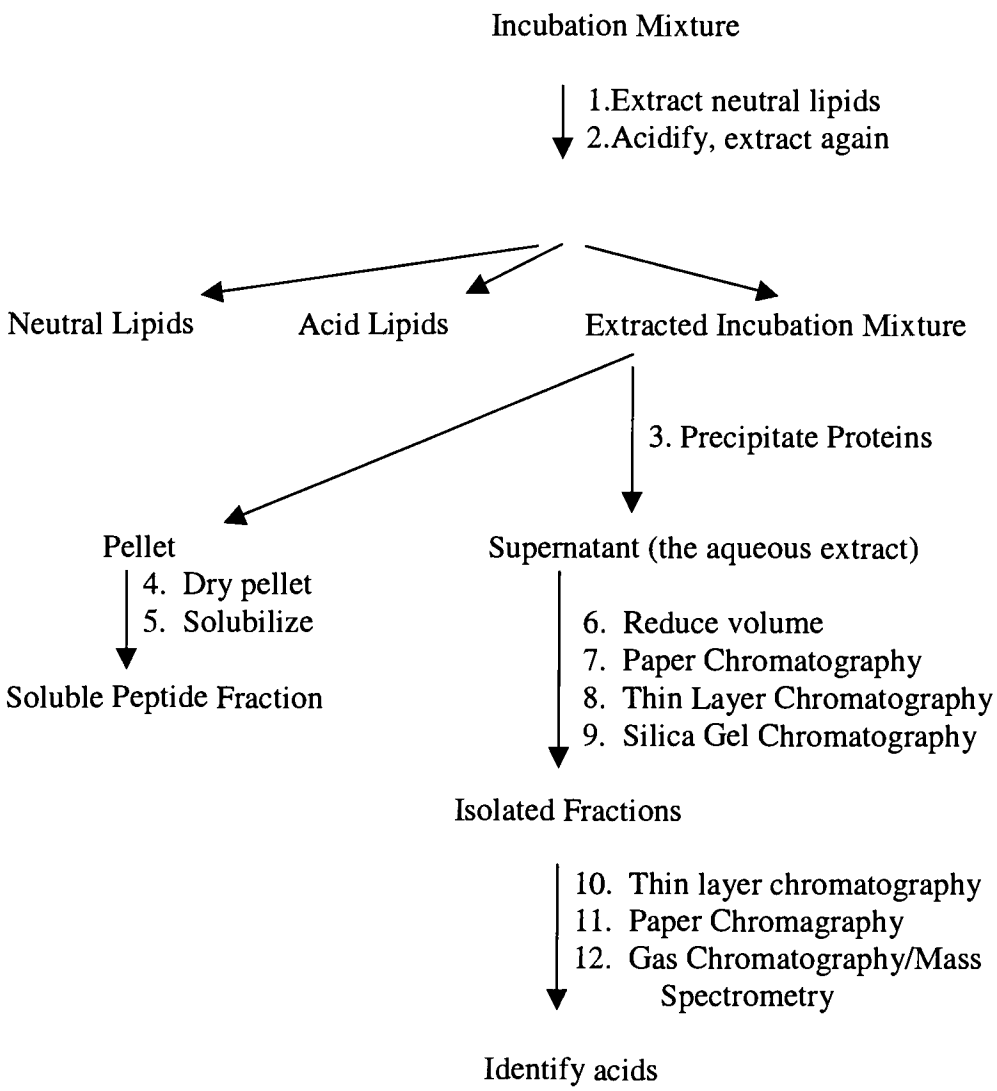

Figure 6. Protocols for the isolation and characterization of lipid and acid components in the incubation mixture.

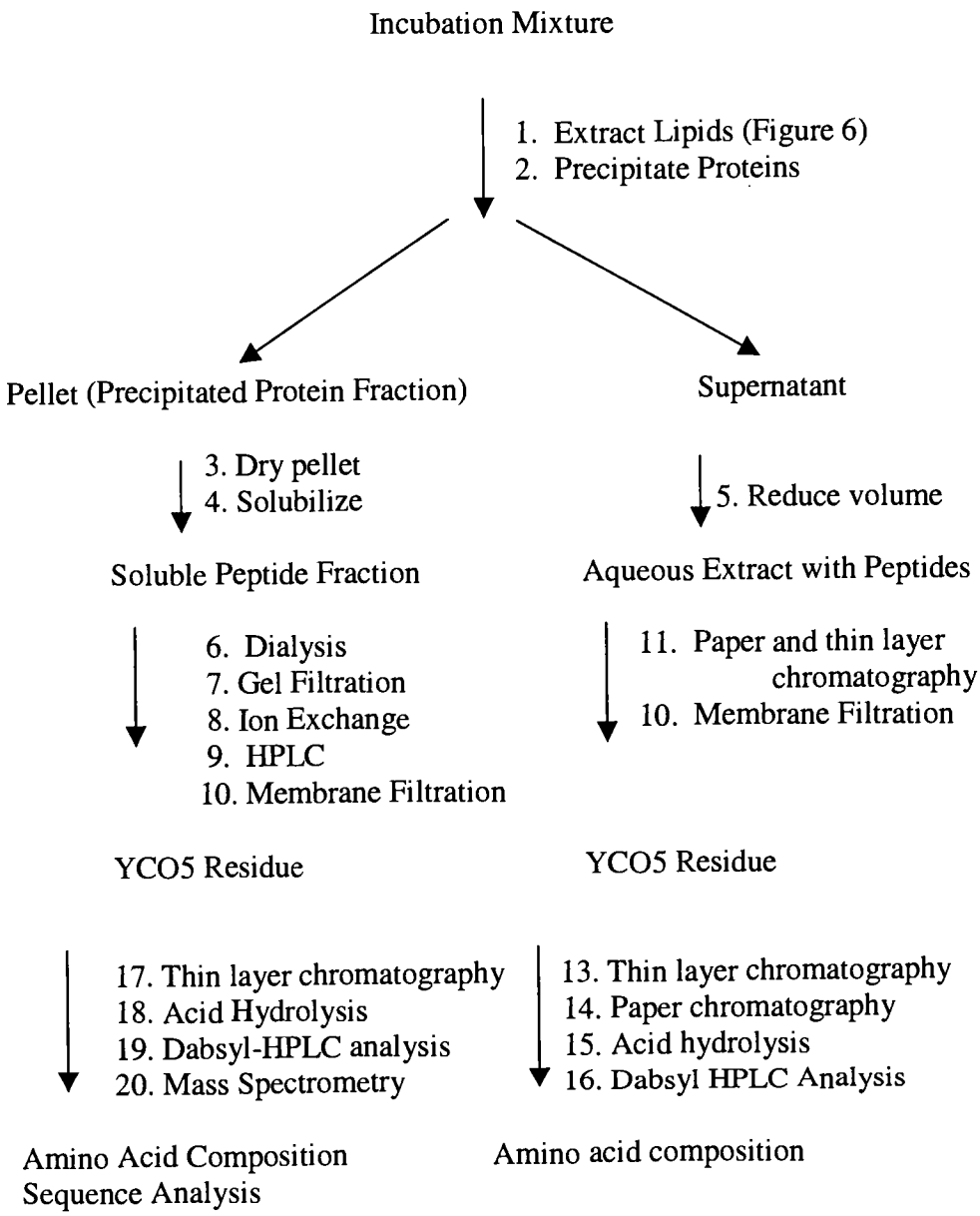

Figure 7. Protocols for characterization of peptides in both the soluble and aqueous extract fractions.

volume of 10.5 ml. In larger incubations additional buffer was omitted. The Erlenmeyer flasks were sealed with rubber stoppers; small incubations (10.5 ml total volume) used 125 ml Erlenmeyer flasks while large incubations (60-120 ml total volume) used 500 ml Erlenmeyer flasks. The amount of ATP in the incubation mixtures was kept at the ratio of 16.7 μmoles/5 ml supernatant. The flasks were incubated for two hours and fifty minutes in a 37°C oscillating water bath. These conditions had been established for optimal incorporation of mevalonate-2-^{14}C into a neutral lipid fraction. At the end of the incubation period, 50 ml of petroleum ether (distilled at 30-60°C) was added and the incubation mixture was kept at -20°C until further analysis. Initial time samples were obtained by rapidly freezing (0 to 15 minutes) the incubation mixture in a dry ice/acetone bath immediately after the addition of mevalonate-2-^{14}C, then adding petroleum ether and storing as before.

Large incubation mixtures for isolation of labeled fractions utilized the same protocols, with amounts of label and ATP adjusted according to the volume of supernatant used. Typically, 50 ml of the postmitochondrial fraction was used in a preparative incubation. Two hundred mls of distilled petroleum ether were added to preparative incubations containing 50 ml postmitochondrial supernatant at the end of the incubation period. or to frozen initial time samples as soon as freezing was complete. The incubation mixtures were stored at -20°C immediately after addition of the petroleum ether. Control samples contained no postmitochondrial supernantant but contained equivalent amounts of 0.M phosphate buffer, ATP, and mevalonic acid-2-^{14}C, and were subjected to the same incubation, extraction, and isolation procedures. The specific procedures for the isolation of labeled fractions are explained in the following sections. An inactivated protein control sample showed all added mevalonic acid-2-^{14}C remained unchanged (data not shown) throughout the incubation and isolation procedures.

Extraction of neutral lipids from the incubation mixture

Incubation mixtures were thawed and extracted with distilled (30-60°C) petroleum ether (Figure 6, step 1). For analytical incubations, utilizing 5 ml of postmitochondrial supernatant, a total of 200 ml petroleum ether was used; 50 ml petroleum ether was added to the incubation mixture and the flask gently swirled for 10-20 seconds. The extract was decanted to a second flask containing 30 gms of anhydrous sodium sulfate, and the extraction procedure repeated three more times, combining the extracts from each flask. For preparative experiments, a total of 800 ml distilled petroleum ether was used for each incubation mixture and dried over 120 gm anhydrous sodium sulfate. The extracts were filtered through Whatman No.1 paper to a second flask along with petroleum ether washes of the sodium sulfate. The petroleum ether was carefully evaporated on a steam bath for 45 minutes leaving the neutral lipid fraction. The activity of each aorta preparation (crude homogenate) was assessed by noting the amount of mevalonate-2-^{14}C derived label in this fraction by scintillation counting, using the toluene fluor. Different aorta preparations were compared in this manner. The volume of the petroleum ether extract could also be reduced by careful distillation below 55°C and the reduced volume extract stored at -20^{o} C until used for further analysis.

Extraction of the acid lipid fraction from the incubation mixture

The incubation mixture, after extraction of the neutral lipids, was acidified by addition of the protein precipitant trichoroacetic acid or by adjusting the pH to 2 with 6 N HCl (Figure 6, step 2). The acidified incubation mixture was then extracted with distilled petroleum ether. The same protocol for extraction of the neutral lipids was

used for the extraction of the acid lipid fraction. After careful removal of the petroleum ether on a steam bath, the residue remaining in the flask was taken up in two ml of petroleum ether and stored at -20°C until used for further analysis.

Precipitation of protein from the incubation mixture

The protein fraction of the incubation mixture was isolated (Figure 7, step 2) by adding cold 95 percent ethanol to a final concentration of 80 percent ethanol, then allowing the mixture to stand at -20°C overnight. The mixture was centrifuged at 1500 x g in a GSA rotor in a Sorvall centrifuge with the chamber kept at 5°C. The supernatant was carefully decanted and saved for the preparation of the aqueous extract. The protein pellet was washed first with 95 percent ethanol and then 100 percent ethanol, centrifuging in Cortex tubes using a SS34 rotor at 5000 x g for 10 minutes with each wash The supernatants from these ethanol washes were saved and combined with the original supernatant. The protein pellets were carefully dried, weighed, and stored at -20°C. For the purposes of this study this protein is referred to as precipitated protein fraction (Figure 7, steps 3,4).

In the initial stages of this study, proteins were precipitated by the addition of 10 percent (w/v) trichloroacetic acid (TCA) to a final concentration of 4.3 percent and the protein pellet isolated by centrifugation as above. The TCA-precipitated pellets were washed with water and 95 percent ethanol; the supernatants were combined with the original supernatant. The protein pellet was dried, weighed and stored frozen as above. Ethanol was chosen as the preferred precipitant over TCA because the presence of TCA complicated subsequent analyses of the protein supernatant. Ethanol, in contrast, can be easily removed by evaporation.

Preparation of the aqueous extract and the soluble proteins

The steps in the preparation and characterization of the aqueous extract are shown in Figure 6 (steps 1, 3, 6-12). The aqueous fraction remaining after precipitation of proteins is the combined supernatant and ethanol washes of the protein pellet. This combined fraction is taken to dryness under vacuum using a rotary evaporator, and contains unreacted mevalonic acid as well as the soluble components of the postmitochondrial fraction. This fraction is the dried aqueous extract.

Several solvents were used to bring the dried aqueous extract into solution to further characterized the labeled components: ethanol, ethyl acetate, water, and glacial acetic acid. Glacial acetic acid proved to be the best solvent and was used on a routine basis. For column chromatography, distilled, deionized water was used to dissolve the sample. Once the material was in solution, it was referred to as the aqueous extract.

For preparative experiments, employing 50 ml of postmitochondrial supernatant, 10 ml of solvent was used to transfer the aqueous extract to test tubes for freezer storage. Resolubilizing as well as freezing and thawing the aqueous extract would occasionally result in some insoluble materials. Such material was removed by brief centrifugation in a clinical centrifuge at room temperature as a general protocol. These insoluble materials were not radioactively labeled as assayed by scintillation counting.

The protocol for the isolation of the soluble peptides is included in Figure 7. The protein precipitate from the incubation mixture was air dried, crushed to a fine powder, and stored -20°C until used. The dried protein was solubilized in water and any insoluble material was removed by centrifugation for 15 minutes at 5000 x g

in a Sorvall centrifuge. The temperature of the chamber was kept at 5°C. Subsequent investigations of the precipitated proteins utilized only the supernatant fraction from this step. This fraction is referred to as the soluble peptide fraction. Typically, the dried, precipitated protein from a 50 ml of the postmitochondrial supernatant was solubilized in 10 ml water.

The soluble peptide fraction was first dialyzed against water (Figure 7, step 6). Spectrapor 6 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cutoff of 2000 was used. The soluble protein fraction from a 50 ml post mitochondrial supernatant incubation was dialyzed against one liter of water for 48 hours at 5°C. The dialysate was transferred and stored frozen. The dialysant, outside the membrane, was reduced in volume on a rotary evaporator. The water bath temperature for large aqueous samples was kept at 45-50°C during evaporation.

The material remaining after evaporation of the dialysant of the soluble protein fraction is referred to as the soluble peptide fraction. In later experiments, sequential filtration was employed using Amicon membranes (Amicon Corporation, Danvers, MA) in place of the dialysis step for the soluble peptide fraction.

Determination of protein concentration

A modified Lowry method (115, 116) was routinely used to estimate the amount of protein in samples. Bovine serum albumin (BSA) was used as the protein standard, at a concentration of 0.5 mg/ml in water. The protein sample, standard (5 to 100 μgm protein) or blank volume was 1.0 ml. One ml of reagent containing one part of copper tartrate solution (0.1% copper sulfate, 0.2% potassium sodium tartrate, and 10% sodium carbonate), one part 0.8 N sodium hydroxide, and two parts water were added to the protein or blank sample. The samples were allowed to

stand at room temperature for ten minutes, then 0.5 ml of diluted (one part to five parts water) Folin-Ciocalteau reagent (from Sigma Chemical Co., St. Louis, MO) is added with rapid vortexing. The absorbance at 750 nm was read 30 minutes later.

The Bradford method of protein determination was also used (117) using commercially available Bradford reagent (BioRad Laboratories, Inc., Richmond, CA), and bovine serum albumin (stock solution (0.5 mg/ml in water) as a standard. This method was used with the soluble proteins. The total volume of the protein sample and the standard sample was 0.8 ml; water was used, if necessary, to bring the final volume to 0.8 ml. The standard solutions contained 2 to 20 μgm of protein, and BSA was again used as the standard. The sample volume, containing either standard (2 to 20 μgm protein) or protein from arterial sources, was made up a final volume of 0.8 ml with water; samples are then vortexed with 0.2 ml of the dye reagent. The samples are read at 595 nm within one hour after addition of the dye. The dye reagent is used without dilution and potentially interfering substances are included in standard and blank solutions if they are present in the protein sample.

The ultraviolet absorbance at 260 and 280 nm of samples was determined with the Hewlett Packard diode array spectrophotometer. This information was used to monitor the spectral characteristics of fractions from column chromatography and was not used to determine the quantity of protein.

Gel filtration chromatography

Bio-Gel P gels (BioRad Laboratories, Richmond, CA) were used as column supports in liquid chromatography. This support consists of crosslinked polyacrylamide beads which are defined in their ability to resolve particular molecules within a specific molecular weight range or exclusion limit. In this study,

Bio-Gel P columns were used, as described below, to characterize the soluble peptide fraction from the precipitated protein (Figure 7, step 7). Fractions from the column were collected with a Pharmacia Model Frac-100 fraction collector and were approximately 6.5 ml in volume for all procedures. All glass columns were used in the chromatographic steps, and chromatographic procedures were carried out at room temperature. The column solid support was washed, prior to sample application, with eluting buffer or water until there was no absorbance reading at 260 and 280 nm in the eluant. Water used throughout this study was deionized and glass distilled before use.

Bio-Gel P100 chromatography

A column bed of 28 cm x 2.5 cm was prepared with Bio-Gel beads using 0.1 M potassium phosphate buffer, pH 7.0, containing 0.006 M $MgCl_2$. The protein sample was dissolved in a small volume (two to three ml) of water and applied to the column, followed by an equal volume of water. Elution was carried out with the same phosphate buffer at a flow rate of 1.5 ml per minute. The absorbance at 260 and 280 nm was measured for each fraction, and aliquots of each fractions were counted in Aquasol to measure the radioactivity present.

Bio-Gel P6 chromatography

The dimensions of the Bio-Gel P-6 column were 25.0 cm x 1.0 cm and the beads were packed in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.006 M $MgCl_2$. The protein sample was dissolved in water and applied to the column followed by an equal volume of water. The flow rate was 1.3 ml per minute. The absorbance at 260 and 280 nm was determined for each fraction and aliquots were

taken from fractions to measure radioactivity by liquid scintillation counting in Aquasol.

Bio-Gel P2 chromatography

A 33 cm x 1.5 cm Bio-Gel P-2 column was packed in water, and washed with water until there was no absorbance at 260 and 280 nm in the eluent. Protein samples were dissolved in two ml water for application to the column; this was followed another two ml of water applied to the column. Elution continued with water and the flow rate was set at 0.8 ml per minute. Radioactivity and ultraviolet absorbance of each fraction were determined as described above.

Ion Exchange Chromatography

Anion exchange chromatography

Cellex E (Bio-Rad Laboratories) was used as the exchange media, packed into 1 cm x 20 cm column (Figure 7, step 8). The column was washed with 1 M LiCl to remove contaminants, as monitored by ultraviolet absorbance at 260 and 280 nm, then with water to remove any residual LiCl. The sample, dissolved in two ml water, was applied to the column followed with two ml water; the sample was at pH 7.0. The column was eluted using a linear gradient of 0 to 1 M LiCl, in a total volume of 800 ml. A flow rate of one ml per minute was maintained. Aliquots of fractions were counted in Aquasol to measure the radioactivity by means of liquid scintillation counting; the absorbance at 280, 260, and 250 nm of each fraction was determined.

Cation exchange chromatography

Bio-Rex-70 (Bio Rad Laboratories) was used as the cation exchange media in a 1.3 cm x 20 cm column (Figure 7, step 8). LiCl (1 M) was used to wash the column until all ultraviolet absorbing materials were removed; water was then used to remove residual LiCl. Proteins were dissolved in water and the sample (3.1 ml, at pH 7.0) was applied to the column, followed by two mls water. A linear salt gradient of 0 to 1 M LiCl, in a total volume of 800 mls, was used to elute the column. Fractions (6.5 ml volume) were scanned for absorbance at 280, 260 and 250 nm and aliquots of each fraction were counted in Aquasol to measure radioactivity by a liquid scintillation counter.

Membrane Filtration

Both the soluble protein fraction and the aqueous extract residues from the preparative incubation mixtures were dissolved in 20 ml water for membrane filtration purposes (Figure 7, steps 10, 12). This process was carried out stepwise with each sample using an Amicon stirred cell apparatus (Model 543-70) and nitrogen gas pressure at 60 psi.

The sample was first filtered through an Amicon YM1 membrane (Millipore American Corporation, Bevelry, MA) with a molecular weight cutoff (MWCO) of 1000 daltons. After passage of the solution through the filter, the material remaining on the filter (YM1 residue) was saved by addition of small amounts (1-2 ml) of water. Several washes were combined to remove all of the YM1 residue. The solution passing through the filter is referred to as the YM1 filtrate. This filtrate was subsequently passed through an Amicon YCO5 (500 MWCO) membrane to produce the YCO5 residue and the YCO5 filtrate. The YCO5 residue was also transferred

with several ml of water. This solution was stored at -20°C when not in use. The filtrate samples were reduced in volume as necessary with a rotary evaporator and stored -20°C for subsequent investigations. Membrane filtration gave the same separation results as prior experiments with column chromatography and dialysis, and was used directly with either the soluble fraction or the aqueous extract.

Thin layer chromatography

Thin layer chromatography was used as both an analytical and preparative procedure to separate components in both peptide and aqueous extracts (see Figures 6 and 7). Silica gel G ("Adsorbosil Plus 1"), containing a $CaSO_4$ binder, were obtained from Alltech Corporation (Deerfield, IL). Cellulose plates (Avicel, a microcrystalline cellulose) were obtained from Analtech Corporation (Newark, DE). All plates were 20 x 20 cm unless otherwise indicated. Samples were applied to the plate using a glass syringe or disposable glass capillary tubes. The origin was set at 2.5 cm from the bottom of the plate. Sample solvent was removed during application with a stream of warm air from a hair dryer.

Development of the thin layer plates was carried out in glass chambers lined with Whatman #1 or #3 paper, sealed with plastic wrap, and covered with weighted glass lids. Plates were supported during development by metal frames. Development was allowed to continue until the solvent front reached a point 2.0 cm below the top of the plate. The solvent was removed from the thin layer plates, after development, in the air draft of the laboratory hood.

Thin layer chromatography of carboxylic acids

The aqueous extract (Figure 6, steps 8, 10) was used as a source of water soluble carboxylic acids. This extract was obtained from incubated or initial time samples of postmitochondrial supernatant incubated with mevalonate-2-^{14}C. Silica Gel G plates were used and plates were developed in an acid solvent, n-butanol/chloroform/acetic acid/water, 100/100/4/30 using the organic phase; or a basic solvent, ethanol/concentrated ammonia/water, 160/20/20. Acids were detected on the plate by spraying with a solution of 0.4% bromocresol green in ethanol at pH 7.0. Under these conditions acids appear as yellow spots on a blue background. The sensitivity of this method of detection is one μgm of sample. The thin layer chromatography of standards is shown in Figures 8 in an acid solvent and in Figure 9 in a basic solvent.

Thin layer chromatography of amino acids and peptides

Silica Gel G plates were used in the separation of amino acids and peptides (Figure 7, steps 13, 17). Plates were developed in n-butanol/acetic acid/water, 4/1/5, using the upper, organic layer as the developing solvent. Amino acids and peptides were detected by spraying the plate with a ninhydrin-cadmium reagent. This reagent contains 0.2 percent ninhydrin dissolved in a solution containing 0.5 gm cadmium chloride, 40 ml acetic acid, 50 mls water in a final volume of 500 ml acetone (118). Colored spots appear after heating the plate in a 110°C oven for 10 minutes. Ninhydrin sensitive materials appear as predominantly pink spots on a white background. The sensitivity of this method is also one μgm of material.

Figure 8. Thin layer chromatography of *trans*-methylglutaconate shunt intermediates in an acid solvent. The organic phase of the mixture of n-butanol/chloroform/acetic acid/water (100/100/4/30) was used. Detection was with bromocresol green. Fifty μgms of each sample was applied: (A) ß-hydroxy-ß-methylglutaric acid, (B) β-methylglutaconic acid, (C) β-hydroxybutyric acid, (D) acetoacetic acid, (E) dimethylacrylic acid and (F) mixture of standards

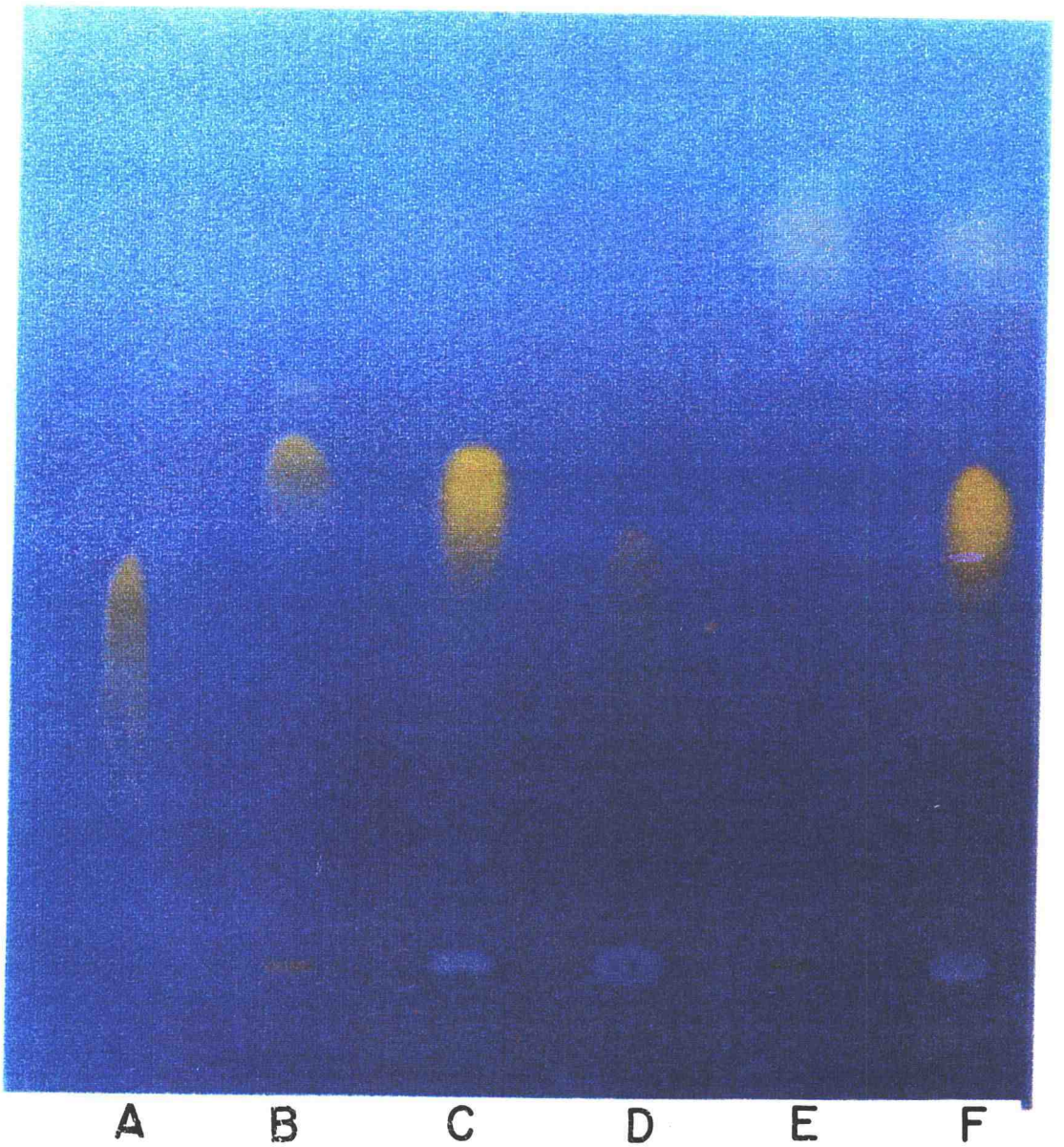

Figure 8

Figure 9. Thin layer chromatography of the *trans*-methylglutaconate shunt acids in a basic solvent. The solvent used was ethanol/water/ammonia, 160/20/20 Fifty μgms of each acid was applied and detection was with bromocresol green: (A) ß-hydroxy-ß-methylglutaric acid, (B) β-methylglutaconic acid, (C) β-hydroxybutyric acid, (D) acetoacetic acid.

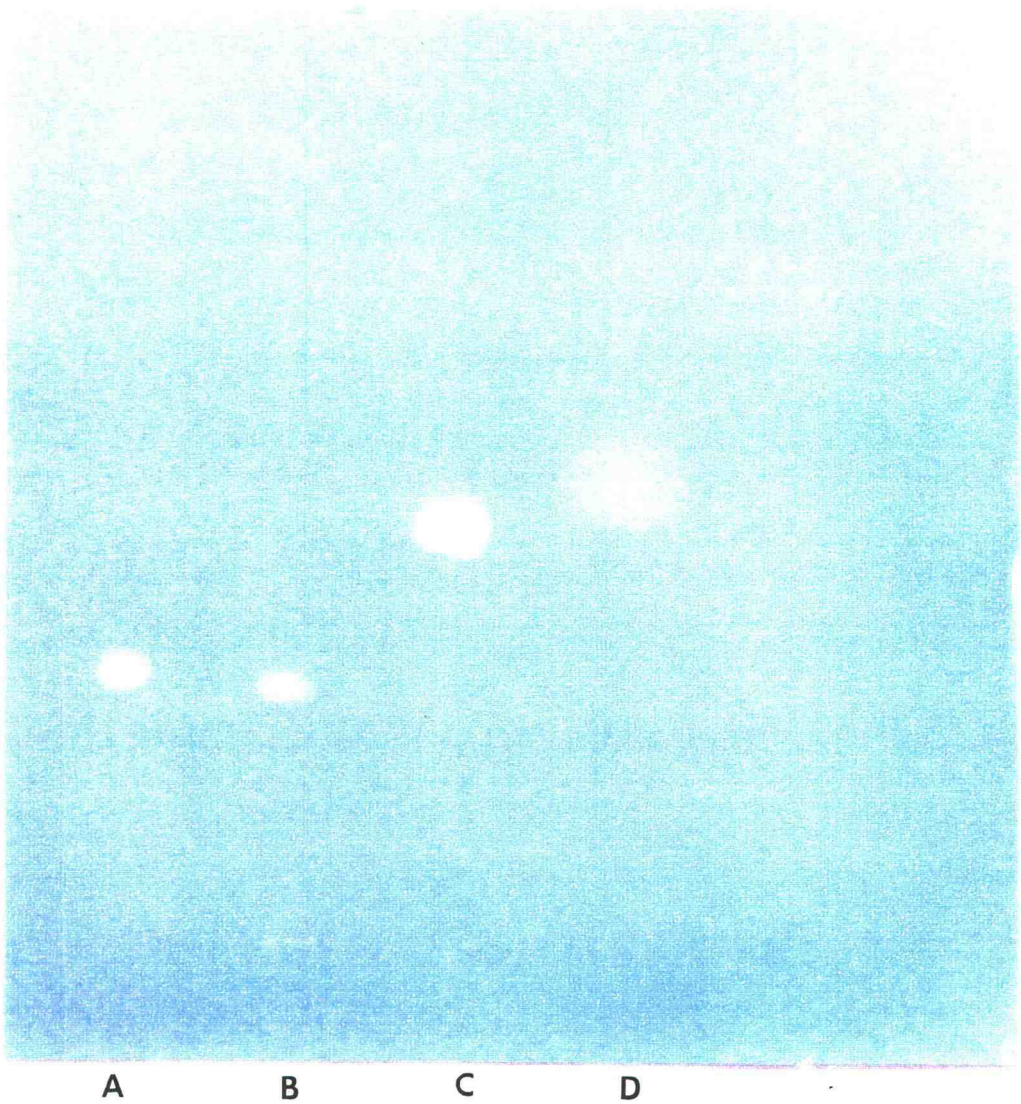

Figure 9

Thin layer chromatography of amino acids and peptides

Silica Gel G plates were used in the separation of amino acids and peptides (Figure 7, steps 13, 17). Plates were developed in n-butanol/acetic acid/water, 4/1/5, using the upper, organic layer as the developing solvent. Amino acids and peptides were detected by spraying the plate with a ninhydrin-cadmium reagent. This reagent contains 0.2 percent ninhydrin dissolved in a solution containing 0.5 gm cadmium chloride, 40 ml acetic acid, 50 mls water in a final volume of 500 ml acetone (118). Colored spots appear after heating the plate in a 110°C oven for 10 minutes. Ninhydrin sensitive materials appear as predominantly pink spots on a white background. The sensitivity of this method is also one μgm of material.

Preparative thin layer chromatography

Preparative thin layer chromatography (Figure 7, step 13) was used to separate the peptide components of the aqueous extract that were obtained by membrane filtration, so that quantities of peptide sufficient for further analysis could be obtained. The YCO5 residue was used as the sample. Samples for preparative separation by thin layer chromatography are applied as a 0.5 cm wide, 15 cm streak at the origin. The origin band was located 2.5 cm from the bottom of the plate. Up to 1.2 mg of protein were applied to a single plate; development was the same as described for analytical procedures. An aliquot of the preparative sample was run on the same plate for detection purposes so that the material in this lane could be sprayed with ninhydrin and the resultant color development could be used as a guide in selecting the preparative band. Bands of interest were removed by scraping the silica from the plate into glass funnels lined with Whatman #1 fluted filter paper. The elution solvent was then added dropwise to the silica to remove the target sample.

The eluting solvent was removed either by evaporation (rotary evaporator) or under a stream of nitrogen gas. Several solvents or a combination of solvents were employed in the elution of material from silica but experience with these solvents demonstrated that glacial acetic acid was the solvent of choice for the first elution of chromatographed components. The silica was then eluted with a mixture of tertiary amyl alcohol and 12 N HCl in a ratio of 15:1. The amount of each solvent used was 4.0 ml for the each band removed from the preparative plate.

Paper chromatography

General procedures

Paper chromatography was utilized to isolate components of various fractions and to separate and identify amino acids, carboxylic acids, and nucleotides(Figure 6, steps 7, 10; Figure 7, steps 11, 13). Whatman No. 1 paper was routinely used in analytical separations and Whatman No. 3 paper was used in preparative experiments. In procedures using descending solvent migration, the mobile phase was then placed in the glass trough which was mounted at the top of a chromatography chamber. The trough was held in place by a glass ledge in the chamber itself or by an apparatus within the chamber. The paper chromatogram was weighted in the glass trough with a glass rod before the addition of the mobile phase. After sealing the jar with plastic wrap and a weighted glass cover, development proceeded until the solvent migrated to a point within 2.5 cm below the top edge of the paper. In ascending solvent techniques the mobile phase was placed directly in the bottom of glass jars or in a glass tray, and chromatograms paced upright in the solvent. Sewing the vertical edges together with cotton thread or stapling the edges

together (in non-acid solvents) allowed construction of cylinders that provided for solvent migration without the need for support apparatus. Analytical and preparative samples were applied as described for thin layer chromatography. The mobile phase on paper chromatograms was removed by placing the chromatograms in the air draft of the laboratory hood; this would generally remove all the developing solvent. Amino acids and carboxylic acids were detected by spraying with ninhydrin or bromocresol green, respectively, as described for thin layer chromatography (page 36).

Components in both paper and thin layer chromatography could be characterized and compared by computing their Rf value on a particular chromatogram. The Rf value is the ratio of the distance (measured from the origin to the center of the particular spot) a component has migrated compared to the distance (also measured from the origin) to which solvent has migrated (the solvent front). The use of Rf values from several different systems and techniques allowed for the deduction of the identity of the component of interest.

Paper chromatography of organic acids

Sheets of Whatman No. 1, 21x50 cm, were used in a descending technique, with n-butanol/isobutanol/water/90 percent formic acid, 30/60/90/30 (organic layer) as the solvent. Bromocresol green (0.04 per cent in ethanol) was used as the detection agent for these chromatograms. A second solvent system (119), n-butanol saturated with 1.5 N NH_4OH, was used in ascending chromatography. Papers run in this second solvent system were sprayed with 0.04 percent bromocresol purple

after exposure to an atmosphere saturated with a 3 percent solution of ammonia. Chromatograms of standard acids are shown in Figures 10 in the acid solvent and in Figure 11 in a basic solvent.

Paper chromatography of amino acids and peptides

For separation and identification of component amino acids in peptides, two dimensional protocols were used. In one approach (120) a sample of hydrolyzed peptide was applied to one corner of a 23x50 cm sheet of Whatman No. 1 paper. Development (descending) in the first direction was done with water-saturated phenol in the presence of 0.3 percent ammonia. The chromatogram is prepared on Whatman No. 1 paper. Development (descending) in the first direction was done with water-saturated phenol in the presence of 0.3 percent ammonia. The chromatogram is then rotated 90^{o} so that the chromatogram can be developed in the second direction in the ascending mode. The solvent used in the second direction was n-butanol/acetic acid/water, 4/1/5, using the organic phase. In later experiments, a second two-dimensional solvent system was used.

The second method for two dimensional separation of amino acids is that of Redfield (121), . Both directions were done in the ascending mode. For the first dimension, a mixture of methanol/water/pyridine, 60/20/20 was used and development was continued for 14 hours. After drying, the chromatogram is again rotated 90° and developed in n-propanol/water/diethylamine, 85/15/3 for 14 hours. The chromatograms were dried thoroughly before spraying. Figure 12 shows the chromatography of amino acid standards by this method.

Figure 10. Paper chromatography of the *trans*-methylglutaconic shunt acid standards in an acid solvent: (A) ß-hydroxy-ß-methylglutaric acid, (B) ß-methylglutaconic acid (C)ß-hydroxybutyric acid, and (D) dimethylacrylic acid.

Figure 10

Figure 11. Paper chromatography of *trans*-methylglutaconic standard acids in a basic solvent. The solvent was n-butanol saturated with 1.5 N NH_4OH. Left to right, the samples are: (A) ß-hydroxy-ß-methylglutaric acid, (B) ß-methylglutaconic acid, (C) ß-hydroxybutryic acid, (D) sodium acetoacetate, (E) dimethylacrylic acid.

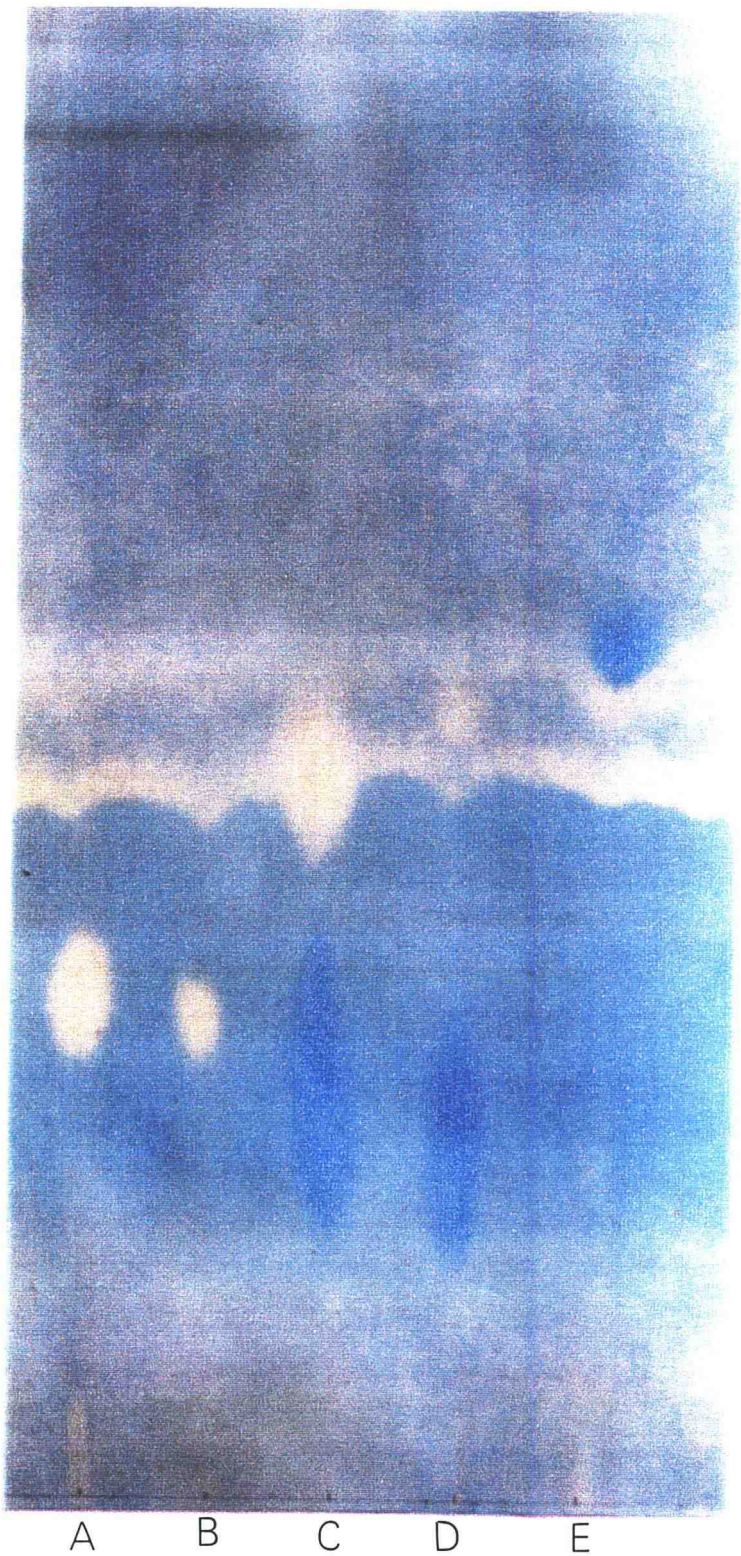

Figure 11

Figure 12. Two dimensional paper chromatography of amino acid standards. Identity of each standard is noted on the figure. The order and direction of each solvent is also noted in the figure by arrows. Solvent I was methanol/water/pyridine, 60/20/20, and Solvent II was n-propanol/water/diethylamine, 85/15/3. Arrow denotes origin

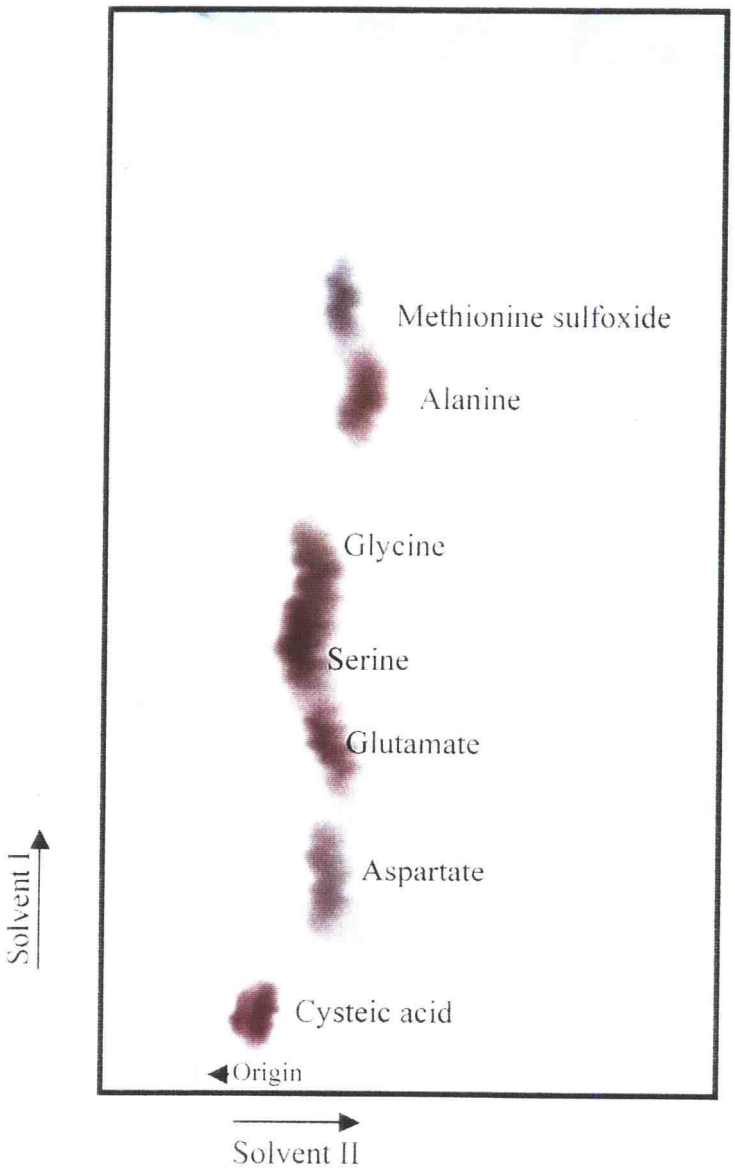
Methionine sulfoxide
Alanine
Glycine
Serine
Glutamate
Aspartate
Cysteic acid
Origin
Solvent I
Solvent II

Figure 12

Preparative isolation of peptides

Paper chromatography was used to separate the radioactively labeled peptides on a preparative basis. The fraction from membrane filtration was streaked as a thin band 7 cm from the bottom edge of a 23x50 cm sheet of Whatman No. 3 paper. A small aliquot of the preparative sample was also applied as marker sample, and development by descending technique with water saturated phenol as the mobile phase continued for 18 hours. The marker band was sprayed with ninhydrin as previously described and the position of radioactivity labeled components was determined with the Bioscan imaging scanner.

Bands of interest on preparative paper chromatograms were cut out of the paper using the ninhydrin response or location of radioactivity as a guide. These bands were further cut into 5 mm squares and placed in a 250 ml Erlenmeyer flask. The component(s) of the band were eluted with water or glacial acetic acid. This extraction procedure was epeated four times to insure removal of desired material. repeated four times to insure removal of desired material. The combined extracts for each band were reduced in volume on a rotary evaporator and stored at -20°C when not in use.

Performic Acid Oxidation of Peptides

Acid hydrolysis (see below) is a conventional method to hydrolyze peptides into the component amino acids of the selected peptide. Since cysteine and cystine are destroyed during acid hydrolysis procedures, it is necessary to convert these amino acids to a stable residue before hydrolysis. Treatment with performic acid will convert cysteine and any disulfide bonds to cysteic acid residues prior to acid

hydrolysis procedures. In this research a vapor phase method (122) was used to oxidize any cysteine or cystine residues to cysteic acid residues. The target peptide was dissolved in water or glacial acetic acid if not already in solution. The peptide solution was transferred to a 10 x 75 mm test tube which had been previously rinsed with concentrated nitric acid and pyrolyzed to remove any residual materials. The solvent in the tube was then removed using a stream of nitrogen gas. The tube was then stabilized in an upright position in a small beaker and the beaker placed in a glass or plastic dessicator. A mixture of 20 ml 98 percent formic acid and one ml 50 percent hydrogen peroxide was prepared and immediately transferred to a clean glass petri dish in a glass desiccator along with the dry sample tubes. The desiccator was sealed by vacuum and peroxidation continued overnight at room temperature. The petri dish was then removed from the desicator and the sample tubes placed under vacuum for 30 min to remove any residual vapors, and to remove any condensation in the tubes. The peptide sample was then ready for any further investigation.

Acid hydrolysis of peptides

Peptides that were isolated from both the soluble peptide fraction (Figure 7) and the aqueous extract (Figure 6) were analyzed by vapor phase hydrolysis with 6 N HCl (123). This procedure will provide information about the component amino acids of the peptide in question. If necessary, the peptide was first dissolved in water or glacial acetic acid and a suitable aliquot transferred to a clean 10 x 75 mm test tube and brought to dryness under a stream of nitrogen gas. As a general rule, at least 10 μgms of protein, as assayed by the Lowry method, was transferred for analysis. The tube containing the dried peptide sample was placed in an all glass vacuum desicator along with a separate beaker containing 5.0 ml of 6 N HCl. The

desicator is evacuated for 15 minutes prior to placing in a 110°C oven overnight (12 to 14 hours). At the end of this time period the desicator is removed from the oven and the tubes transferred to a second desicator to be dried for approximately 30 minutes under vacuum. The dried tubes containing the hydrolyzed peptide sample are covered with parafilm and stored at -20°C until further use. The hydrolyzed peptide was then ready for analysis by paper chromatography or dabsyl HPLC methods (see below).

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to purify peptides from the soluble protein fraction and to identify amino acid derivatives. An Isco Model 2360 gradient programmer and an LKB 2150 HPLC pump system were used in conjunction with an Isco V_4 variable wavelength detector and a Beckman 163 variable wavelength detector. For purification of the peptide(s) by HPLC, the sample was injected and the detector response used as a guide to switch the flow of effluent to collection tubes. For peptide purification purposes, one detector was set at 280 nm and the second detector at 214 nm. Dabsyl derivatives were monitored at 460 nm. Solvents and gradients used are described in the results section.

For purification of peptides a Vydac reversed phase (modified with C18 groups, octadecylsilane) column of dimensions 4 x 250 mm was used. For dabsyl amino acids a Phenomenex Ultracarb 5 ODS (octadecylsilane) column (4.6 x 250 mm) was employed. Quantitation of amino acids was done by comparison of chromatograms of amino acid standards run the same day. Areas under the standard peaks were compared to peaks identified in samples according to their retention time and appearance on the chromatogram.

Preparation of Dabsyl derivatives of amino acids

Dabsyl amino acid derivatives were prepared according to Malencik (124) as described below. For comparision with results of analyzed peptides, an amino acid standard mixture (Sigma Chemical Company, number AA-S-18) was used. This procedure allowed for identification and quantitation of amino acids in the peptide being analyzed. When peptides were hydrolyzed, an amino acid standard was also hydrolyzed under identical performic acid or vapor phase HCl hydrolysis conditions; as before, glass tubes for hydrolysis were prewashed with concentrated nitric acid and pyrolyzed in a glass cleaning oven for 3 hours before use. Amino acid standards were prepared so that 20 µl of the final solution contained 400 picomoles of each dabsylated amino acid.

Derivatives of amino acids were prepared using 4-dimethylaminoazobenzene-4'-sulfonyl chloride (Dabsyl-Cl, from Sigma Chemical Co., St. Louis, MO) as the derivatizing agent. The strong absorbance of Dabsyl-derivatized amino acids at 460 nm allow for the sensitivity of this method to be in the nanogram (of protein) range (125). For this work, at least 10 µgm of peptide to be analyzed was hydrolyzed first. In some cases the peptide was also treated with performic oxidation (see above) before acid hydrolysis. The hydrolyzed peptide samples were dried under vacuum after vapor phase HCl hydrolysis. A blank tube was included in the hydrolysis experiment for background determinations. To each sample tube was added 50 µl of buffer containing two parts 20% sodium carbonate and seven parts 200 mM boric acid (v/v). Next, 100 µl of freshly prepared Dabsyl-Cl solution (5.2 mg/4ml acetonitrile) was added with vortexing. Tubes were then covered with parafilm and placed in a 60°C heating block for 10 minutes. A solution of equal

parts ethanol and 0.1 M sodium phosphate buffer (pH 7.0) was then prepared and 350 µl of this mixture was added to each tube with more vortexing. The derivatized amino acids were then ready for HPLC analysis. Dabsylated amino acids were covered and stored at -20°C when not in use.

Mass spectrometry of soluble peptides

The development of mass spectroscopy (MS) application in biochemistry has resulted in the introduction of various techniques for use in characterizing biomolecules, especially proteins and peptides. The soluble protein fraction was used as a source of peptides for analysis in this study. A brief overview of the methods used in this work follows. For a more complete discussion of methods and theory see (129).

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) utilized a laser beam directed onto a sample that is suspended or dissolved in a matrix. In mass spectrometry the analyte or sample must be volatilized. In this work, the sample is a peptide or group of peptides isolated from the soluble peptide fraction (see Figure 7). The matrix is selected on the basis of its ability to absorb the laser radiation energy. Ionization and volatilization occurs by absorption of the laser radiation by the matrix. When this happens, both sample and matrix with are volatilized. The now volatilized and ionized sample is directed to a time-of-flight (TOF) mass spectrometer for analysis.

Matrices used in this study were 2-5-dihydroxybenzoic acid (DHA) or α–cyano-4-hydroxycinnamic acid (CCA). The peptide sample used for analysis was dissolved in 0.1 percent trifluoracetic acid. A solution of the sample was then

overlaid on the matrix sample prior to laser irradiation. Mass spectra were produced by irradiating the sample with a 337 nm laser; the ion source was set at 21 kv.

Electrospray ionization (ESI) was used both alone and in conjunction with liquid chromatography (LC). This technique uses methodology to produce analyte ions from a solution of the analyte. The sample solution is introduced into a heated vacuum chamber from an ion spray needle maintained at high voltage. The resulting charged droplets are introduced to a nebulizer gas and heated to strip solvent ions from the analyte ions. These analyte ions are then directed to a mass analyzer (by TOF) by electrostatic forces. With ESI used alone, without chromatography, the analyte solution is injected directly into the sample chamber and subjected to TOF analysis and subsequent tandem mass spectrometry.

With liquid chromatography mass spectrometry (LCMS) the sample solution is chromatographed on a 5 μ reversed phase column (C18, octadecyl silane), 1 mm x 100 mm. The liquid chromatography (LC) instrumentation consisted of a Perkin-Elmer ABI 140B pump system, Rheodyne 8125 injector and a 5 μl sample loop. Flow rate was set at 40 μl/minute; a 15-minute gradient program running from 5 to 95 percent acetonitrile in water was used to elute the peptides. The effluent from the column, containing the sample of interest, became the sample for electrospray ionization. Tandem mass spectrometry was used to take the selected peak and subject it to secondary fragmentation, from which a sequence of the peptide could be deduced.

The potential of the ion spray needle was placed at 5000 + 4700 V to produce positive ions and –4500 volts for negative ions. The potential of the orifice to introduce ions to the mass analyzer was set to 80 volts. The nebulizer gas (air) was set at 42 psi, and a nitrogen curtain gas over the opening to the mass spectrometer was set at 0.6 liters/minute.

The samples were run on a Perkin Elmer Sciex API III plus triple quadropole Ionspray mass spectrometer (Ontario, Canada). The vacuum in the quadropole sector is maintained at a 10^{-7} torr vacuum with no sample and 10^{-5} vacuum with a 150 ns/1.8 kv delay. In these experiments the collision gas was set to 230 x 10^{12} molecules/cm^2 and the collision energy was adjusted to 25 volts.

EXPERIMENTAL RESULTS

In this section, experiments are described, which were carried out in a concerted fashion, exploring the pathways of mevalonate metabolism in a post mitochondrial preparation of normal bovine aorta. These processes impact the development of the atherosclerotic plaque, and except for exploration of the biosynthesis of cholesterol, have received little attention in the search for the etiological patterns of atherosclerosis in normal vascular tissue.

Incubation of the postmitochondrial supernatant

Mevalonate-2-^{14}C (0.5 μci) was incubated with 50 ml of the postmitochondrial supernatant. The amount of radioactivity in the neutral, soluble protein, acid petroleum ether, and aqueous extract fractions was determined. The results are summarized in Table 1. Control experiments when postmitochondrial supernatant was omitted demonstrated that no label is incorporated into the petroleum ether extract. All mevalonic acid-2-^{14}C remained, unchanged, with the aqueous extract in the control samples.

While both ethanol and trichloroacetic acid were used as protein precipitants, no differences in the subsequent results were observed. In a separate experiment, when inactive postmitochondrial supernatant was incubated with adenosine triphosphate (ATP) and mevalonate-2-^{14}C, there was no incorporation of radioactive label into the subsequently isolated components of various fractions. The added mevalonate-2-^{14}C was recovered completely in the aqueous extract and thin layer chromatography established that the labeled material was not changed(results not shown). This result was consistent throughout the experimental work, so that only exposure of

Table 1. Distribution of radioactivity from mevalonate-2-^{14}C. The fractions were obtained from initial time (0 to 15 minutes) or incubated (two hours and fifty-five minutes) postmitochondrial supernatant incubated with 0.5 μci ($1.11x10^6$ dpm) mevalonate-2-^{14}C.

	Counts per minute			
Sample	Neutral Fraction	Soluble Protein	Acid Ext.	Aqueous
Initial time	--	$1.27x10^4$	--	$6.3x10^5$
Incubated[1]	$6.3x10^4 \pm 771$	$1.74x10^4 \pm 513$	1497±20	$6.93x10^5 \pm 1.8x10^4$
Buffer control[2]	--	--	--	$7.1x10^5$, $8.3x10^5$

[1] n=3 samples, standard deviation
[2] n=2, both values listed

mevalonate-2-^{14}C to the viable postmitochondrial supernatant resulted in changes in the starting material.

Characterization of the neutral lipid fraction

The neutral fraction has been shown (78) to contain acyl glycerols (primarily diacylglycerols) with the greatest radioactivity incorporated into the medium chain n-fatty acids containing eight (caprylic acid) or ten (capric acid) carbons.

During the initial studies on the incorporation of radiolabel into the neutral lipid fractions, it was observed that considerable variability occurred in the amount of radioactivity incorporated into the neutral fraction for identical incubation mixtures. The variability was caused by the loss of volatile unidentified components in the neutral lipid fraction. Standardizing the conditions of evaporation removed the variability of amount of incorporated label observed in the neutral fraction. The volatility of the labeled material is observed when aliquots of a neutral lipid petroleum ether extract were counted under several conditions: (1) without removal of the petroleum ether (2) evaporation of the petroleum ether from an aliquot and counted immediately and (3) evaporation of the extract and allowing the residue to stand open at room temperature overnight. The incubation mixture in this experiment contained five ml of postmitochondrial supernatant and 0.5 μci of mevalonate-2-^{14}C. The results are summarized in Table 2. It appears that approximately 17 percent of the label is lost when open to the air.

Table 2. Volatility of the radioactively labeled components in the neutral extract. Aliquots of the neutral extract were either dried on steam bath or allowed to air dry overnight. Amounts were corrected to express total sample radioactivity. Values were compared to the petroleum ether extract that was not dried.

	Counts perminute		
Sample	Petroleum Ether Extract*	Steam Bath	Air dry overnight
Initial time (n=2)	377±117	384±118	292±127
Incubated (n=4)	6202±137	6072±178	5074±509
Control	-0-	-0-	-0-

*Petroleum ether counted without evaporation of solvent.

The acid petroleum ether extract

A second petroleum extract, the acidified petroleum ether extract, was also characterized. The postmitochondrial supernatant, after incubation with mevalonate-2-^{14}C, was acidified with trichloroacetic acid used to precipitate the protein. When ethanol was used to precipitate the protein, 6 N HCl was used to adjust the pH to 2. Petroleum ether was used to extract the acidified layer and treated in the same manner as the neutral lipid extract to assess for incorporated radioactivity. Table 1 shows the results of the acid extraction procedure. In the control and initial time samples there was no radioactivity observed in the acid petroleum ether extract.

Thin layer chromatography of the acid petroleum ether extract

The nature of the labeled component in the acid petroleum ether fraction was studied using both thin layer and paper chromatography. The sample for thin layer chromatography was dissolved in ethanol and chromatographed in an acid n-butanol/chloroform/acetic acid/water, 100/100/4/30, using the organic layer) solvent. The radiochromatogram is shown in Figure 13, with the label migrating near the solvent front with Rf of 0.95. The labeled area on the plate was removed, extracted and re-chromatographed in a basic (ethanol/ammonia/water, 60/20/20) solvent, and the radiochromatogram of the thin layer plate is shown in Figure 14. In the basic solvent, the radioactive material now appears as a sharp band at the origin and a second, more diffuse band, Rf of 0.45. For comparison, mevalonic acid-2-^{14}C standard is shown in Figures 15 (acid solvent) and 16 (basic solvent). Figure 17 shows a blank scan to illustrate background noise. The detector response in all the radiochromatograms shown in this report is dependent upon the total amount of label detected by the instrument. Therefore, with more highly labeled samples, little

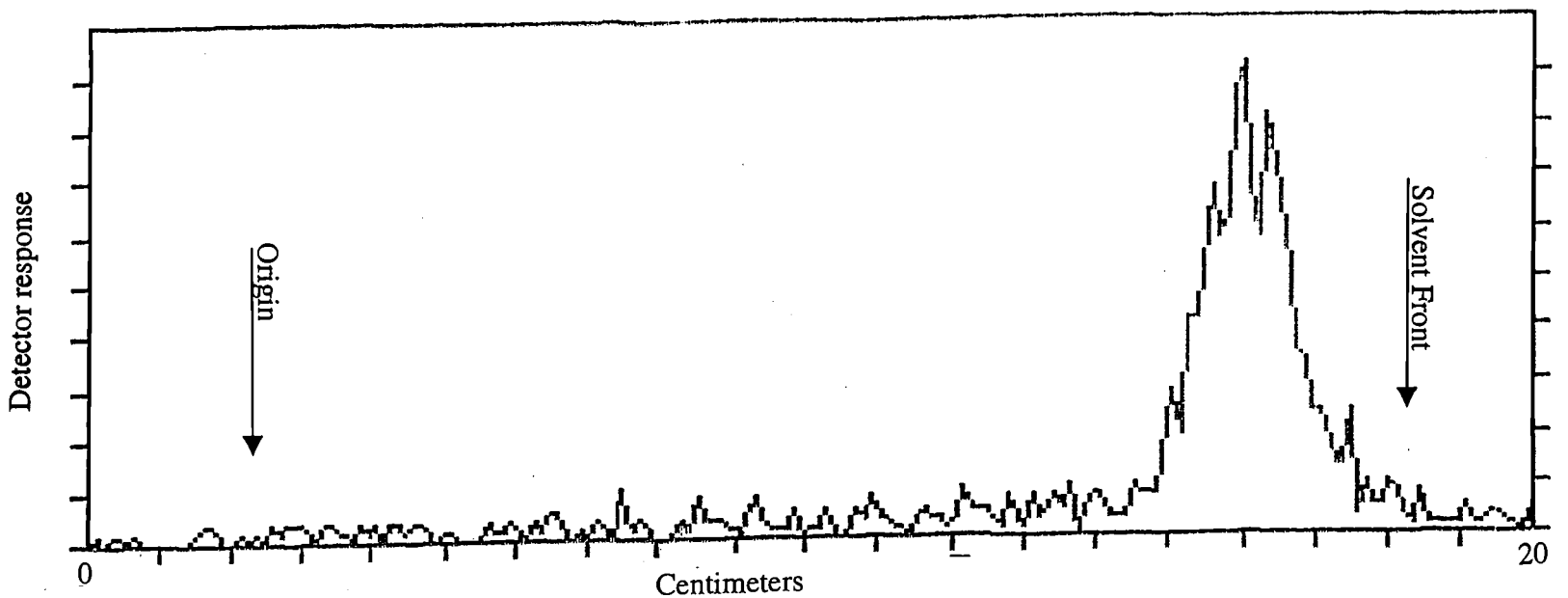

Figure 13. Radiochromatographic scan of thin layer chromatography of the acid petroleum extract in an acid solvent. Origin and solvent front are marked with arrows. The bottom scale represents centimeters along the edge of the plate. The data presented show the location of the radioactivity after thin layer chromatography of the sample. The solvent is water saturated n-butanol/chloroform/acetic acid, 100/100/4.

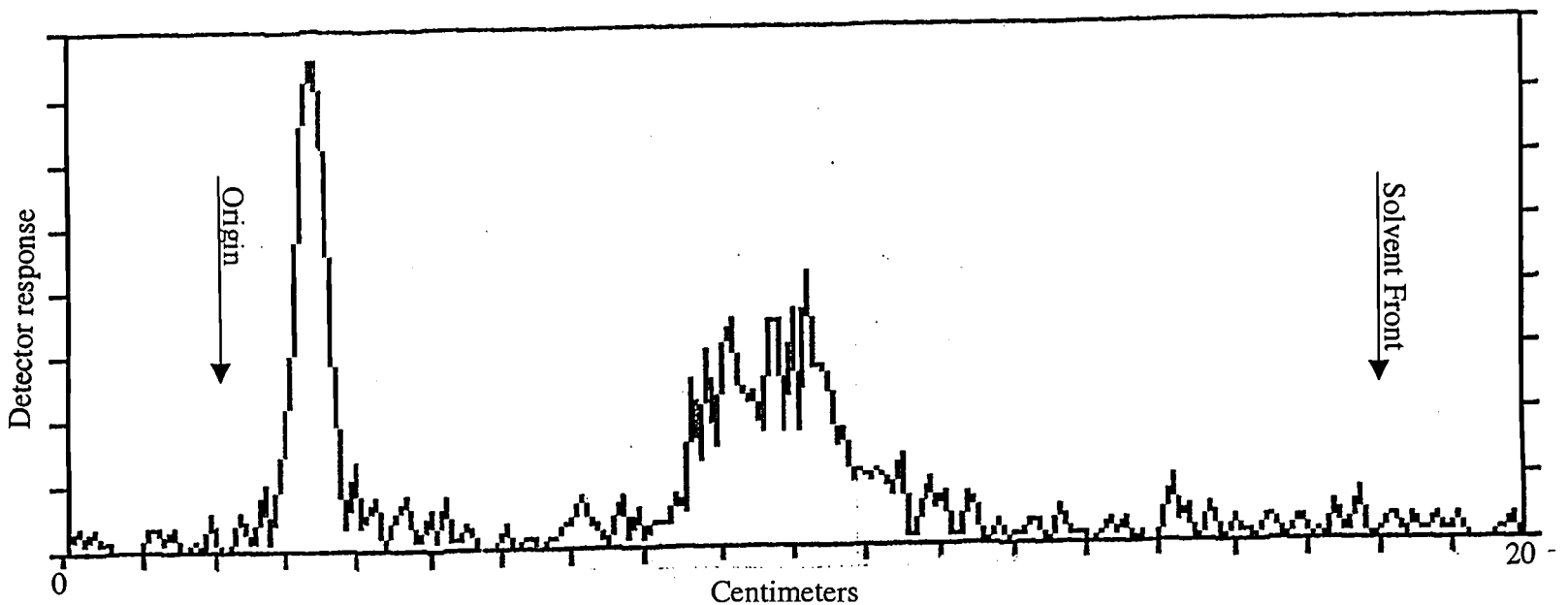

Figure 14. Radiochromatographic scan of thin layer chromatography of the acid petroleum extract in a basic solvent. Origin and solvent front are marked with arrows. The bottom scale represents centimeters along the edge of the plate. The data presented show the location of the radioactivity after thin layer chromatography of the sample. The solvent is ethanol/ammonia/water/ 60/20/20.

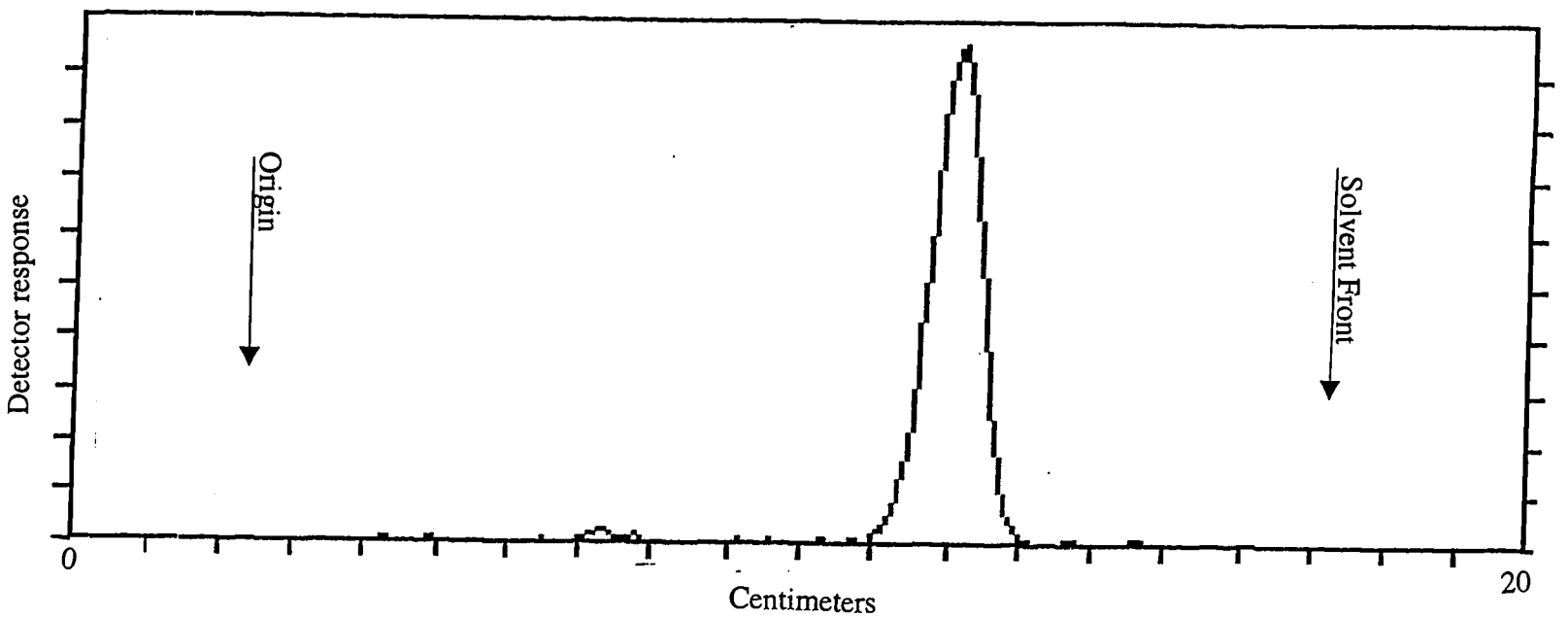

Figure 15. Radiochromatographic scan of thin layer chromatography of standard mevalonic acid-2-^{14}C in an acid solvent. Arrows mark the origin and the solvent front. The bottom scale represents centimeters along the edge of the plate. The data show the location of radioactivity on the thin layer plate in the sample lane. The solvent is n-butanol/chloroform/acetic acid/water, 100/100/4/30.

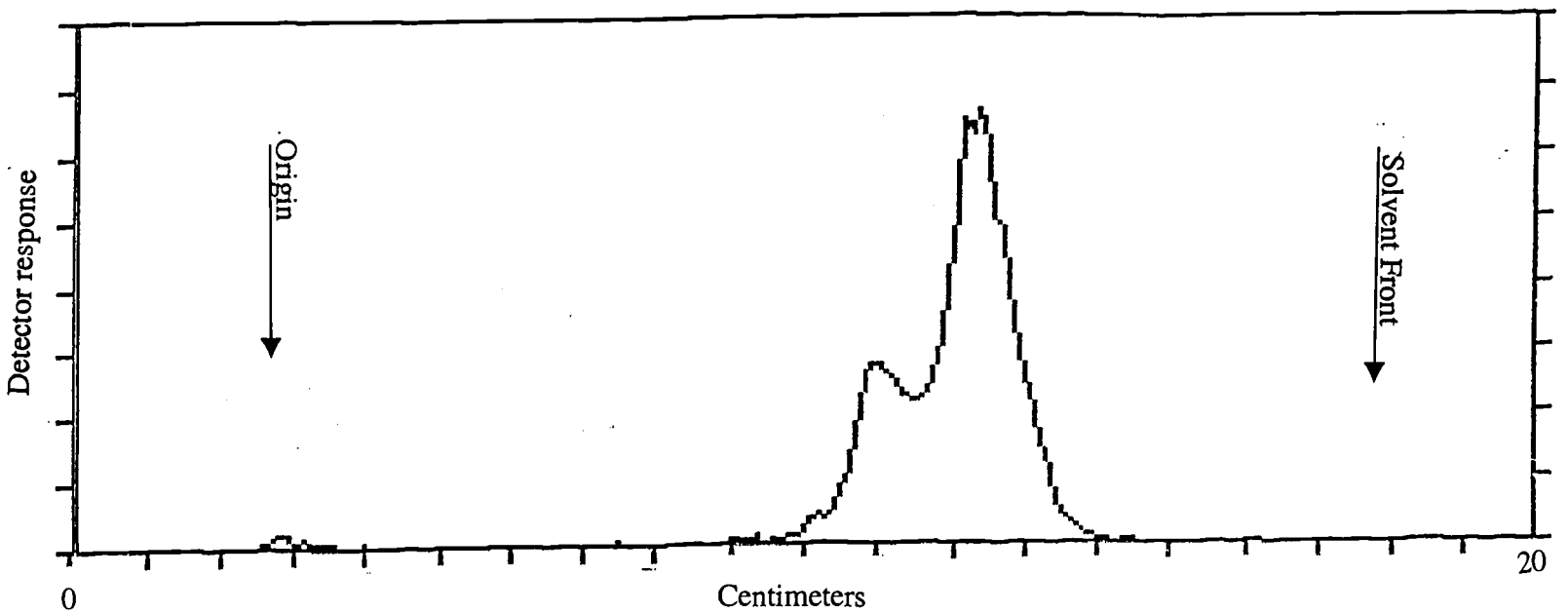

Figure 16. Radiochromatographic scan of thin layer chromatography of standard mevalonic acid-2-^{14}C in a basic solvent. Arrows mark the origin and the solvent front. The bottom scale represents centimeters along the edge of the plate. The data show the location of radioactivity on the thin layer plate in the sample lane. The solvent is ethanol/ammonia/water, 60/20/20

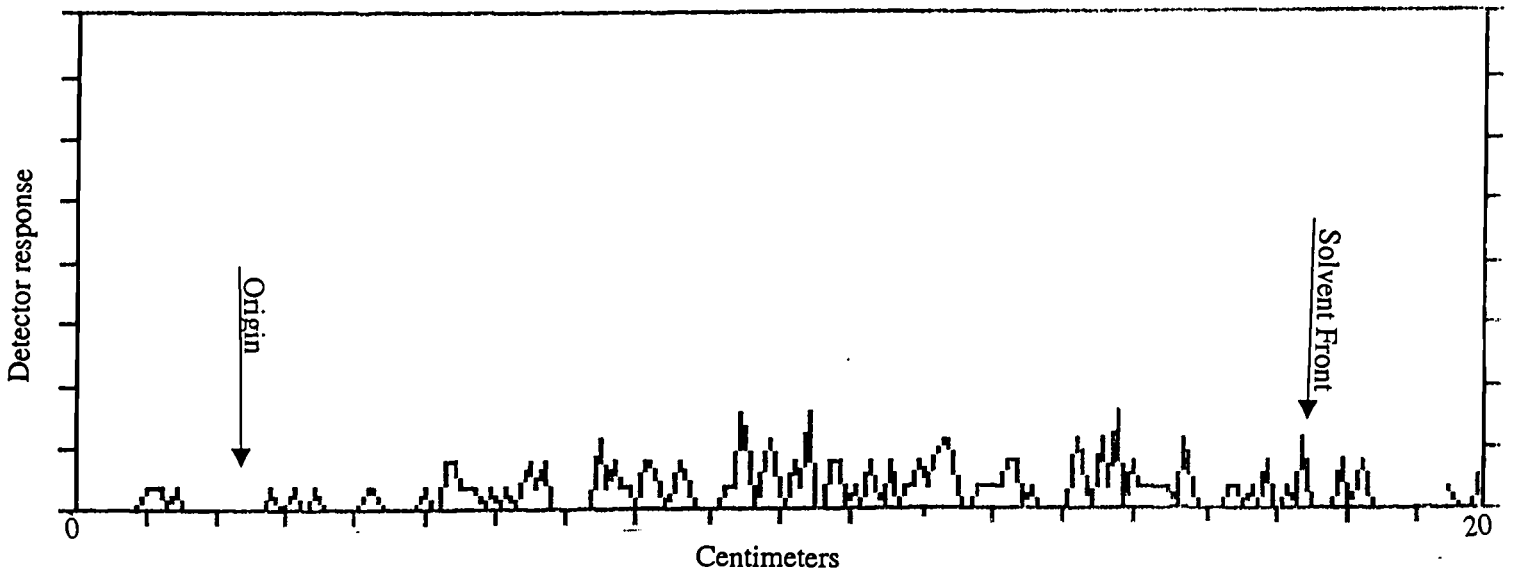

Figure 17. Radiochromatographic scan of a thin layer chromatograph with no radioactivity in the sample lane (background scan). The arrows mark the origin and the solvent front, while the bottom scale is measured in centimeters. The data represent the scan observed when no radioactive label is present on the thin layer plate.

background noise is detected on these chromatograms. The acid petroleum ether extract changes chromatographic behavior, depending on the acidity of the solvent. The mobility of the label in an acid solvent (Figure 13) suggests a protonated, less polar form of an acid compound. The behavior in basic solvent suggests more than one component.

Paper chromatography of the acid petroleum ether extract

Paper chromatography of the acid petroleum ether extract was carried out in three chromatograms. In the first, the extract was chromatographed in a system that separates short and medium chain carboxylic acids. The solvent was n-butanol saturated with 3 N ammonia; and the paper was run in the ascending mode. Detection was with bromocresol green and the Rf values of the standards and the acid extract are summarized in Table 3.

The same solvent was used in the second chromatographic analysis and the migration of the labeled acid petroleum ether extract was compared to longer chain acids; C_{12} (lauric), C_{14} (myristic), C_{16} (palmitic), $C_{18:1}$ (oleic) and $C_{18:2}$ (linoleic). All of these acids migrated to a distance with Rf of 0.75 while the radioactivity migrated to a distance of Rf of 0.87.

A third paper chromatogram, also run in n-butanol saturated with 3N ammonia, used dimethylacrylic acid as the sample in amounts of 1 μgm to 1 mg. This acid was selected because of its participation in the *trans*-methylglutaconic shunt and its migration behavior on both thin layer and paper chromatograms. The Rf of dimethylacrylic acid is 0.40 in amounts to 250 μgm; larger quantities migrate more slowly and exhibit excessive tailing. The radiochromatographic scan of this sample on the paper chromatogram (not shown) indicated a single radioactive spot with an R_f

Table 3. Rf values for medium chain acids and the acid petroleum ether extract with paper chromatography. The solvent was n-butanol saturated with 3N ammonia. 50 µgm of each standard was used and standards were run separately. Rf was calculated as noted for each detection method. For mevalonate-2-^{14}C standard, 2000 cpm were applied. For the acid petroleum ether extract, 500 cpm were applied.

Sample	Rf (bromocresol green)	Rf (radioactivity)
Butyric acid (C_4)	0.29	--
Caproic acid (C_6)	0.53	--
Caprylic acid (C_8)	0.64	--
Capric acid (C_{10})	0.67	--
Mevalonic acid	--	0.17
Acid petroleum ether extract	--	0.83

of 0.83, which was also coincident the acid component detected with bromocresol green. Thus the component(s) of the acid lipid extract was not dimethylacyrlic acid or an n-chain monocarboxylic acid containing from two to 18 carbons; it also did not appear to be a hydrocarbon-like compound. The acid component(s) were not further characterized.

The *trans*-methylglutaconate shunt

As part of the concerted effort to identify the mevalonate-related pathways in arterial tissue, investigations were directed toward developing methods which would permit the isolation of intermediates of the *trans*-methylglutaconate shunt, and subsequent identification of any label incorporated from mevalonate-2-^{14}C. Isolation of a fraction containing small soluble components—as the shunt intermediates—was achieved with the precipitation of proteins from the postmitochondrial supernatant after petroleum ether extraction of both neutral and acid lipids. The supernatant and pellet washes remaining after centrifugation were combined and reduced in volume to obtain the aqueous extract.

Isolation of a fraction containing small soluble components—as the shunt intermediates—was achieved with the precipitation of proteins from the postmitochondrial supernatant after petroleum ether extraction of both neutral and acid lipids. The supernatant and pellet washes remaining after centrifugation were combined and reduced in volume to obtain remove any remaining mevalonate-2-^{14}C. The isolation protocols are diagrammed in Figure 6.

Silica gel column chromatography

The aqueous extract was subjected to silica gel column chromatography to analyze for the presence of intermediates of the *trans*-methyl glutaconate shunt. The sample for chromatography was a portion of the incubated aqueous extract of post mitochondrial supernatant; chromatography was carried out as described in the methods section. The elution of known acids of the *trans*-methylglutaconic shunt was determined by chromatography of standards on the column, prior to running the aqueous extract. The location of fractions containing the standard acids was determined by titration with standardized base, according to the method of Gamble et al. (133). Selected fractions were pooled, dried, and the residues used as samples in paper chromatography. Fractions 3-5 contained dimethylacrylic acid; fractions 6-9 contained β-methyl-ß-glutaconic acid; fractions 31-34 contained β-hydroxybutyric acid; and fractions 35-39 contained both mevalonic acid and ß-hydroxy-ß-methylglutaric acid. These findings were confirmed by mass spectrometry (not shown). It was necessary to run the standards on the column so that components from subsequent samples could be identified by a combination of chromatographic techniques and mass spectrometry.

The silica gel elution profile for standard mevalonic acid is shown in Figure 18. It can be seen that the mevalonate appears starting at fraction 33 and that the bulk of the radioactivity is eluted in this peak. A sample of aqueous extract was applied to a second silica gel column and chromatographed under standard conditions. The column profile is shown in Figure 19 . The bulk of the radioactivity eluted between fractions 24 and 48. Two peaks were resolved at the beginning of the elution profile,

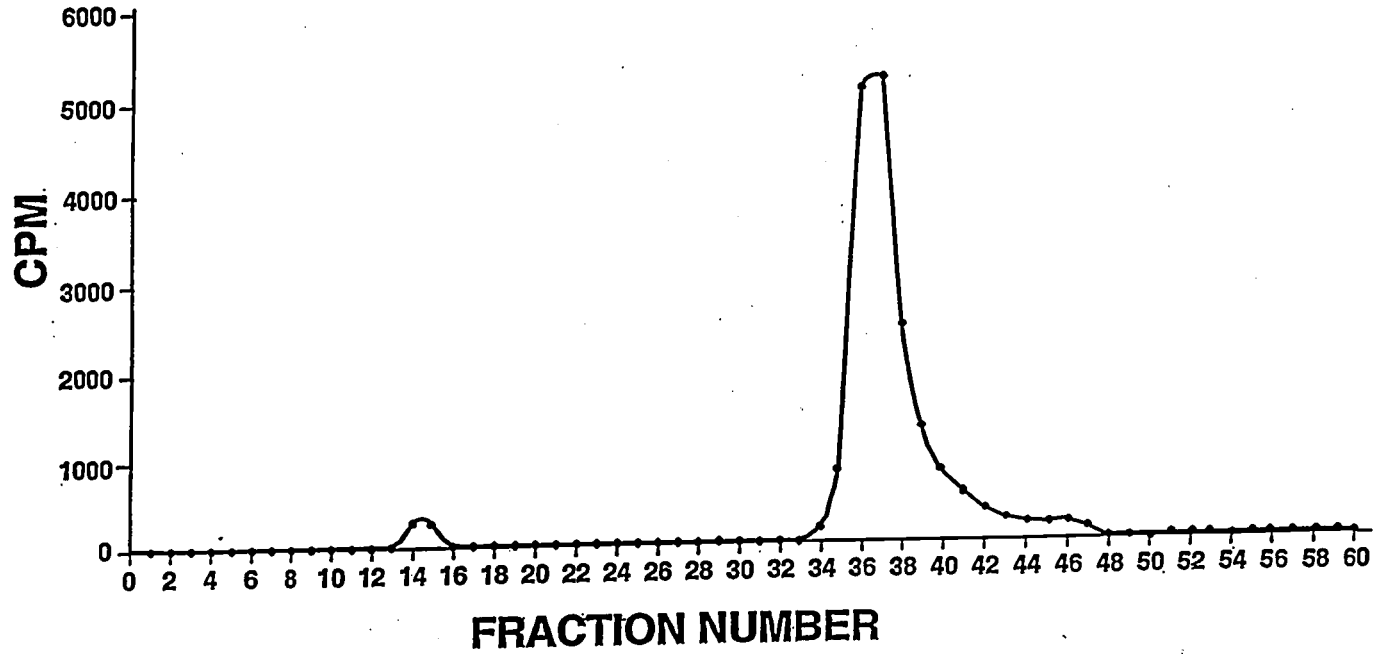

Figure 18. Silica gel column chromatography of mevalonic acid-2-^{14}C. The column profile compares fraction number and radioactivity in each fraction. The column was eluted with a gradient of t-amyl alcohol in chloroform.

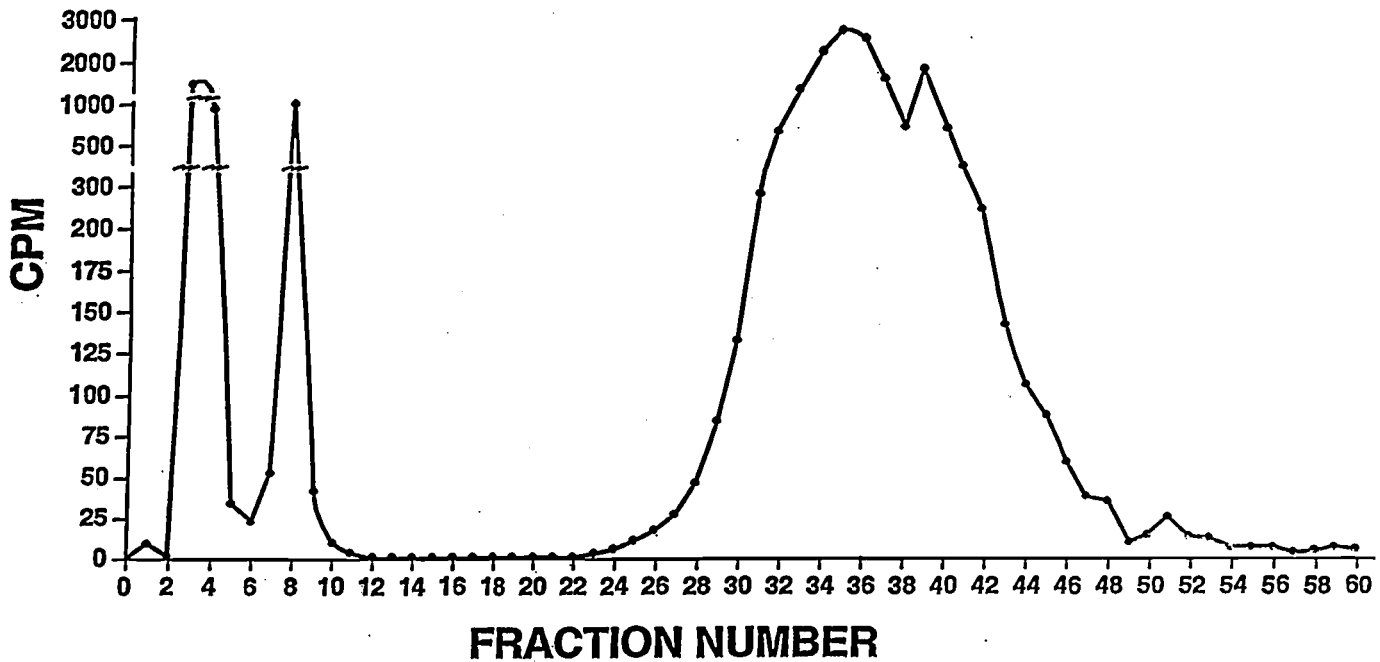

Figure 19. Silica gel column chromatography of the aqueous extract. The column profile compares fraction number and radioactivity in each fraction. The column was eluted with a gradient of t-amyl alcohol in chloroform.

coincident with the elution of dimethylacrylic acid and β-methyl-ß-glutaconic acid, respectively. Fractions 31-33 contained hydroxybutyric acid and fractions 34-35 contained ß-hydroxy-ß-methylglutaric acid, as confirmed by mass spectrometry, giving evidence that these acids are intermediates in the *trans*-methylglutaconate shunt, and that the shunt is active in vascular tissue. The fractions corresponding to the known standards from the column chromatogram shown in Figure 19 were also identified by paper chromatography (see below). These findings again confirmed the earlier identification of these acids in the *trans*-methylglutaconate shunt. This is the first observation, to our knowledge, in any tissue, of the existence of these acids as shunt intermediates.

Paper chromatography of silica gel chromatography fractions

The identification of the labeled components in the aqueous extract that were separated by silica gel column chromatography was pursued by combining the column fractions of interest and subjecting them to paper chromatography. The fractions were combined on the basis of the column chromatographic behavior of the known shunt acids, and excess solvent removed by evaporation. The residue from each set of combined fractions was subjected to paper chromatography in ethanol/7.5 N-ammonium hydroxide/water, 80/10/10. The results of chromatography of the *trans*-methylglutaconic shunt standards was used as a guide (Methods section, Figure 9) for removal of bands on the paper. The bands were cut out and counted. While the majority of radioactivity eluted with the bands was associated with mevalonic acid and ß-hydroxybutyric acid, there was label present in the dimethylacrylic acid, and ß-methylglutaconic acid, confirming the results observed from Figure 19 and mass spectrometry.

Thin layer chromatography of the *trans*-methylglutaconate intermediates

The separation of *trans* methylglutoconate shunt acid standards by thin layer chromatography is shown in Figures 6 and 7 in the Methods section. Since these thin layer chromatography protocols had been developed to separate the shunt intermediates, this method was used to characterize the acid components of aqueous extract. The radiochromatogram is shown in Figure 20. There is radioactivity observed in components with R_f values equal to β-hydroxy-ß-methylglutaric acid, dimethylacrylic acid, and β-methylglutaconic acid in addition to mevalonic acid. In a second experiment, the bands on the thin layer plate were selected according to the mobility of the *trans*-methylglutaconic standard acids in acid solvent. The silica bands were removed and counted and the results are summarized in Table 4 . The results indicate that radioactivity from mevalonate-2-^{14}C appears in β-hydroxy-ß-methylglutaric acid, dimethylacrylic acid, and β-methylglutaconic acid, while any radioactivity in ß-hydroxybutryic acid remained associated with mevalonic acid.

Determination of alcohol dehydrogenase activity in the postmitochondrial supernatant

Dimethylallyl alcohol is oxidized to dimethylacrylic acid in the proposed *trans*-methylglutoconate shunt, implying alcohol dehydrogenase participation. In this proposed pathway (Figure 4) dimethylallyl pyrophosphate is first hydrolyzed to dimethylallyl alcohol. Two oxidative steps convert this substrate first to dimethylallyl aldehyde (shown as 3-methylcrotonaldehyde in Figure 4) and next to dimethylacrylic acid. Therefore, alcohol dehydrogenase activity in the postmitochondrial supernatant was assayed using dimethylallyl alcohol, isopentenol and ethanol as substrates. For this assay, 0.1 ml of postmitochondrial supernatant was used, containing 0.7 mg protein. The results, summarized in Table 5, show that

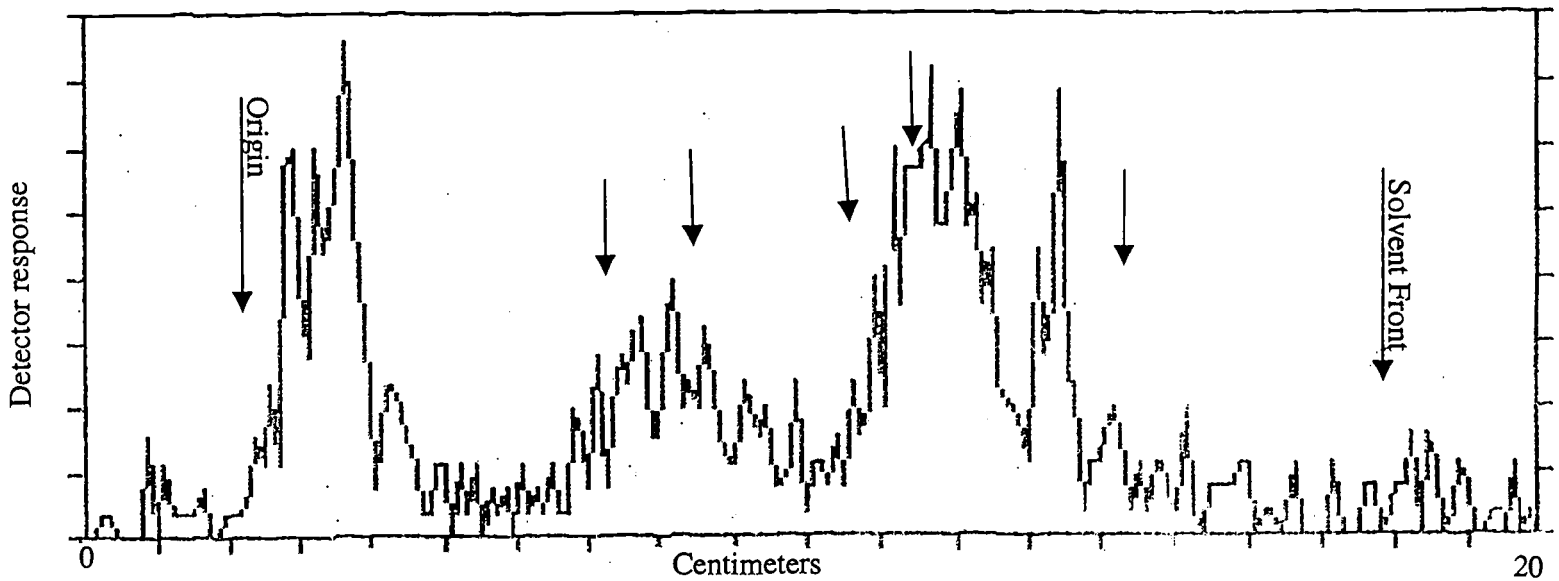

Figure 20. Radiochromatographic scan showing separation of the trans-methylglutaconate shunt intermediates in the aqueous extract by thin layer chromatography. The data show the location of radioactivity in the sample lane of the plate. Unlabeled arrows indicate, from left to right, the migration of β-hydroxy-β-methylglutaric acid, β-hydroxymethylglutaconic acid, β-hydroxybutyric acid, mevalonic acid, and dimethylacrylic acid. The solvent was the organic phase of n-butanol/chloroform/acetic acid/water, 100/100/4/30.

Table 4. Thin layer chromatography of the *trans*-methylglutaconate shunt acids. Bands were removed according to Rf values and counted by scintillation counting. The solvent wasn-butanol/chloroform/acetic acid, 100/100/4

Compound	R_F	CPM ± Std. Dev.
Unidentified	0.0	900 ± 132
β-hydroxy β-methyl glutaric acid	0.11	905 ± 34
Unidentified	0.29	3305 ± 228
β-methylglutaconic acid	0.48	247 ± 14
β-hydroxy butyric acid, mevalonic lactone	0.60	2528 ± 160
Unidentified	0.74	447 ± 89
Dimethyl acrylic acid	0.85	315 ± 95
Unidentified	0.95	159 ± 57

Table 5. Alcohol dehydrogenase activity in the postmitochondrial supernatant. Alcohols were assayed for alcohol dehydrogenase activity in two concentrations.

Substrate	Concentration μM	NADH[1]
Isopentenol	198	18.3 ± 1.6
	396	36.3 ± 1.8
Dimethylallyl alcohol	198	23.4 ± 1.5
	396	30.4 ± 1.4
Ethanol	198	10.0 ± 3.7
	396	9.5 ± 1.4

[1] μmoles formed/minute, n=3.

the least activity was exhibited with ethanol. The control, run with no substrate, exhibited no acitivity.

Isolation and characterization protein and peptides

Identification of the peptide fraction

Thin layer chromatography of the aqueous extract (Figure 20) showed a substantial amount of radioactivity at the origin. This material did not correspond to any of the *trans*-methylglutaconate shunt intermediates in terms of chromatographic behavior. Therefore this aqueous extract was then subjected to thin layer chromatography using conditions that would separate phospholipids, to investigate the possibility of incorporation into this lipid class. The solvent was chloroform/methanol/petroleum ether (distilled at 30°-60°C)/acetic acid/boric acid, 40/20/30/10/1.8. The radioactivity in the aqueous extract was separated into a peak at the origin and a residual mevalonic acid peak. The incorporated label remained at the origin.

In a second thin layer chromatogram, conditions for separation of the citric acid cycle intermediates were used (126). Cellulose thin layer plates (Avicel microcrystalline cellulose from Analtech, New Jersey) were used in a solvent composed of diethyl ether/formic acid/water, 140/40/20. The results are summarized in Table 6. Both of the thin layer chromatography techniques showed no incorporation into known standards run at the same time; however, the results did indicate the label may be present in amino acids or similar component(s). Ninhydrin, a usual indicator for free amino groups, gave a positive response to the

Table 6. Thin layer chromatography of citric acid cycle intermediates. The acids were run for comparison with the aqueous extract. Standards were applied at 50 μgm each. The aqueous extract sample contained 1000 cpm while mevalonate-2-^{14}C contained 2000 cpm.

Sample	R_f	Detection[1]
Succinic acid	0.17	BG
Mumaric acid	0.86	BG
Malate acid	0.49	BG
α-Ketoglutaric acid	0.69	BG
Citric acid	0.40	BG
Dimethylacrylic acid	0.90	BG
β-Hydroxymethyl glutaric acid	0.0	BG
Aqueous extract (label)	0.02, 0.77	Bioscan
Mevalonate-2-^{14}C (standard)	0.77	Bioscan
Aqueous extract (control)	0.77	Bioscan

[1] Detection was with bromocresol green (BG) or with the Bioscan radioactivity scanner.

label-containing bands (excluding mevalonate-2-^{14}C), a result which led to experiments characterizing the labeled material as peptide in nature. The buffer control appeared to contain only mevalonic acid-2-^{14}C

Paper and thin layer chromatography to identify the peptide fraction

The labeled material in the aqueous extract, migrating near the origin, gave a positive response to ninhydrin and but did not migrate like any of the standard acids tested on the chromatogram (summarized in Table 6). Therefore, paper chromatography with known amino acid standards was run along with a portion of the aqueous extract obtained from the incubated postmitochondrial supernatant. A solvent system of n-butanol/acetic acid/water, 5/1/1, provided for the separation of amino acids, mevalonic acid-2-^{14}C, and the labeled portion of the aqueous extract. This data is summarized in Table 7. Thin layer chromatography in the same solvent system gave similar results and these results are summarized in Table 8. Ninhydrin was again used as the detection agent in these chromatograms. It can be seen that the ninhydrin response and the incorporated label in the aqueous extract appeared coincident in both paper and thin layer chromatography systems.

Isolation and characterization of the aqueous extract peptides

Characterization of peptides from thin layer chromatography

Preparative thin layer chromatography of the aqueous extract was then used to obtain larger amounts of the peptide fraction. The aqueous extract from 100 ml of incubated postmitochondrial supernatant was applied to a thin layer plate, and chromatographed in n-butanol/acetic acid/water 120/30/150, using the organic layer.

Table 7. Paper chromatography of amino acids and the aqueous extract. Samples (50 μgm of standards) in ascending manner. The solvent is butanol/acetic acid/water, 5/1/1. The Rf values were determined with detection with ninhydrin or scanning for radioactivity. The buffer control contains mevalonic acid-2-^{14}C (2000 cpm) mevalonic -2-^{14}C standard contained 1000 cpm. The aqueous extract contained 500 cpm.

Sample (radioactivity)	Rf (ninhydrin response)	Rf
Alanine	0.29	—
Aspartate	0.20	—
Cysteine	0.09	—
Leucine	0.65	—
Phenylalanine	0.57	—
Threonine	0.27	—
Mevalonic acid-2-^{14}C	—	0.75
Aqueous extract	0.21	0.19
Buffer control	—	0.76

Table 8. Thin layer chromatography of the aqueous extract and amino acids. Standards and the aqueous extract were chromatographed in n-butanol/acetic acid/water, 4/1/1. The buffer control contains mevalonic acid-2-^{14}C (2000 cpm); mevalonic acid-2-^{14}C, 1000 cpm; aqueous extract, 500 cpm.

Sample	Rf (ninhydrin)	RF (radioactivity)
Alanine	0.49	—
Aspartic acid	0.30	—
Cysteine	0.21	—
Leucine	0.70	—
Phenylalanine	0.66	—
Threonine	0.41	—
Buffer control	—	0.85
Aqueous extract	0.29	0.29, 0.85
Mevalonic acid -2-^{14}C	—	0.85

The preparative plate was divided into five areas, or bands, for extraction. The result of radioactivity scanning with the Bioscan instrument and the ninhydrin response was used as a guide. The silica was removed from the plate, extracted and the residue, after removal of solvent, was used for further analysis. Band I, the band nearest the origin, contained the ninhydrin positive label starting at the origin to about 2 cm above the origin and was the only section that contained incorporated label. Therefore, Band I was selected for further investigation.

The results of paper chromatography of Band I from thin layer chromatography and standard amino acids in a basic solvent (n-butanol/ethanol/ammonia, 1/1/1) is summarized in Figure 21. Ninydrin detected the position of each amino acid and any amino acid/peptide components of Band I. Radioactivity was assayed by counting strips cut from the chromatography lane. The ninhyrin positive material from Band I remained near the origin, while any mevalonic acid -2-^{14}C migrated to a Rf of 0.67.

Membrane filtration of the aqueous extract

Further purification of the aqueous extract was accomplished by filtration of the aqueous extract through membranes with defined pore sizes. This step removed residual starting material(s) and salts. Two step filtration was used, employing a Nucleopore stirred cell pressurized with nitrogen gas. The extract was filtered first through an Amicon YM1 membrane (MWCO of 1000 Daltons)to obtain the YM1 residue. The filtrate was used on a YCO5 (500 D MWCO) membrane to obtain the YCO5 residue and the final filtrate. Residue remaining on both the YM1 membrane and the YCO5 membrane were taken up in several washes of water. The results of membrane filtration, in terms of protein and radioactivity, is summarized in Table 9.

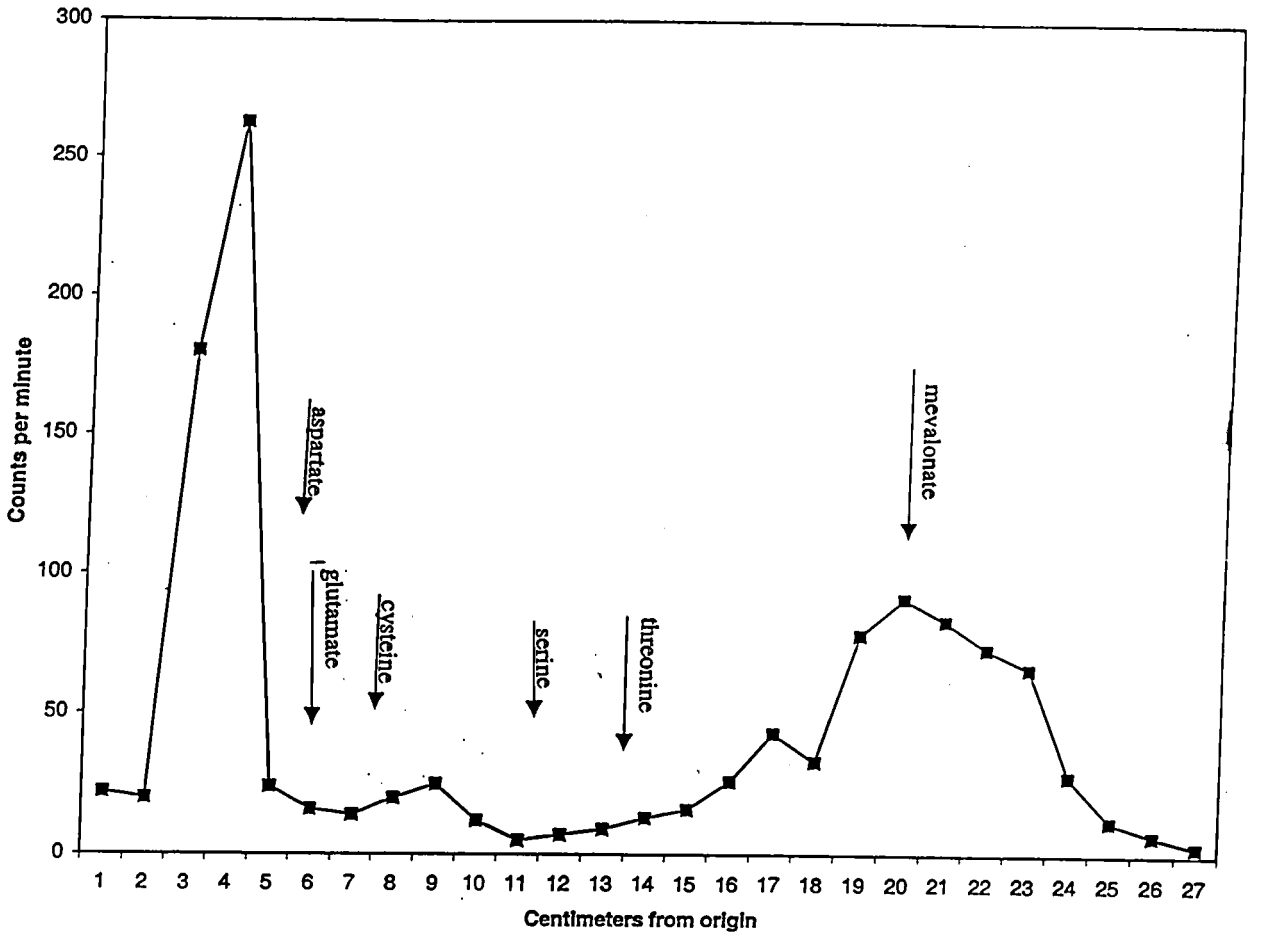

Figure 21. Distribution of radioactivity with paper chromatography of Band I. The sample was Band I obtained from thin layer chromatography. The solvent was n-butanol/ethanol/ammonia, 1/1/1. The sample lane was cut into 0.5 cm widths and the strips counted in the scintillation counter. Arrows indicate migration of standards, which were detected with ninhydrin,

Table 9. Membrane filtration of the aqueous extract. Two step filtration through 1000 MWCO (YM1 membrane) and 500 MWCO (YCO5 membrane) is summarized in terms of separation of protein and radioactivity. The YM1 filtrate served as the sample for the YCO5 filtration. The original sample is the combined aqueous extracts representing two liters of post mitochondrial supernatant incubated with a total of 2.0 μci of mevalonate-2-^{14}C. Protein is expressed in milligrams (mg).

Sample	Protein (total mg)	cpm (total)
Aqueous extracts	53.9	3.6×10^6
YM1 residue	3.1	3.1×10^4
YM1 filtrate	50.3	2.6×10^6
YCO5 residue	17.2	3.4×10^5
YCO5 filtrate	31.7	2.9×10^6

The majority of the radioactivity passed through both membranes, while most of the protein remained on the YCO5 membrane.

The membrane samples were compared by thin layer chromatography in the acid solvent, as shown in Figure 22. The radioactivity in both the YM1 filtrate and YCO5 filtrate appeared in band migrating identically to mevalonate-2-^{14}C. The YM1 filtrate and the YCO5 residue contained a labeled band at the origin as well as some residual mevalonate. The peptide bands were detected with ninhydrin reagent; the spots usually appear pink to purple in color.

The results of the sequential membrane filtration and the location of the labeled material by thin layer chromatography led to preparative thin layer chromatography of the YCO5 residue. The residue, in a concentrated solution, was applied to a thin layer plate and developed in the same solvent. The results are shown in Figure 23. Four areas, or bands, were selected on the basis of the location of radioactivity (Band 1, at origin), and ninhydrin reactivity (Bands 2, 3, 4). The bands were scraped off the plate and extracted with acetic acid. Figure 23 shows how bands were selected. A summary of the recovery of protein (Lowry) and radioactivity in each band is shown in Table 10. While mevalonate-2-^{14}C is presumed to pass through the YCO5 filter, the control experiment with filtration of the aqueous extract demonstrated that some mevalonate remains on the filter with the residue sample. This mevalonate can be separated from the bands of interest during the preparative thin layer chromatography step, since mevalonate migrates faster than the band of interest. The results show that Band 1 contained the most protein and label and was subjected for further study. Bands 2, 3, and 4 were not characterized any further. For this study, the subsequent experiments dealt with a sample that had been incubated for two hours and fifty-five minutes only. The control sample had no label or ninhydrin response.

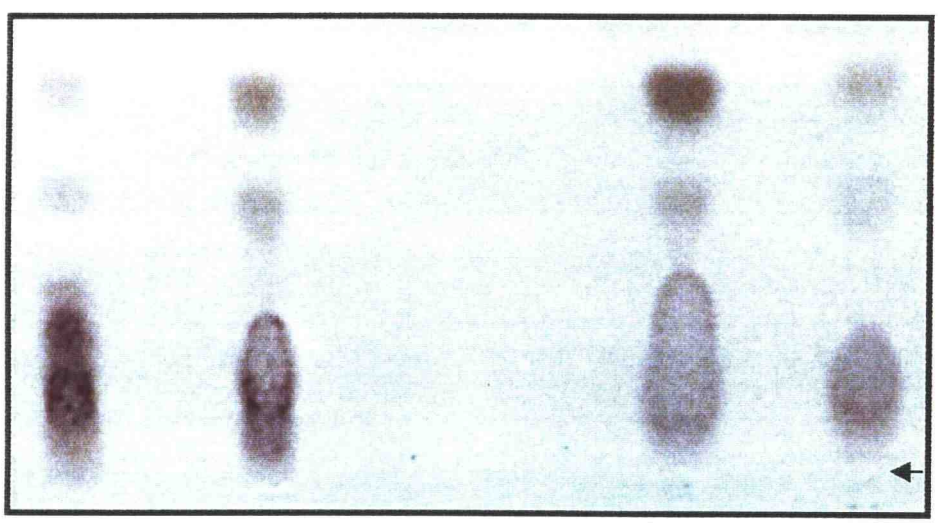

Figure 22. Thin layer chromatography of membrane filtration fractions. Left to right: the aqueous extract, the YM1 filtrate, the YCO5 residue, and the YCO5 filtrate. Arrow designates the origin; the solvent front is not shown. The solvent is n-butanol/acetic acid/water, 5/1/1.

Figure 23 . Preparative thin layer chromatography of the YCO5 residue. Numbers on the figure refer to bands removed from the plate and the bands are delineated with lines. The right lane is an analytical sample and the left lane is the preparative band. The line below Band 1 is at the origin; the solvent front is not shown. The solvent is n-butanol/acetic acid/warier, 5/1/1. The arrows designate the origin.

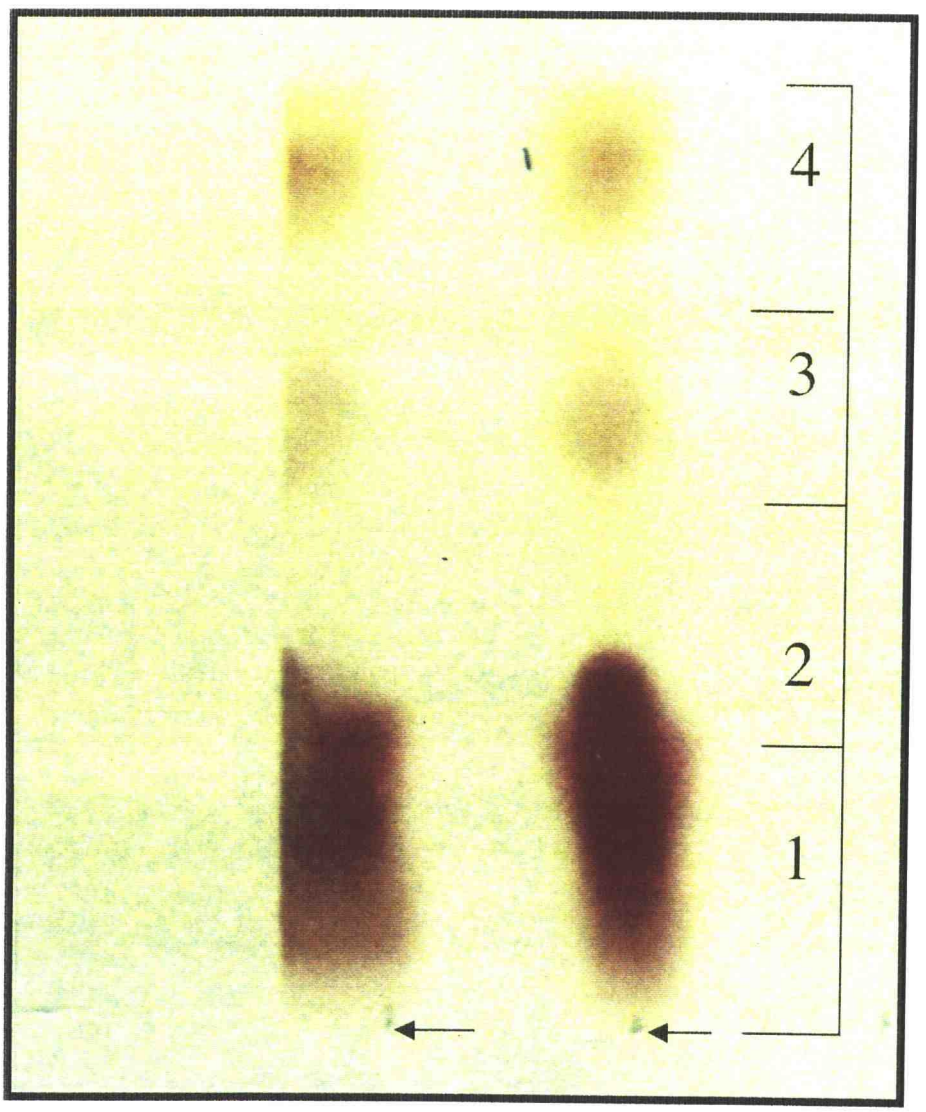

Figure 23

Table 10. Preparative thin layer chromatography of the YCO5 residue. The YCO5 residue was chromatographed in butanol/acetic acid/water 4/1/5 on silica plates, and the bands removed and extracted as described in Methods. Protein was determined by the Lowry (116) method.

Sample	Rf	Protein (total)	% (Protein)	Radioactivity
YCO5 Residue	—	2.3 mg	100	4.4×10^5
Band 1	0.04	0.900	39	1.0×10^5
Band 2	0.15	0.310	13	—
Band 3	0.28	0.075	3.3	—
Band 4	0.40	0.180	7.8	—

Paper chromatography of band 1 from preparative thin layer chromatography

Water saturated phenol (120) was selected as the mobile phase to separate the components of Band 1 obtained from preparative thin layer chromatography. Again, the sample to be separated was applied as a narrow band and solvent was allowed to migrate, in a descending manner, to within 3 cm of the far edge. The paper was thoroughly dried; a marker band (5 µl of sample band) was run at the same time. This band was cut off and sprayed with ninhydrin, and radioactivity was located on this same strip with counting with the Bioscan instrument. Five bands (I-V, I at origin) were selected on the basis of the presence of label and/or ninhydrin response. Bands I, II, and III were ninhydrin positive. Bands I, IV and V were labeled and their location on the chromatogram are summarized in Table 11B. The radioactive material was not mevalonate-2-^{14}C based on the migration of mevalonate on paper with water-saturated phenol as the solvent. In addition, on a separate paper chromatogram using two-dimensional paper chromatography of the preparative material, Band I label stayed coincident with the ninhydrin response, and the label in Bands IV and V remained as a single spot on the chromatogram, and coincident with the ninhydrin response. Finally, no other ninhydrin bands or radioactivity were detected by two dimensional chromatography.

At this point in the research project, the components of Band I obtained from preparative thin layer chromatography were likely to be of low molecular weight, between 500 and 1000 daltons, based on the prior separation on the Amicon YCO5 membrane. Therefore the bands obtained from phenol paper chromatography of Band I (from preparative thin layer chromatography) were expected to be of low molecular weight as well.

The bands from phenol paper chromatography were cut out and numbered Phenol Band I (at origin) to Phenol Band V (at solvent front). Phenol Bands II and III were not radioactive and were detected with ninhydrin only. Phenol Bands IV and V were radioactive, but were not detected with ninhydrin. The substance in each band was eluted with water, the solutions dried under vacuum and used in perfomic acid and vapor phase HCl acid hydrolysis experiments. The buffer control sample contained no ninhydrin positive material or label throughout these experiments. Performic acid oxidation was introduced to detect the presence of cysteine in any of the bands. Methionine is converted to methionine sulfoxide and cysteine to cysteic acid, after exposure to performic acid.

Cysteine is destroyed in acid hydrolysis as are tryptophan, and to some extent, tyrosine. The lack of absorbance at 280 nm and some preliminary results with fluorescence detection with HPLC experiments excluded tyrosine and tryptophan as constituents.

The presence of cysteine in the isolated bands was determined by first treating a portion of the isolated band (from phenol paper chromatography) with performic acid vapor overnight. Part of this performic-acid treated sample was then subjected to vapor phase HCl hydrolysis overnight. The control, performic acid treated, and performic acid plus HCl vapor phase hydrolysis were then chromatographed on paper in two dimensions. The first dimension was with liquefied phenol, while the second dimension was in butanol/acetic acid/water, 4/1/1. The constituent amino acids were identified by comparison with chromatograms of standard amino acids run at the same time.

The radioactive label detection (as determined by radiochromatographic scan) results are shown in Table 11A. The amino acid composition results, as determined by two-dimensional chromatography are summarized in Table 11B. Phenol Band II

Table 11A. Summary of location of incorporated label from mevalonate-2-^{14}C by paper chromatography. The sample for each section below was a band selected from phenol paper chromatography. Each band was treated with performic acid or performic acid plus HCl hydrolysis. Control samples received no treatment. Bands II and III were not labeled. The samples were then analyzed by two dimensional paper chromatography. The Rf values below show the location of the label after each development in the first solvent (Solvent I, water-saturated phenol) and second solvent (Solvent II, n-butanol/acetic acid/water, 5/1/1).

	Rf Values of Label in Each Sample from Radiochromatographic Scan					
Sample	Control		Performic Acid		Performic Acid/HCl	
Solvent	I	II	I	II	I	II
Phenol Band I	0.19	0.25	0.19	0.26	--	
Phenol Band II	--		--		--	
Phenol Band III	--		--		--	
Phenol Band IV	0.65	0.34	--		--	
Phenol Band V	0.94	0.34	0.92	0.47	0.97	0.35

Table 11B. Summary of results of amino acid analysis of peptides described in Table 11 A Amino acids were detected with ninhydrin and identified by comparison with known standards. Control samples were not treated. The other samples were treated with performic acid or performic acid and vapor phase hydrolysis with HCl.

Value	Control	Performic Acid	PerformicAcid/HCl
A. Phenol Band I			
Amino Acids	glu asp	glu asp	glu asp ala cysteic lys
B. Phenol Band II			
Amino Acids	glu	glu	glu
C. Phenol Band III			
Amino Acids	glu	glu	glu ala cysteic his tyr met
D. Phenol Band IV			
Amino Acids	—	— gly	cysteic asp glu ser gly ala
E. Phenol Band V			
Amino Acids	—	—	glu asp ala val lys ser gly met

contained only glutamic acid. The buffer control sample did not contain any ninhydrin-positive material when carried through the same procedures. Phenol Bands I, III, IV, and V released amino acids under HCl vapor phase hydrolysis conditions, but not performic acid conditions. Phenol Bands II and III contained no radioactive label. The radioactivity in Phenol Band I was released only after both performic acid and HCl treatment, while the label in Phenol Band IV was released after treatment with performic acid only. The label in Phenol Band V did not appear to be affected by the acid treatments.

Amino Acid Analysis by Dabsylation

Two of the peptides obtained from phenol paper chromatography (Table 11A and 11B), Phenol Bands II and IV, were also subjected to both performic acid and vapor phase HCl hydrolysis and derivation with dabsyl chloride, a reagent that chromatographic step (93). For estimation of molar ratios of amino acids present in samples, a standard mixture of amino acids was treated identically to the samples and chromatographed under the same set of conditions on the same day. Peak identities and areas were obtained by comparison of chromatograms. Samples were prepared for analysis by performic acid treatment and a combination of performic acid treatment and vapor phase HCl hydrolysis. Dabsyl derivatives, frozen when not in use, remained stable for at least several months. Final volume of the dabsylated sample was 500 μl and 20-40 μl were taken for analysis. The amino acid content on a molar ratio basis was calculated by using the μvolt response/pmole ratio of the amino acid standards.

The peptides obtained from the phenol paper chromatography were given a group name, the Nketewa peptides. Nketewa means small in the Twi language of Ghana, and the term was chosen because of its appropriate descriptive nature of the

peptides and the advisor's Fulbright experiences of Ghana. Nketewa I refers to phenol band I and so on.

Nketewa peptides II and IV were subjected to dabsylation after preliminary treatment with perfomic acid or performic acid plus vapor phase HCl hydrolysis. This approach resulted in three dabsylated samples for each Nketewa peptide: one, control (no treatment); two, treated with performic acid; and three, performic acid plus vapor phase HCl hydrolysis. Amino acids are observed when HCl and performic acid are used to treat a blank tube (125). Therefore, these control values obtained from blank tubes treated at the same time the Nketewa peptides were treated are used to correct for background values on the vapor phase HCl values. The perfomic acid values were used to correct the HCl vapor phase hydrolysis values. The results are summarized in Table 12.

The molar ratios of the amino acids observed can be calculated from this data. Nketewa II was expected to have only glutamic acid, and the dabsyl results confirm that expectation. The Nketewa IV values, by setting the cysteic acid value to one and normalizing the other molar amounts to this value resulted in an amino acid molar ratio of cys/glu/ser/gly/ala/val, 1/3/2/1/1/1, rounded to the nearest integer.

Table 12. Results of dabsyl amino acid analysis. Nketewa peptides II and IV were analyzed. Results are expressed in picomoles observed after correction of background values. Samples were either treated with performic acid only (Column 2) or performic acid and vapor phase acid hydryolysis (HCl)(column 3).

A. Nketewa Band II

Observed Amino Acid	Performic Acid	Performic/HCl
glu	200	402

B. Nketewa Band IV

Amino Acid	Performic Acid	Performic/HCl
cys	—	57
glu	7	157
ser	—	124
gly	8	79
ala	—	79
val	33	59

This result agrees favorably with the results shown in Figure 11B where the amino acid composition of Nketewa IV was found to contain, by two-dimensional paper chromatography after similar hydrolysis treatments, five of the acids observed by hydrolysis followed by dabsylation: cys, glu, ser, gly, and ala. The two results differed in that the dabsyl analysis suggested the presence of valine in peptide while the paper chromatographic analysis (Figure 11B) did not.

Characterization of the soluble peptide fraction

The results of the studies on the lipid extracts and the aqueous extracts clearly demonstrated that mevalonate was being utilized by the postmitochondrial supernatant. Label derived from the starting material appeared in the two lipid extracts, as well as several peptides. Further studies then were directed toward characterizing the components of the soluble protein fraction.

The precipitated protein was free of the original mevalonate-2-^{14}C. This result occurred in initial time, incubated, and inactive post-mitochondrial supernatant experiments. Buffer control samples (no supernatant) were treated along with incubated and initial time samples in order to correct for any background interference in subsequent experiments.

After precipitation and washing with water and ethanol, the protein pellet was air dried and homogenized to a coarse powder to assure complete drying. The soluble peptides were taken up in 10 ml water and particulate material removed by low speed centrifugation, and are referred to as the soluble protein fraction. The postmitochondrial supernatant contained from 7.2 to 7.5 mg protein/ml (Lowry). Approximately 350 mg dry protein could be obtained from ethanol precipitation of 50 ml of supernatant.

Gel filtration of the soluble peptide fraction

Biogel P-100 column chromatography of the soluble protein fraction is shown in Figure 24. This gel has an exclusion limit of 100,000 D The sample for the chromatogram was 3 ml of solubilized protein obtained from 50 ml of post-mitochondrial supernatant. A total of 300 ml water was used to elute the column. The fractions were analyzed for protein by the Bradford method. The separation of several protein peaks were observed. The labeled material, however, eluted as a single peak. Using the chromatogram as a guide, fractions 14-22 (containing ^{14}C label) were pooled, dialyzed and the dialysant reduced in volume and chromatographed on a Biogel P-6 column. Biogel P-6 has an exclusion limit of 6,000 D. This chromatogram is shown in Figure 25. Labeled fractions were pooled and used for analysis by ion exchange chromatography.

Ion exchange chromatography of the soluble fraction

Anion exchange chromatography of the pooled, concentrated fractions obtained from gel filtration was performed on a Cellex-E resin (BioRad Laboratories) column. A gradient of 0 to 1 M LiCl in water was used as the as the eluent. The chromatogram is shown in Figure 26. Since the protein fraction did not appear to bind to the column, the labeled protein fractions were again pooled, concentrated and used as sample for cation exchange chromatography on BioRex-70 resin (BioRad Laboratories). The results are shown in Figure 27. The radioactivity and protein appeared in fractions two through four, as shown. The overall results of the ion exchange chromatography indicate that this protein fraction was not tightly bound to anion or cation exchange columns. The labeled protein fraction was again pooled and taken down to a small volume.

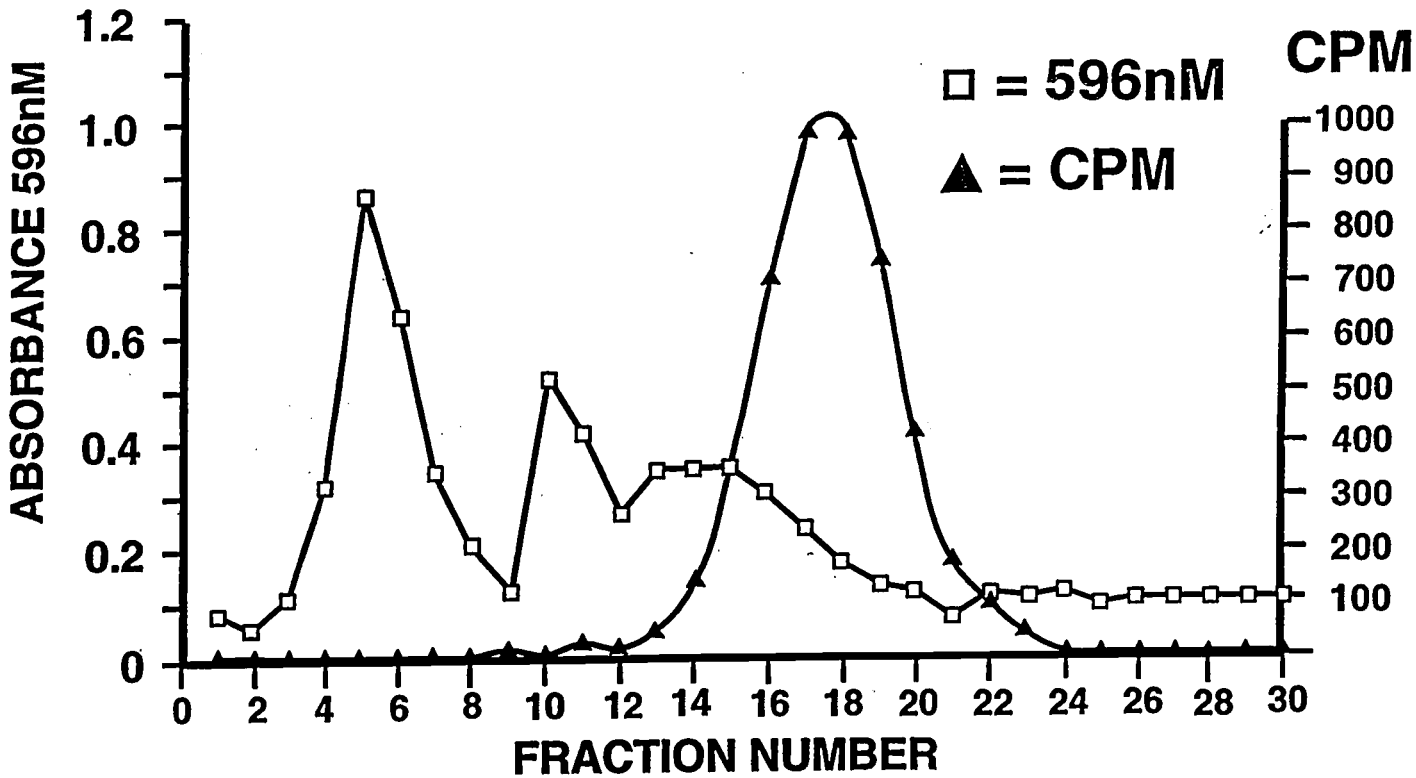

Figure 24. BioGel P-100 chromatography of the soluble peptide fraction. The chromatogram illustrates the elution of protein [as analyzed for protein by the Bradford (117) method] and radioactivity (cpm). Water was used as the eluent.

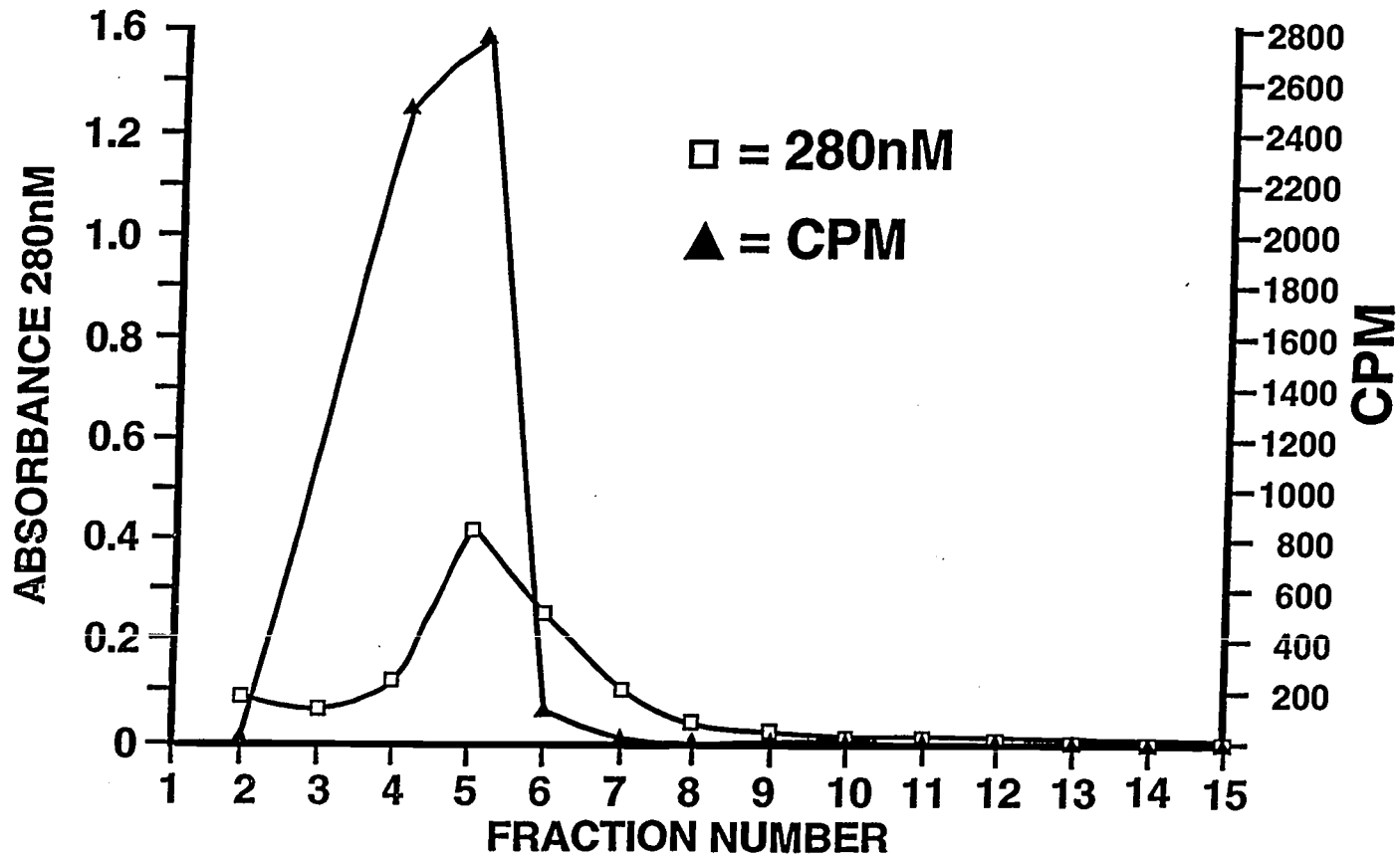

Figure 25. BioGel P-6 chromatography of labeled fractions. The pooled labeled fractions obtained from BioGel P-100 (Figure 24) were applied to the column. Fractions were analyzed for protein (absorbance at 280 nm) and for radioactivity (cpm). The column was eluted with water.

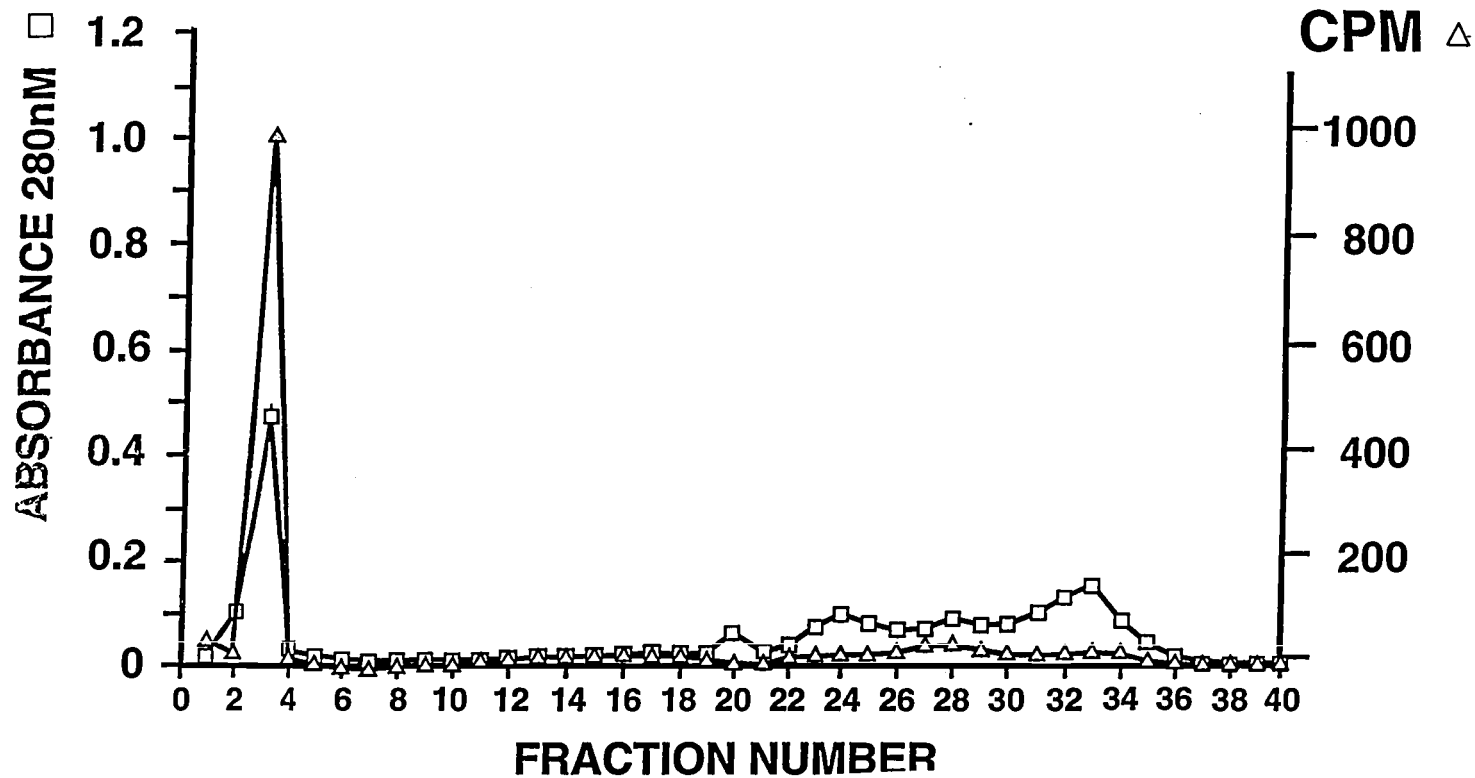

Figure 26. Cellex–E anion exchange chromatography of the labeled fractions. The combined fractions from BioGel P-6 chromatography (Figure 25) were applied to the column and eluted using a LiCl gradient in water. Samples were analyzed for protein (absorbance at 280 nm) and for radioactivity (cpm).

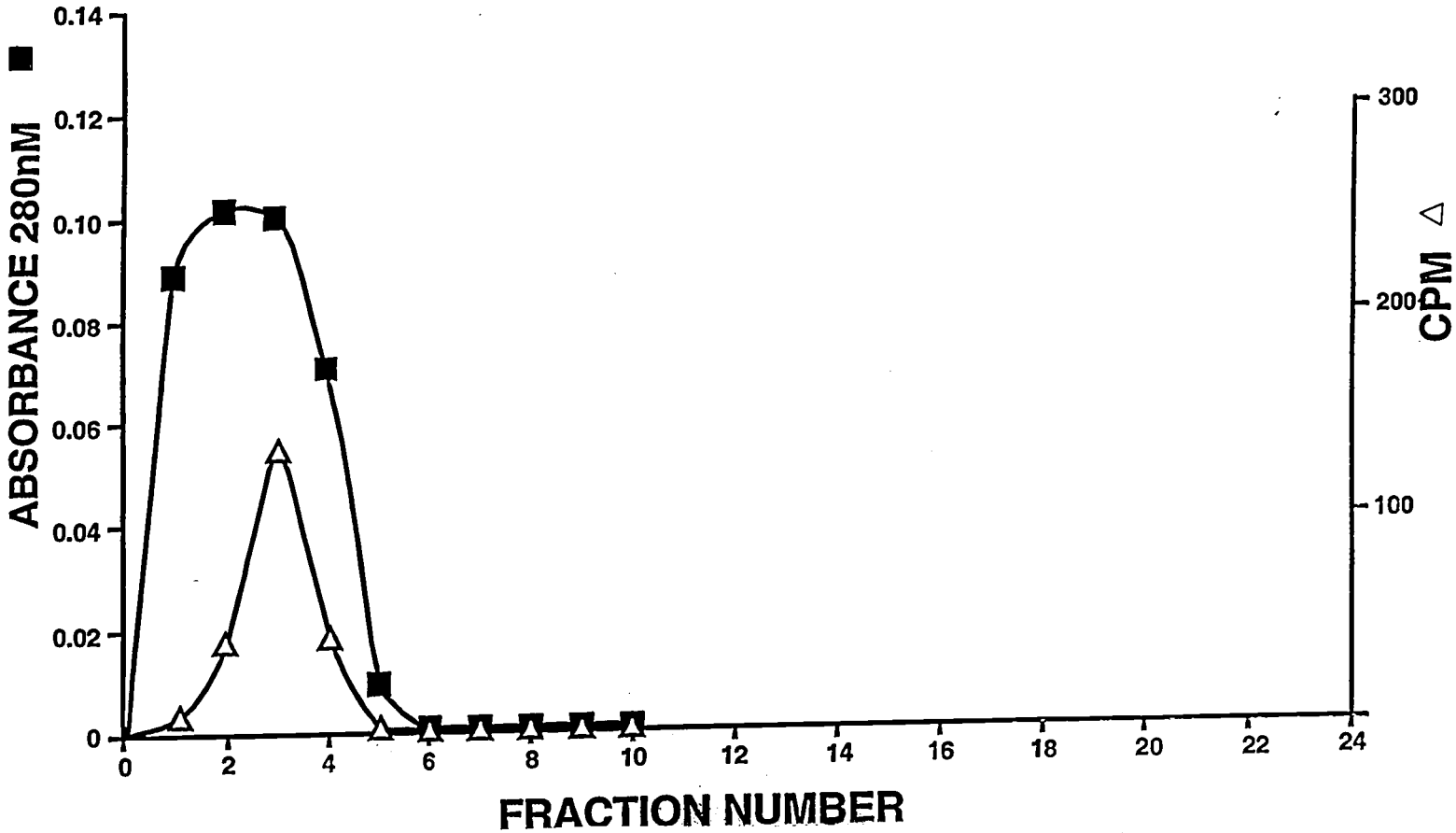

Figure 27. BioRex-70 cation exchange chromatography of the soluble peptide fraction. The combined fractions from anion exchange chromatography (Figure 26) were applied to the column and eluted with a LiCl gradient in water. Fractions were analyzed for protein (absorbance at 280 nm) and for radioactivity (cpm).

HPLC of the soluble peptides

The material isolated by gel filtration and ion exchange chromatography was subjected to HPLC on a C_{18} Vydac column (modified with octadecyl silane, C18 groups). The column was eluted with a nonlinear gradient of 10% trifluoroacetic acid in water and 0.067% trifluoracetic acid in acetonitrile. The 8.3 minute peak eluted at approximately 30% acetonitrile. The HPLC trace is shown in Figure 28.

Each peak from HPLC was collected and taken to dryness. The first two peaks that eluted contained nucleotides and an amount of peptide too small to be useful for further analysis. The peak eluting at 8.3 minutes was selected for further analysis as it appeared that sufficient protein was present. The material in this peak was subjected to vapor phase HCl hydrolysis and subsequent dabsylation, and analysis by HPLC.

The results of this analysis are summarized in Table 13. Thus the material in this peak appeared to contain peptide(s) which released amino acids upon hydrolysis. The term Nketewa was also applied to this material, and is referred to as Nketewa A. No amino acids were found in a control sample (no hydrolysis) or reagent blank (no peptide).

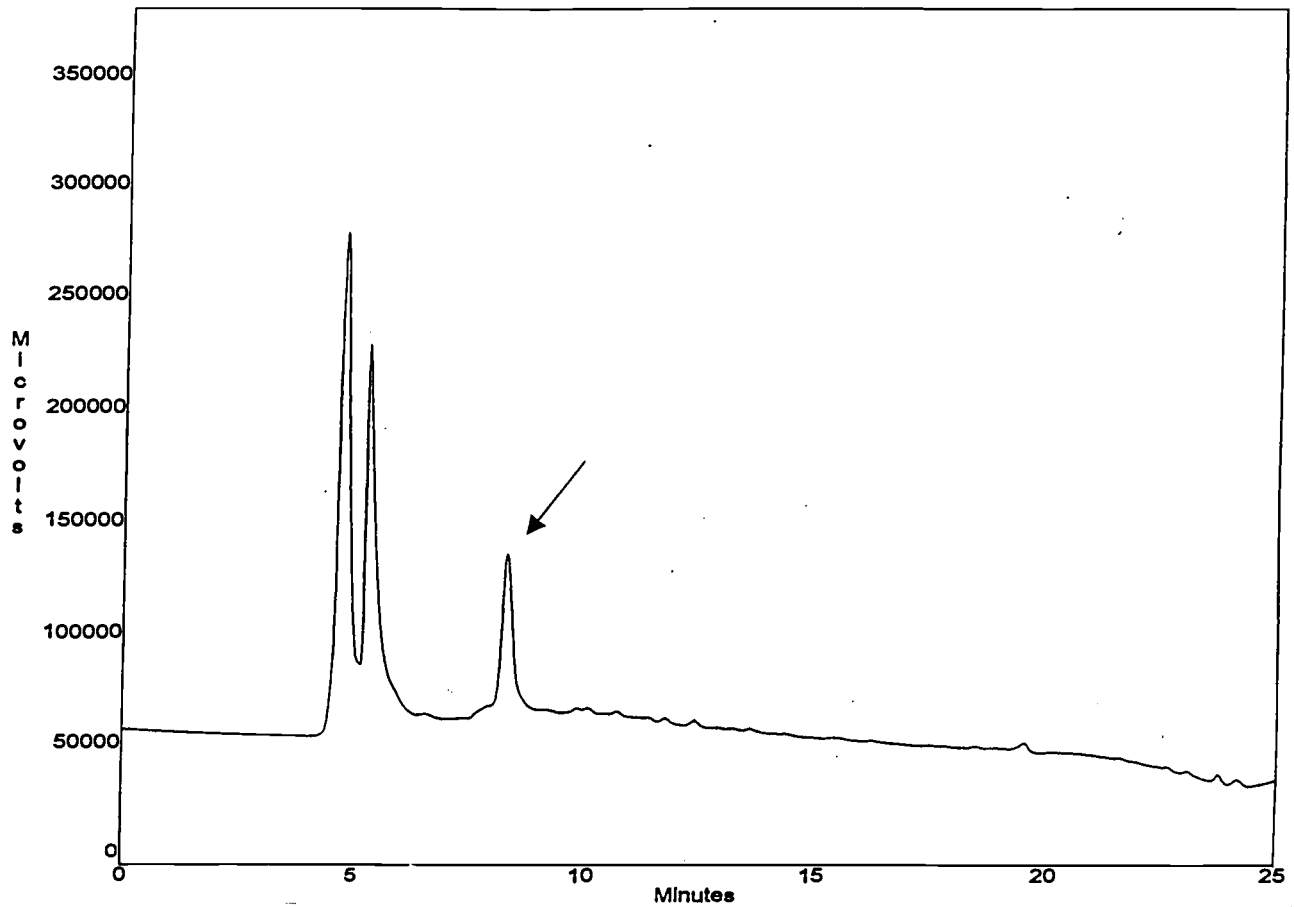

Figure 28. HPLC of the soluble peptide fraction. The soluble peptide fraction was chromatographed on a reversed phase column. The microvolt response is based on the absorbance at 280 nm. The sample was eluted in a gradient of acetonitrile in 0.1% triflouroacetic acid. The sample at 8.3 minutes was used in subsequent analyses.

Table 13. Dabsyl amino analysis of the soluble peptide. The sample, Nketewa A, for analysis was the peptide obtained by HPLC. Amino acid content is expressed as a percent of total amino acid content.

Amino Acid	Mole %
Aspartic	21.28
Glutamic	15.67
Serine	3.78
Threonine	3.32
Glycine	16.01
Alanine	9.12
Proline	4.24
Valine	3.75
Arginine	1.95
Methionine	2.49
Isoleucine	2.87
Leucine	3.86
Phenylalanine	2.77
Lysine	5.91
Histidine	1.29
Tyrosine	1.69

Mass spectrometry of the soluble peptides

The soluble fraction, like the aqueous fraction could be purified by membrane filtration, using the same protocol. The soluble extract was filtered first through the YM1 membrane to give a YM1 residue and a YM1 filtrate. This filtrate was used with a YCO5 membrane to obtain the samples YCO5 residue and the YCO5 filtrate.

The YCO5 residue was next subjected to preparative thin layer chromatography, to give three ninhydrin bands, termed Nketewa 1 (at origin), Nketewa 2 and Nketewa 3.

Nketewa 1 was removed from the plate, extracted, and characterized further by mass spectrometry and sequencing experiments. The sequencing was performed by the Central Services Lab, at the Gene Research Center at Oregon State University. For sequencing purposes, the peptide N-myristolated protein kinase C inhibitor (Sigma Chemical Company, St. Louis, MO) was used as a negative control. This peptide has a myristoyl group covalently attached to the N-terminal amino group. The Edman degradation (127) is the basis for sequencing protocols and requires a free amino end terminus of the peptide. The control peptide, with a sequence of N-myristoyl-phe-ala-arg-lys-gly-ala-leu-arg-gln-NH_2 gave negative results, as expected. Bradykinin, as a positive control, gave a sequence of arg-pro-pro-gly-phe-ser-pro-phe-arg, which is reported by the supplier (Sigma Chemical Co., St. Louis, MO). The Nketewa 1 peptide could not be sequenced could not be sequenced. Similar results were obtained when cyanogen bromide cleavage (128) was attempted on the Nketewa 1 peptide. Cyanogen bromide cleaves peptides on the carboxy side of methione residues (128). The result is a new N-terminal amino acid residue which was on the carboxy side of the original methionine residue. This new peptide

is now available for sequencing. The original peptide is bound to a membrane so does not interfere with this sequencing process, and has a new carboxy terminal residue, homocysteine. The positive control was α-melanocyte stimulating hormone (N-acetyl-ser-tyr-met -glu-his-phe-arg-trp-gly-lys-pro-val-NH_2). The Nketewa peptides of the aorta, both from the aqueous extract and the soluble protein fraction did not give product(s) when subjected to cyanogen bromide cleavage or Edman degradation.

Positive controls were also used in the mass spectrometry experiments. The mass spectrum (MALDI) of α-melanocyte stimulating hormone is shown in Figure 29, indicating the correct molecular weight of this peptide.

The Nketewa 1 peptides(from Band 1 of thin layer chromatography) were analyzed by MALDI mass spectrometry, shown in Figure 30, to give information about the mass(es) of the peptide(s). The Nketewa 1 sample was then used in liquid chromatography and one peak was selected for further analysis for sequence information. The LC/MS (tandem mass spectrometry) of the selected peptide, mass 1038.1, is shown in Figure 31. There was insufficient data to obtain information about the other peptides observed in the liquid chromatography of the Nketewa 1 sample.

The protocol for determining the sequence of a peptide from tandem mass spectrometry is not always straightforward, because of the variable fragmentation pattern that occur when peptides are treated in this manner (for a review, see 129). The amino acid residue may fragment from the main peptide from either the amino terminus or the carboxy terminus. In addition, the residue may fragment at different points in the amino residue

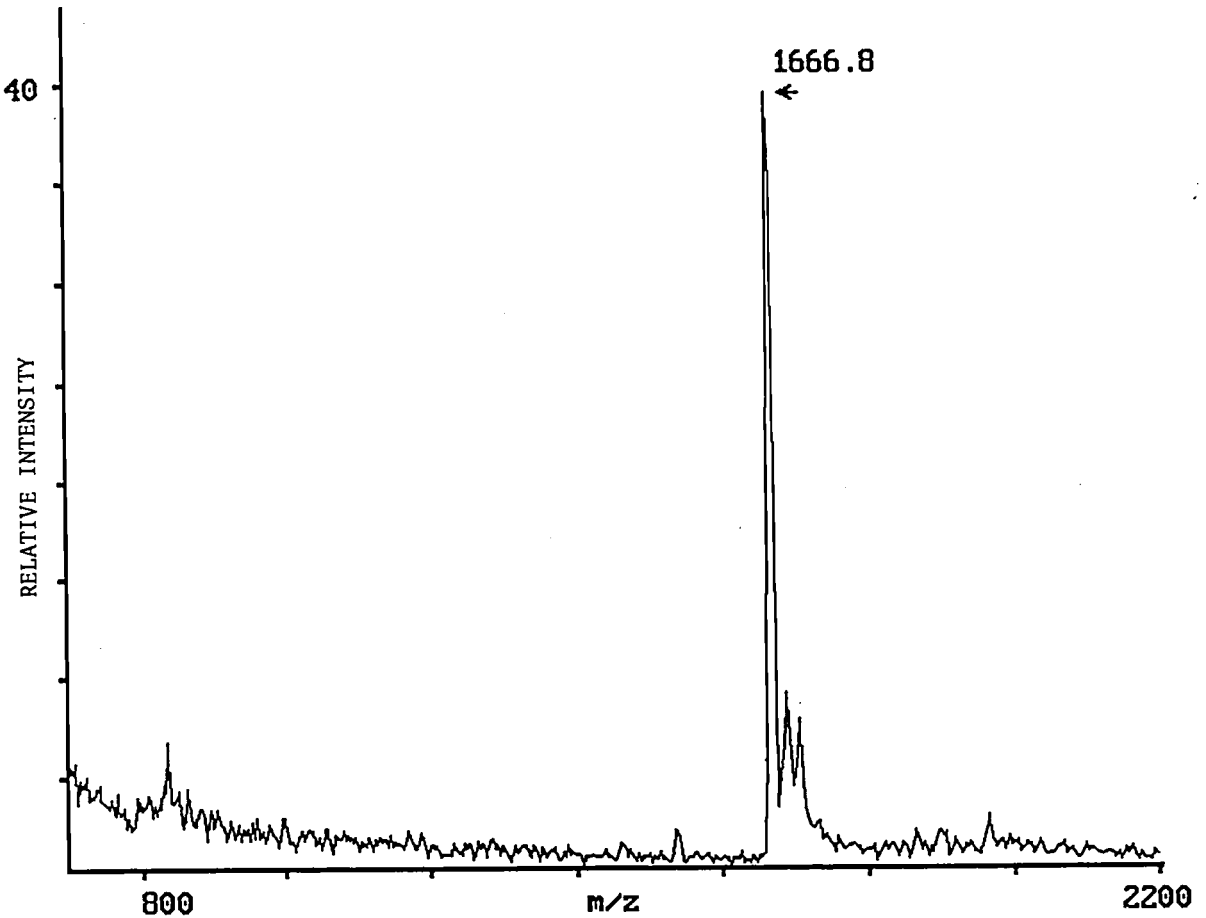

Figure 29. MALDI mass spectrum of α-melanocyte stimulating hormone.

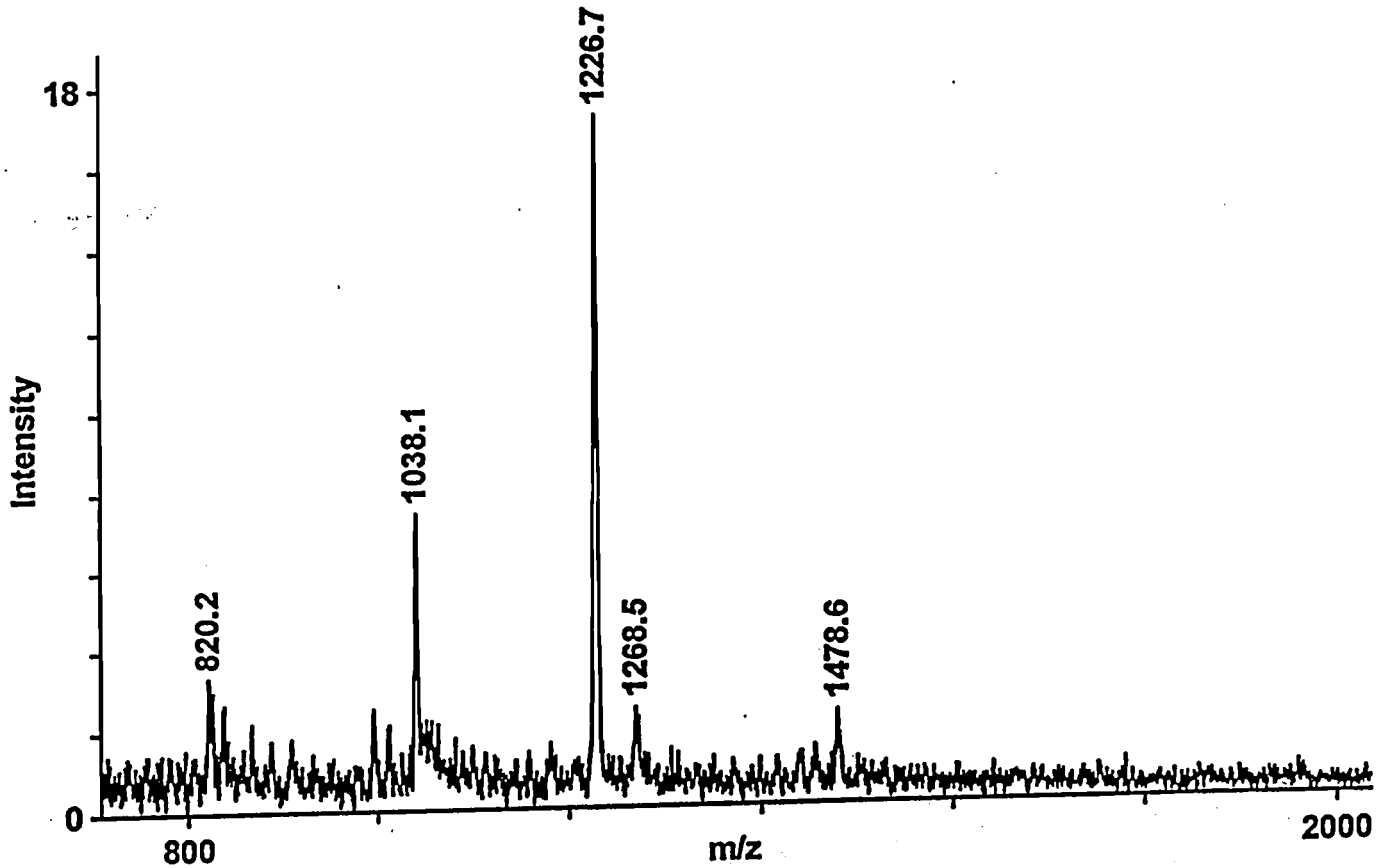

Figure 30. The MALDI mass spectrum of the Nketewa peptides. The sample for the analysis was band 1 obtained from thin layer chromatography of an HPLC sample.

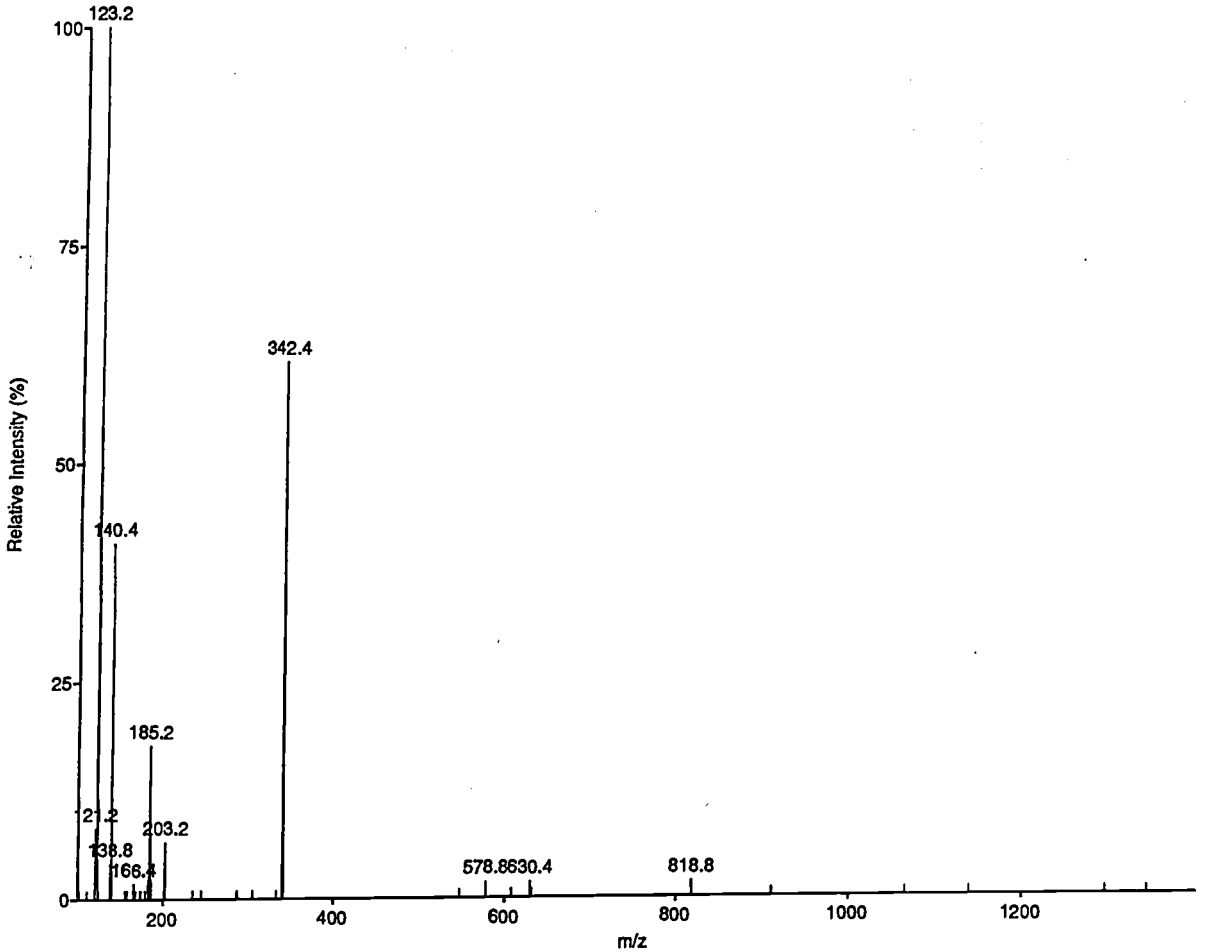

Figure 31. Tandem mass spectrum of the 1038 Dalton Nketewa peptide. The peptide was selected first from liquid chromatography mass spectrometry and then analyzed by tandem mass spectrometry.

itself, giving rise to the potential of various masses for the same amino acid residue. Since the study is done in an acidified matrix, protonation of the residue takes place. In this study, the amino acid composition had been determined for this peptide by vapor phase HCl hydrolysis followed by two dimensional paper chromatography. This gave (at least) an amino acid composition of ser, gly, ala, val, met, phe and cys.

The data from Yates et. al (134) was used as a guide in assigning the identity and composition of each fragment from the tandem mass spectrum. Table 14 shows the deduced peptide sequence. The information about the amino acid composition of the peptide strengthened the decision making when assigning residue position in the peptide. The data also indicated the presence of a fragment with the mass of a cysteine residue with a farnesyl moiety attached. The deduced sequence was his-gly-val-cysteine(farnesyl)phe-ala-ser-met, or with one letter codes for the amino acids, HGVCFASM.

A search was made to identify this Nketewa peptides using the BLAST and SWISSPORT databases, but no significant match was found; a search of the EMBL-Heidelberg data base indicated the sequence was present on chromosome 9, HRPK-295 D22. The data from the database searches is summarized below. A similarity of genetic sequences in the EMBL-Heidelberg data gave three potential matches (note one letter codes are used for amino acid residues):

(1) 1 H G V C F A S M 8
| | | | | | | |
48 H G E C F A S M 41 (residue number in similar sequence)

Percent similarity: 87.5 Found in human genomic sperm library

(2) 1 H G V C F A 6
| | | | | |
15307 H G V C F A 15312 (residue number in similar sequence)

Phase 1

Percent similarity: 100.0 Found in Homo Sapiens clone DJ0865F17; HGTS Phase

(3) 1 H G V C F A 6
| | | | | |

Table 14. Deduction of the sequence of the 1038 Dalton Nketewa peptide from tandem mass spectrometry. The amino acid composition data was also used. The sequence was deduced from the amino terminal end of the peptide.

Proposed Sequence	Peak in Spectrum (Daltons)	Notes
His	138.1	
His-gly	166.4	
His-gly (+H_2O)	185.0	
His-gly-val	342.4	
His-gly-val-cys (farnesyl)	630.4	
His-gly-val-cys (farnesyl)phe-ala	818.8[1]	(phe-ala = 188)
His-gly-val-cys (farnesyl)phe-ala-ser-met	1038	

[1]Peak of mass 578 corresponds to loss of a fragment from the peptide from the carboxy terminal end. This lost fragment corresponds to phe-ala-ser-met.

DISCUSSION AND CONCLUSIONS

Atherosclerosis is a dynamic, progressive disease of the large arterial vessels characterized by the development of thrombogenic and occlusive lesions (plaques) in the vessel wall. Plaque formation is the result of accumulation of lipids into macrophages ("foam cells") in the subendothial layer and the migration of smooth muscle cells into the intimal layer and their subsequent proliferation. Calcification, the formation of a fibrous and often fragile cap, the accumulation of extracellular matrix materials, and the deposition of lipids including free and esterified cholesterol contribute to the complexity of the lesion. These concurrent processes are not only the result of external environmental conditions but also the involvement of the components of the vessel wall itself.

Understanding the biochemistry of the vessel wall is integral to the elucidation of the etiology of atherosclerosis. Cholesterol, because of its recognition as a characteristic early component of lesion composition, and because of the regulation of cholesterol uptake into cells (46,48) has been central to atherosclerosis research. Cholesterol is supplied from external resources and is also synthesized by the cell from a remarkable six-carbon precursor, mevalonic acid. This molecule is also the precursor of a variety of other molecules, including heme A, ubiquinone, dolichols, isopentenyl adenine (a modified nucleoside) and the isoprenoid modifications of proteins; mevalonate is also required for cell proliferation.

Mevalonate-related processes are directly related to atherogenesis as well as normal functioning of the arterial wall. This investigation has addressed three topics that are related to mevalonate metabolism, using a cell-free, postmitochondrial supernatant prepared from bovine aorta. Central to the research process described here was the design of protocols and the combination of protocols to collect the experimental results.

The first question addressed by this research asks if neutral lipid and acidic lipid components, which have been biosynthesized from mevalonate, can be further characterized. This question is an extension of earlier research (78, 95) performed in this laboratory. The second question asks if the intermediates of the *trans*-methylglutaconate shunt can be isolated from this preparation, as it is known (78) that the postmitochondrial supernatant is capable of incorporating the radioactivity in mevalonate-2-C^{14} into C_8 and C_{10} acids in diacylglycerols. The third question asks if mevalonate can be incorporated into isoprenoid modifications of peptides and proteins.

The Neutral and Acidic Petroleum Ether Extracts

As noted above, the neutral and acid lipid petroleum ether extracts of the incubated postmitochondrial supernatant contained radioactivity derived from mevalonate-2-^{14}C. Incorporation of mevalonate-2-C^{14} into both neutral lipid and acidic lipid extracts required adenosine triphosphate but no other cofactors. The volatility of some of this incorporated material in the neutral lipid fraction was

observed when at least 10 percent of the radioactivity is lost from this fraction when left open to the atmosphere. Dimethylallyl alcohol is a required intermediate in both the *trans*-methylglutaconate shunt and the isoprenoid biosynthetic pathway. This branched chain alcohol and its isomer, isopentenol are both volatile and, as pyrophosphorylated esters, are intermediates in the isoprenoid pathway. The formation of isopentenyl pyrophosphate by arterial tissue has been demonstrated (43). Hydrolysis of these intermediates to the free alcohol is possible with pyrophosphatases and phosphatases, which are present in arterial tissue (95). Thus the appearance of these short chain alcohols in the neutral lipid extract would not be unexpected. In addition, prenylation of proteins requires the prenols farnesol and geranylgeraniol, also in pyrophosphorylated forms, again possible substrates for ester hydrolysis. This prenyl protein modification is required for a variety of cellular functions and the control of other cellular functions. Attempts to identify this material by mass spectrometry methods were not successful due to the amount of extraction solvents employed and the similarity of the extraction solvents used to the neutral lipid extract.

Incubation of the mevalonate-2-^{14}C demonstrated that about 6 percent of the added label appears in this neutral lipid fraction. Since only the R form of mevalonate is used in metabolic processes, and the commercially available labeled mevalonate-2-^{14}C is in the RS form, incorporation may be as high as 12 percent. The significance of these products in normal or atherosclerotic tissue cannot be

determined until they are identified, but these products may be important in biosynthetic pathways or catabolism of isoprenoid(s).

It is known (95) that the neutral fraction contains diacylglycerols as well as the above unidentified component(s). Diacylglycerols can act as detergents for the transport of cholesterol (136) and have been shown to participate in intracellular signaling pathways (137). Diacylglycerols are associated with the activation of protein kinase C (137), a process that leads to increased production of extracellular matrix and cytokines. The *de novo* synthesis of diacylglyerols and subsequent signaling events is also associated (138) with enhanced vascular contractility, permeability, and cell proliferation. All these diacylglycerol- associated processes are present in normal functioning tissue. Cholesterol accumulation occurs in atherogenesis. Accumulation of extracellular matrix impacts the disease process by enhancing fibrotic cap formation. Cytokines contribute to the chronic inflammatory state of lesions. Calcification of arterial tissue results in the formation of insoluble deposits in the arterial wall.

The extracted products were not limited to the volatile components and the diacylglycerols. A second radioactive component was extracted from an acidified, incubated postmitochondrial supernatant. This component displayed different chromatographic behavior in two solvents (acidic and basic) on thin layer plates. The radioactive component moved with the solvent front in an acidic solvent and stayed at the origin with a basic solvent, as detected by radiochromatographic scanning of the plates. The fact that this product was extracted only from an

acidified mixture implies a protonated molecule, while the extraction and chromatographic behavior describe the hydrophobic nature of the component. Acidification of the mixture may also have released this component from a second molecule, but there is no other information to suggest this. The results obtained by paper chromatography demonstrated that this component was not one of the straight chain (2-18 carbons) carboxylic acids tested, nor was it the *trans*-methylglutaconate intermediate, dimethylacrylic acid. Attempts to identify this component by mass spectrometry also were not successful, again due to the nature of the extraction solvents and the relative quantity used. This component represents a product of mevalonate metabolism yet to be identified. The production of acids from mevalonate-derived molecules is not unexpected (for example, the *trans*-methylglutaconate shunt). Other researchers have observed that farnesol is converted to a number of dicarboxylic acids as a degradative strategy when farnesol accumulates in the cell (138). The acid lipid extract may represent the removal of unwanted isoprenoid products in the cell, or may be an unesterified product released on treatment with acid. The results from studying the trans-methylglutaconate shunt intermediates showed that the acid petroleum ether extract product(s) were not from this pathway.

The Trans-methylglutaconate Shunt Intermediates

The *trans*-methylglutaconate shunt is a series of reactions (Figure 4 in the Introduction, page 16) that convert mevalonate to acetyl CoA. It has been

indirectly shown to operate in other tissues (131-133) and ^{14}C obtained from mevalonate –2-^{14}C appears both in expired CO_2 (75) and fatty acids (76). The *trans*-methylglutaconate shunt intermediates have never been directly observed, to our knowledge. To demonstrate the presence of the shunt intermediates in arterial tissue, it was necessary to employ two systems of thin layer chromatography and two systems of paper chromatography, so that a range of chromatographic behavior could be used to identify the intermediates. Likewise, silica gel chromatography to separate small carboxylic acids, after the method of Gamble *et al* .(133), was used to isolate and identify the postulated shunt intermediates found in the post mitochondrial supernatant. The silica gel column for this protocol could be standardized with known intermediates so their location on the column chromatogram was established. Utilizing the fractions containing the postulated intermediates allowed for characterization by thin layer chromatography and paper chromatography. Mass spectrometry analysis confirmed the elution of standards in the standard column profile, and GC/MS showed the presence of several intermediates. The use of the combined protocols demonstrated that radioactivity from mevalonate-2-^{14}C was recovered in *trans*-methylglutaconic acid, β-hydroxybutryic acid, β-hydroxy-β-methylglutaric acid, and dimethylacrylic acid. The amount of incorporated label in the intermediates, compared to that found in the neutral lipids, is approximately five percent. However, the functioning of this pathway may be significant in its synthesis of diacylglycerols, as discussed above. The low synthesis of cholesterol in vascular tissue (78) may indicate that other

metabolites of mevalonate are more important, such as the biosynthesis of diacylglycerols and other isoprenoids. Over 90 per cent of the radioactivity from mevalonate-2-^{14}C found in the neutral lipid fraction appears in the diacyglycerol fraction. Atherogenic processes would also be affected by limiting the *de novo* biosynthesis of cholesterol and therefore its accumulation in the vessel wall.

The *trans*-methylglutaconate shunt also contains two oxidative steps catalyzed by alcohol dehydrogenase: the conversion of dimethylallyl alcohol first to dimethylacrylaldehyde and a second oxidative step to dimethylacrylic acid. The presence of alcohol dehydrogenase activity was successfully demonstrated in this report. Dimethylallyl alcohol and isopentenyl alcohol both were more active than ethanol in an alcohol dehydrogenase assays. This enzymatic step is necessary for the conversion of the early isoprenoid pathway intermediates to dimethyacrylic acid. This additional evidence supports the presence of the *trans*-methylglutaconate shunt in the postmitochondrial supernatant. It should be noted that dimethylacrylic acid is also an established intermediate for the catabolism of leucine, but such degradation occurs in mitochondria. No labeled leucine was observed in this research.

Therefore, definitive results showing the participation of the shunt intermediates dimethylacrylic acid, *trans*-methylglutaconic acid, and β-hydroxy-β-methylglutaric acid, were described. To our knowledge, this is the first time this has been reported. The *trans*-methylglutaconic shunt provides a means of

transferring carbons from mevalonate acid to biomolecules which impact both normal and diseased states of arterial tissue, as noted above.

Mevalonate Derived Radioactivity in Peptides

Other biomolecules affected by mevalonate are peptides, which are covalently modified with isoprenoid side chains. This postranslational modification was first reported their isolation from yeast (64). Since this discovery in 1979, prenylated proteins include the γ subunit of trimeric G proteins, (small G proteins in the rab, rac, and rho families), nuclear lamins A and B and rhodopsin kinase and other numerous examples (141). The role of prenylated proteins in intracellular signaling may impact those processes implicated in atheroslerosis such as cell proliferation, migration, thrombosis, and the secretion of extracellular matrix material. The research presented in this study demonstrated the incorporation of mevalonate-2-^{14}C label into peptides found in both the aqueous extract and the soluble peptide fraction. The peptides in both fractions were composed of small molecules, and so were collectively called the Nketewa peptides.

The peptides in the aqueous extract could be separated into five fractions (Nketewa I to V) by phenol paper chromatography. One of the isolated components, Nketewa II, was found to contain free glutamic acid. No other free amino acids were observed in these experiments Since previous research also has shown that C_8 and C_{10} carboxylic acid are synthesized by this preparation from

mevalonate, acyl modification of peptides also might occur. N-acylation is not an uncommon occurrence in proteins (127), and small acids (containing 2, 4, 6, and 8 carbons) have been shown to modify proteins (128) by N-acylation. This modification represents a protective as well as membrane association process. N-acylation may account for the inability to sequence the peptides by conventional Edman degradation (127). Peptides are also modified by acyl groups on internal cysteine residues (141-142). However, peptides can be modified by either or both processes of acylation and prenylation with mevalonate derived products.

Other experiments in this laboratory (143) demonstrated that the peptide associated label was not totally stable to acid or base hydrolysis, further strengthening the argument that the label is present in an acyl moiety on the peptide. Treatment with acid or base would release such a modification, while isoprenylation would be stable to either treatment. The label could also be partially released by treatment with methyl iodide (144), a procedure known to release prenyl substituents from proteins. The significance of prenyl labeled proteins in normal tissue and atherosclerosis has been discussed above. Acylation of proteins is another posttranslational modification which affects membrane association of proteins, again reflecting the importance of the modification in cellular activities such as intracellular signaling.

Perhaps the most convincing arguments for the importance of mevalonate derived biosynthetic products comes from research using inhibitors of HMG-CoA reductase. Results from these studies show that depriving cells of mevalonate by

this method demonstrated that a post-mevalonate biosynthetic product is responsible for promoting cell division (145), enhancing thrombosis (146), controlling calcium release (147) and activation of cell signaling pathways (148-149).

We have observed small, radioactively labeled peptides in this research using mevalonate-2-^{14}C as a precursor. A fraction was obtained by HPLC that could be further separated into peptides by thin layer chromatography. One band from this separation was radioactively labeled and shown by MALDI mass spectrometry to contain five separate components. One of these components, with a MW of 1038.1 D was further characterized by LC/MS and tandem MS. A fragmentation pattern was obtained, which along with the amino acid compositional data provided information to deduce a sequence for this peptide: his-gly-val-cys (farnesyl)-phe-ala-ser-met [HGVC(farnesyl)FASM]. This sequence by tandem mass spectrometry also allowed for the farnesyl moiety as a mevalonate derived modification on a cysteine residue. This is the first report of the isolation of a small, prenylated peptide from arterial tissue and demonstrates the ability of this system to synthesize such product(s).

A search of protein sequence databases (SWISSPORT, BLAST, and EMBL-Heidelberg) showed some similar sequences in the human genomic library. The closest match was found in chromosome 9 HRPK-295 D-22, but there was no apparent atherogenic characteristic at this location.

Summary Statement

This research addressed three questions to investigate aspects of arterial wall metabolism. All three investigations used mevalonate-2-^{14}C as precursor to study the questions posed. In each investigation mevalonate was the precursor to (1) neutral and acid lipids, (2) intermediates of the *trans*-methylglutaconic shunt, and (3) radioactivity incorporated into peptides. This acid is a precursor to a variety of biomolecules, which participate in both the participation and control of cell functioning. A review of the literature related to mevalonic acid metabolism of shows that mevalonate-derived biomolecules participate in processes linked with atherogenesis. Important to this research were the design and combination of protocols to answer the questions. Each of these questions related vascular metabolism, using mevalonic acid as the substrate, to processes which may be atherogenic. The summary answers to the questions are listed below.

(1) The neutral lipid extract contained not only diacylglyerols [described previously in 78)] but volatile components. It is suggested these volatile components may be the short chain alcohols, dimethylallyl alcohol and/or isopentenol. The observation that a significant part of the substrate (approximately 12 percent of the correct isomer) is converted to neutral lipids substantiates the conclusion that mevalonate is incorporated into biomolecules other than cholesterol. These biomolecules in

diseased tissue may alter intracellular signaling, cell proliferation, thrombosis, and lesion formation.

(2) The intermediates of the *trans*-methylglutaconate shunt were identified and shown to be radioactive. This is the first report (in any tissue), to our knowledge, of this observation. The activity of this pathway indicates that alternate pathways of mevalonate metabolism are significantly more active than cholesterol biosynthesis when incorporation into the end products (diacylglyerols and cholesterol) are compared. In addition, the function of diacylglycerols include transport of cholesterol, thromobogenesis, and vasodilation. In diseased states, these processes may exacerbate lesion formation.

(3) Isolation of mevalonate –labeled peptides demonstrated the ability of this tissue to modify peptides with acyl or prenyl groups. This research also demonstrated for the first time (to our knowledge) the isolation of small (<2000D) peptides with at least one prenyl modification. In normal or diseased states, prenylated proteins are part of the intracellular signaling process, release of cytokines, expression of an extracellular matrix, and the release of calcium.

(4) Other research has shown that a mevalonate derived product is required for cell division to take place; atherogenic plaque

growth is a combination of these processes, but at a diseased state level.

This research provided methods to obtain results in providing information about the neutral and acid lipid extracts; the isolation and identification of the *trans*-methylglutaconic shunt intermediates; and the isolation and characterization of radioactive peptide, all from a preparation of bovine aorta. The results presented here add to the understanding of arterial wall metabolism in normal tissue and to understanding the etiology of atherosclerosis, in the context of the metabolism of mevalonic acid. In the long run, the answers to the disease question may be as diverse as the development and the manifestations of the disease itself.

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