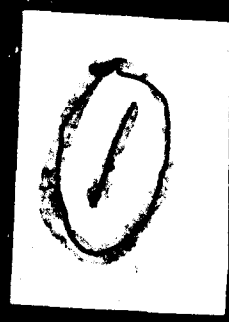
The Smooth Muscle of the Artery

Texas Univ Medical Branch At Galveston Marine Biomedical Inst

1975



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THE SMOOTH MUSCLE OF THE ARTERY

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THE SMOOTH MUSCLE OF THE ARTERY

Edited by

Stewart Wolf

The Marine Biomedical Institute
The University of Texas Medical Branch at Galveston
Galveston, Texas

and

Nicholas T. Werthessen

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Participants

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Preface

This volume contains the edited proceedings of a workshop conference, THE SMOOTH MUSCLE OF THE ARTERIAL WALL, held at Max Planck Haus, Heidelberg, West Germany, on the occasion of the dedication of the new Myocardial Infarct institute of the University of Heidelberg.

The conference was a sequel to one held three years earlier at Lindau, West Germany, on THE ARTERY AND THE PROCESS OF ARTERIOSCLEROSIS. The earlier conference examined available data and conflicting theories on the pathogenesis of arteriosclerosis. A major yield of this gathering was a consensus that smooth muscle cell proliferation in the sub-endothelial space is a fundamental feature of atherogenesis. It was, therefore, decided in a second conference to bring together existing knowledge of arterial smooth muscle and of the structure and function of smooth muscle in general. Accordingly an international and interdisciplinary group of experts were gathered together to assess current knowledge, to attempt reconciliation of disparate data and synthesis of widely scattered information and to identify critical areas of ignorance. As before, the conference was conducted in the form of a dialogue with only a minimal number of formal presentations.

Grateful acknowledgment for financial support of the conference is extended to:

The Government of Baden-Wurttemberg, West Germany

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Special thanks are extended to the University of Heidelberg, to Professor Gotthard Schettler, and to the Max Planck Institute for their generous and gracious hospitality.

The audiovisual responsibilities at the Conference were ably handled by Mr. Harold Druse el and Mr. Robert Robbins of the Department of Medical Illustrations, University of Texas Medical Branch. The illustrations were prepared and some charts redrawn by Edmond S Alexander and Carol Hoecker of the same department. Mrs. Joan Martin undertook the entire process of transcription, typing of edits, redaction and organization of the manuscript with the assistance of Mrs. Diane Townsend, Miss Colleen Hogan, Mrs. Lucille Smith and Ms. Moira Martin.

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WELCOMING REMARKS

Dr. Gotthard Schettler

I am most pleased to have the opportunity to welcome my colleagues and friends, the participants in this Conference. After the successful Lindau meeting three years ago we decided to continue with this multidisciplinary type of Conference. Since we had to organize the Third International Symposium on Atherosclerosis in Berlin it was impossible, and probably not justified at this time, to organize a meeting as long or as large as the former Lindau meeting. We therefore decided to continue in Heidelberg with a "Little Lindau Meeting", a meeting which may be understood as a kind of satellite meeting of our Berlin Conference because our topic will be closely related to atherosclerosis research. However, it will concentrate on a particular topic. This workshop meeting with its intimate atmosphere will provide excellent opportunities for intensive personal contacts and uninhibited discussions. We feel that the flair of the oldest university in our country and Heidelberg itself will be an appropriate and adequate alternative for the ambience of Lake Constance. The slogan of our alma mater is "Semper apertus". Heidelberg has always been very open-minded. The political atmosphere is always exciting, sometimes hot.

As in many other places we have had student violence in Heidelberg. However, as in most other places the situation has stabilized again and the academic youth of our university has returned to the principles of academic engagement and achievement.

Our State Government has made strong efforts to further develop our university. Not far from this building you can see the new University campus of the Heidelberg University. Heidel-

berg has become a center in many fields; especially in physics, chemistry, biochemistry, astronomy and cybernetics. The philosophical sciences, sociology, and the faculty of law have been internationally recognized for a long time and have produced a good number of highly respected authorities.

The faculty of medicine is trying to keep up with international standards. The German Cancer Research Center as well as the Max-Planck Institutes of Heidelberg are well equipped to perform basic research and these institutes keep strong international contacts. This is also true for our medical institutes which are engaged in close cooperation with many foreign universities. Our small Myocardial Infarct Institute which opens officially today also provides the basis for strong cooperation in the field of coronary heart disease and we hope that this Institute will be an adequate place for scientists and students from abroad to perform research on a high level. Furthermore, we hope that this type of cooperation will provide the basis for initiating common research projects.

Heidelberg is one of the very few places in Germany which was not destroyed during the second world war. It is an example of a city living on old traditions. The old castle and the center of the "Altstadt" have been a great attraction for many tourists from all countries. Therefore, Heidelberg seems to be a "must" for most European travel programs. This may also help us to attract more and more friends from science and research to visit and work with us in Heidelberg.

Every fifth inhabitant of this city is from a foreign country and every sixth is from the United States. So I am certain most of you will have no problem getting along in your mother tongue while walking through the old part of the city. The barkeeper and "Schankwirte" especially will answer you in their particular Pfalzisch-English: in the same manner as they used to answer in Pfalzisch-French several hundred years ago when most of their guests came from France.

You, my dear friends, are not asked to express your excitement in the same way as the soldiers of Melac, who particularly enjoyed small fireworks in the castle and thereby created one of the most famous ruins. You are, however, welcome to relax in the over-whelming atmosphere of one of the charming wine-Stuben or student pubs. This advice, however, holds only if the scientific discussions are not going to be too exhausting. The "Roter Ochse" is a good example of one of these places. I tremendously regret that I am not able to demonstrate sabre fencing for you today, since dueling was such an important part of student life in Heidelberg during the last hundred years.

Student disagreements presently are usually expressed in the form of throwing eggs and all kinds of paint spraying, go-ins, and sit-ins. Heidelberg has also been a particularly good example of this type of activity. A more physiological tranquilizer for this area seems to be our wine. I hope, dear friends, you will find enough time and the proper spirit to walk around the Heidelberg area in order to enjoy the hospitality of this city and don't forget to also visit one of the most famous wine areas of our country, the nearby Pfalz. I am sure this is the place to perform very special studies. My colleagues and I are more than happy to help you in this respect in a most professional way. So please, don't forget the slogan of our University, "Semper apertus", which holds good not only for science but also for a good glass of wine.

REPLY

Dr. Stewart Wolf

I would like to thank Professor Schettler for his gracious welcome and express our gratitude to our hosts here in Heidelberg for what looks like not only an intellectually profitable but an enjoyable experience.

There were three people who were unable to come at the last minute but who are listed in your programs: Dr. Benditt from Seattle, Dr. Cookson from Canada and Dr. Butcher from Massachusetts. There may be others. This conference as Professor Schettler has mentioned, grew out of the Lindau Conference that we had three years ago in the beautiful area of Bodensee. That conference was devoted to a general look at the pathogenesis of arteriosclerosis. The effort was to try to reconcile disparate theories and to see where lay the most promising leads for future work.

From that most successful and enjoyable meeting there emerged the conviction that one of the most important elements in the pathogenesis of arteriosclerosis was the nature and behavior of vascular smooth muscle cells. Therefore in planning for this conference we sought participants who had focused their interest on the smooth muscle, not limiting the group to those individuals who had been particularly concerned with arterial smooth muscle as related to the genesis of arteriosclerosis. The objective then of this conference is not to discuss the pathogenesis of arteriosclerosis but to engage in an interdisciplinary discourse on vascular smooth muscle, attempting a synthesis of our knowledge of this very important structure from the point of view of anatomy, function, metabolism and so forth. As we did at Lindau, we hope through interdisciplinary discourse to reconcile disparate data, interpretations and theories.

Chapter 1

STRUCTURAL CHARACTERISTICS, MECHANISMS OF CONTRACTION, INNERVATION AND PROLIFERATION OF SMOOTH MUSCLE CELLS

ULTRASTRUCTURE AND FUNCTION OF VASCULAR SMOOTH MUSCLE

Opening Address by Dr. Andrew P. Somlyo

The major functions of vascular smooth muscle are contraction, thereby mediating vaso constriction, and the synthesis of the extracellular proteins and polysaccharides of the vascular wall. Through this latter function they contribute to the architecture or morphogenesis of the artery. I shall deal in this presentation with contractility. The two main aspects of contractility that have to be related to structure are: (1) Excitation-contraction coupling, the process of triggering contractions and (2) the mechanism of contraction itself.

Calcium and contraction. The role of sarcoplasmic reticulum and mitochondria.

In a striated muscle twitch the excitation-contraction coupling process consists first of the release of calcium by the action potential. Calcium emerges from an intra-cellular storage site, the terminal cisterna of the sarcoplasmic reticulum (157). The rise in intracellular free calcium concentration triggers contraction, which is accomplished by the sliding movement of the thin (actin) filaments along the thick (myosin) filaments (401), accompanied by the breakdown of ATP by the actin activated myosin ATPase (73).

One of the questions facing us, until a few years ago was whether one could demonstrate in vascular smooth muscle an intracellular calcium storage site that could function in a manner analogous to the sarcoplasmic reticulum in striated muscles. To answer that question, it was necessary to show more than the presence of an occasional intracellular tubule in electron micrographs of single section. Such infrequent tubules could represent merely extracellular invaginations rather than the sarcoplasmic reticulum (158). To establish the existence of a functional sarcoplasmic reticulum, it was necessary to demonstrate an intracellular organelle system that was not in direct communication with the extracellular space, yet made sufficiently close contact with the surface membrane to permit its regulation by membrane electrical activity. It was also necessary to show that such

tubules could accumulate the divalent cations that could activate contraction. Each of these criteria have been met in reptilian and in mammalian smooth muscle (80, 81, 358, 370), and it has been proposed that the sarcoplasmic reticulum of vascular smooth muscle contributes to the activation process in a manner similar to that in striated muscle. This suggestion, however, does not imply that the sarcoplasmic reticulum of vascular smooth muscle contributes to the activation process in a manner similar to that in striated muscle. Neither does it imply that the sarcoplasmic reticulum is the sole source of activator calcium in smooth muscle.

The sarcoplasmic reticulum in vascular smooth muscle consists of a communicating system of small tubules (Fig.1) that often form fenestrations about the surface vesicles and, at other points, come to within 10-12 nm of the plasma membrane. The regions of close proximity between the junctional sarcoplasmic reticulum and the plasma membrane are called couplings (Fig. 2), in analogy with the similar structures in cardiac muscle (96, 373). They are traversed by small electron dense structures with a period of approximately 20-25 nm (80, 81, 358). It is thought that the twitch contractions of vascular smooth muscles, triggered by action potentials (371), are mediated through the release of calcium from the sarcoplasmic reticulum at the couplings (370). The use of extracellular markers (e.g. ferritin, colloidal lanthanum, horseradish peroxidase) that enter the extracellular space including surface vesicles, but not the tubules discussed above, confirmed that the system we are dealing with is a true sarcoplasmic reticulum and not one of extracellular invaginations comparable to the T-tubules of striated muscles.

The volume of the sarcoplasmic reticulum is significantly different in different types of smooth muscle. It is only about
RABBIT 2% of the cytoplasmic volume in the rabbit portal-anterior mesenteric vein or taenia coli, while it amounts to 5 - 7.5% of the cytoplasmic volume in the main pulmonary artery and aorta of the same species (80). The functional properties of these different smooth muscles correlate rather well with the proportion of the sarcoplasmic reticulum, as will be discussed by Avril Somlyo (see page 44). One might recall that the terminal cisterna of
FROG the frog sartorious muscle, thought to contain all the calcium necessary for activation of the striated muscle, comprise approximately 5% of the cytoplasmic volume (253).

The uptake of divalent cations by the sarcoplasmic reticulum of intact vascular smooth muscle is most readily demonstrated by incubation of such preparations, prior to fixation, in a physiological salt solution containing strontium (370). Strontium has a higher atomic number, and for a given concentration is, therefore, more electron opaque than calcium, and it can substitute for the latter in a number of physiological processes. Strontium is accumulated by isolated sarcoplasmic reticulum

Figure 1: Sarcoplasmic reticulum in rabbit main pulmonary artery. Transverse section shows the greater amount of sarcoplasmic reticulum in this type of smooth muscle than in portal-anterior mesenteric vein. The more centrally located tubules of sarcoplasmic reticulum continue towards the periphery and make contact (arrowhead) with the surface membrane. (Devine, C.E., Somlyo, A.V. and Somlyo, A.P.: J. Cell Biology 52: 690-718, 1972.) Mag. 37,800 x.

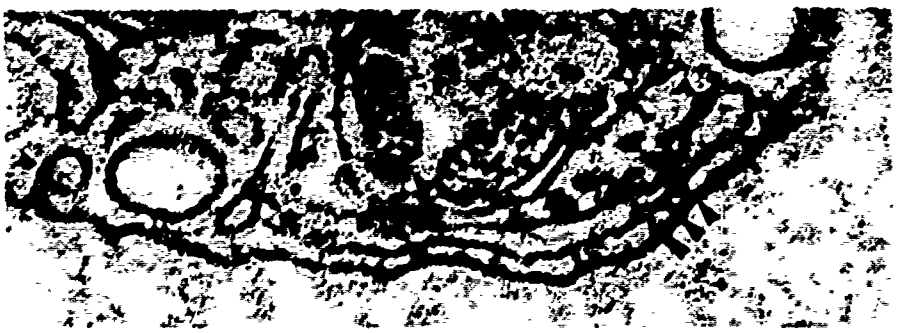

Figure 2: Sarcoplasmic reticulum-surface membrane coupling. This high magnification view of a transverse section of rabbit portal-anterior mesenteric vein shows the close relationship between the two membrane systems, and the periodic densities connecting the gap between the inner plasma membrane and outer lamina of the sarcoplasmic reticulum membrane (arrowheads). (Somlyo, A.P. and Somlyo, A.V., In press.) Mag. 153,000 x.

(from striated muscle) and by mitochondria (from liver) in a manner similar to calcium (125, 402), and can also activate the actomyosin ATPase of striated muscle (85). Incubation of portal-anterior mesenteric vein smooth muscle strips in a solution containing 10 mM strontium is accompanied by some increase in the spontaneous contractile activity (370). When such preparations are fixed, after incubation for 1 hour in this solution, and examined in the electron microscope, the lumen of the sarcoplasmic reticulum is found to contain electron opaque deposits (Fig.3), indicative of the uptake of strontium (370). Similar deposits of strontium can be found in smooth muscles that are not spontaneously active, if the permeability of the surface membrane of these muscles to strontium is increased by incubation in a high K, depolarizing solution. These findings complete the identification of these intracellular tubules as a functional sarcoplasmic reticulum in vascular and other smooth muscles.

The volume of the sarcoplasmic reticulum, as I indicated above, is rather limited in some types of vascular smooth muscle. Because of this, and because of observations on vascular ultrastructure that I shall describe now, we have also considered the possibility that under some conditions, mitochondria may also contribute to the regulation of free intracellular calcium levels (359, 360, 366). This type of ionic regulation by mitochondria has been previously considered, although not proven, in cardiac muscle (63, 151, 198, 327, 422).

The electron microscope observations that mitochondria frequently make close contacts (average 4-5 nm between the two membrane systems) with the surface vesicles lead to the speculative suggestion that the mitochondrial-surface vesicle contacts may be sites where ions are transferred from the cell into the extracellular space (361). Such an ion shuttle presupposes that mitochondria in smooth muscle can accumulate the cations that subsequently are to be transferred into the extracellular space. Therefore, we first proceeded to determine the existence of mitochondrial cation uptake. Incubation of vascular smooth muscle in barium-containing solutions leads to a contractile response and to the appearance of strongly electron opaque intramitochondrial granules that are not found in control preparations (Fig.4). Definitive identification of these granules as deposits of barium was accomplished through use of electron probe x-ray microanalysis (362).

The principles of electron probe x-ray microanalysis and details of these experiments have been described elsewhere (131, 362). The electron beam is focused on an area of interest within a tissue section, and the x-ray photons emitted from the area irradiated by the electrons are recorded. The energies (or wavelengths) of the x-ray photons emitted are characteristic of the

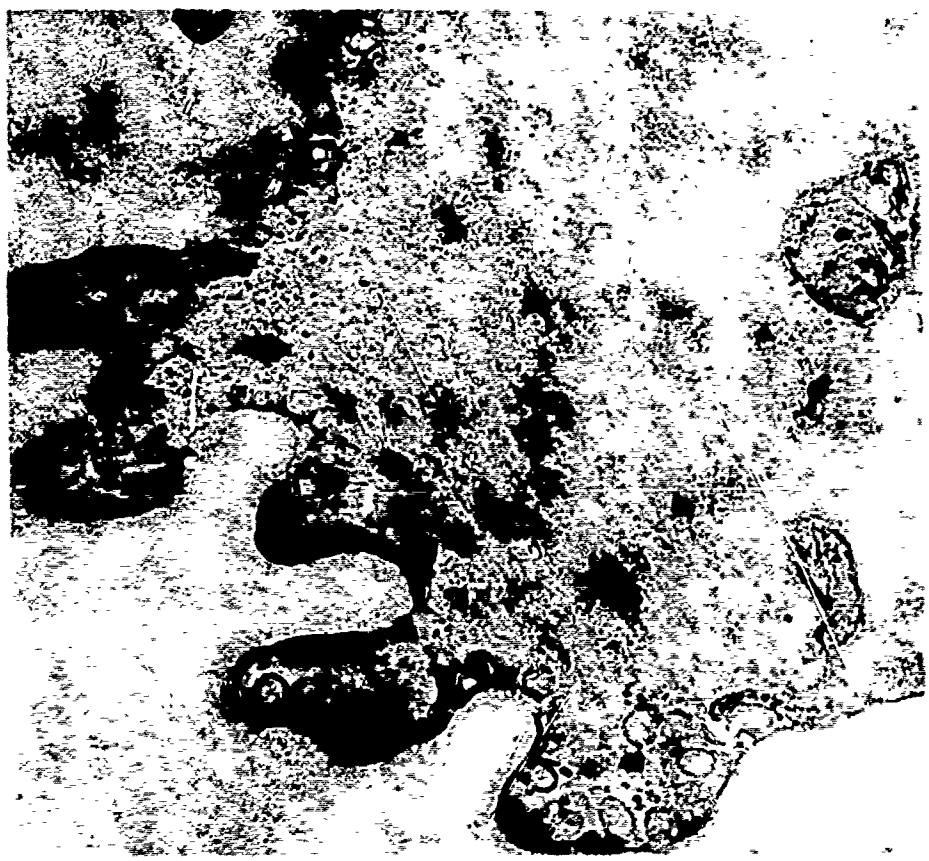

Figure 3: Strontium deposits in the sarcoplasmic reticulum of vascular smooth muscle. Longitudinal-oblique section of guinea-pig portal-anterior mesenteric vein incubated in Krebs' solution containing 10 mM Sr for one hour before fixation with osmium. Electron opaque deposits of strontium are present in the lumen of the sarcoplasmic reticulum of this unstained section. (Somlyo, A.P. and Somlyo, A.V., Unpublished observations.) Mag. 54,000 x.

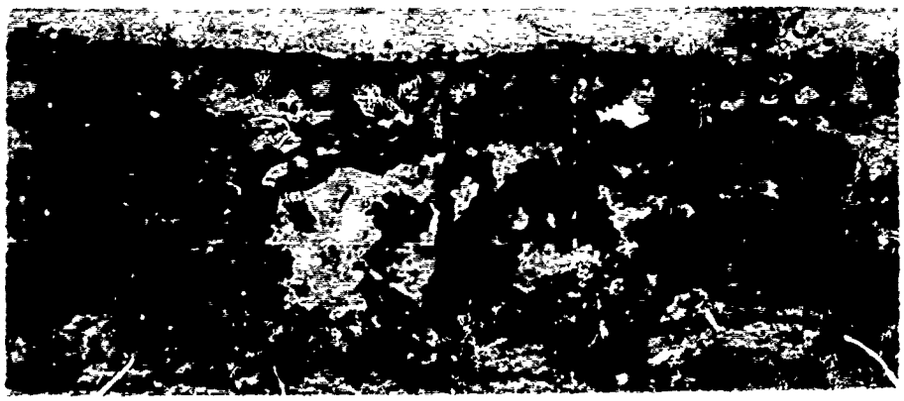

Figure 4: High magnification view of mitochondria containing electron opaque granules, close to the surface vesicles. The granules appear to lie in the mitochondrial matrix space. There is an element of the sarcoplasmic reticulum (arrow) lying between one of the mitochondria and the surface vesicles. Rabbit PAMV incubated for 60 min. in 10 mM Ba^{++}-Krebs' solution. Fixed in 2% osmium tetroxide. Stained en bloc with uranyl acetate. Mag. 94,500 x.

elements within the micro-volume excited by the electron beam. For practical purposes, 10^{-18} grams or more of the heavier elements ($Z > 11$) can be fairly readily identified in tissue sections. Dark mitochondrial granules in smooth muscles that have been incubated, prior to fixation, in barium containing solutions clearly showed the energy peaks characteristic of barium (Fig.5). This x-ray peak was absent over the cytoplasm and over mitochondria that did not contain electron dense granules. In more recent experiments, we have achieved a spatial x-ray resolution of approximately 500A°, and demonstrated the presence of barium in individual mitochondrial granules. Furthermore, through x-ray analysis of sections fixed with glutaraldehyde instead of osmium (to eliminate interference between the osmium M line and phosphorus K line), it has been possible to show that the uptake of phosphorus (presumably in the form of phosphates), is in a Ba/P ratio of approximately 3/4. Occasionally, small x-ray peaks at 3.69 keV, indicative of calcium, were also recorded over such barium containing mitochondria (362).

Strontium has also been identified in the sarcoplasmic reticulum and in mitochondria through electron probe x-ray microanalysis. In vascular smooth muscle incubated in strontium-containing solutions, the electron opaque deposits found in the lumen of the sarcoplasmic reticulum and the granules in mitochondria show the x-ray spectrum characteristic of Sr. In addition to the Sr peak, however, we have frequently also detected a very large calcium peak over mitochondria containing electron opaque granules in vascular smooth muscle loaded with strontium and fixed with osmium vapor (Fig.6).

The observations on the mitochondrial accumulation of Ba, Sr and Ca clearly show that the mitochondria can accumulate divalent cations. These observations, however, are not sufficient evidence for a physiological role of mitochondria in regulating cytoplasmic calcium levels during the contractile cycle, since translocation of calcium may have occurred during chemical fixation. More definitive evidence for or against such a function of the mitochondria in the contractile regulation of smooth muscle will require electron probe x-ray microanalysis of chemically unfixed (frozen sectioned) tissues, currently in progress in our laboratory

Contractile Proteins - Sliding Filament Model

The next question that I shall turn to is the nature of structural information required for establishing the mechanism of contraction in smooth muscle. The presence of the necessary proteins and the length dependence of the contraction of vascular smooth muscle were compatible with the sliding filament model of contraction (363), established for striated muscle, where

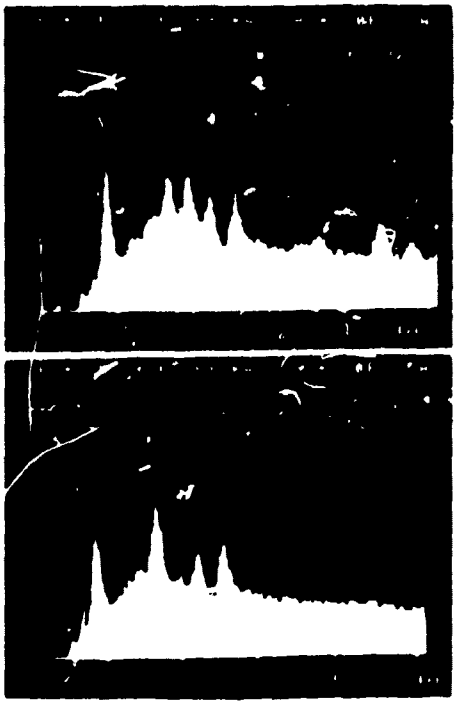

Figure 5

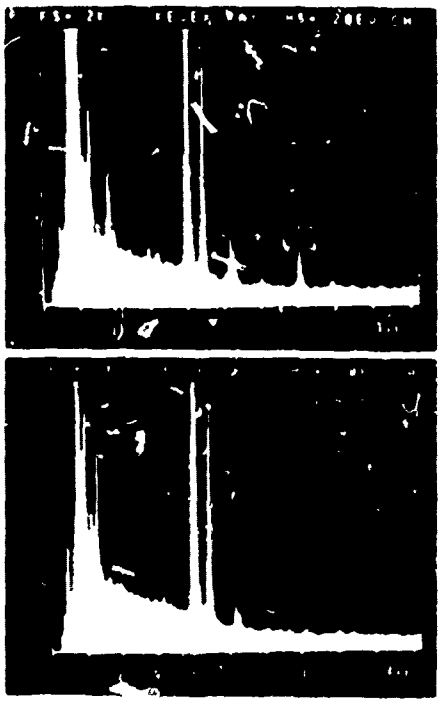

Figure 6

Figure 5: X-ray spectrum of mitochondria containing electron opaque granules in rabbit portal-anterior mesenteric vein incubated in 10 mM Ba-Krebs' solution prior to fixation with glutaraldehyde alone. Upper panel: prominent Ba L_α and L_β and a small, but significant Ca peak are present. Lower panel shows that the characteristic Ba and Ca peaks are absent when the probe is moved off the mitochondrion to the cytoplasm of the same fiber. (From Somlyo, A.P., Somlyo, A.V., Devine, C.E., Peters, P.D. and Hall, T.A., In press.)

Figure 6: X-ray spectrum of rabbit portal-anterior mesenteric vein smooth muscle incubated in strontium-containing solution prior to fixation with osmium vapor. Upper panel: the spectrum over mitochondrial electron opaque granules showing Sr and Ca peaks. Lower panel: the spectrum over the cytoplasm showing the absence of both Ca and Sr peaks. (Somlyo, A.P., Somlyo, A.V., Devine, C.E., Peters, P.D. and Hall, T.A., In press.)

contraction is accomplished through the sliding movement of thin (actin) and thick (myosin) filaments relative to each other. The thin filaments have also been demonstrated in smooth muscle. However, the specific organization of myosin, whether it formed thick filaments that were present in both the relaxed and in the contracted mammalian smooth muscle, has been the subject of considerable controversy (363, 364).

Description of Filaments

Recently improved preparatory techniques and the recognition that swelling of smooth muscle during dissection can easily lead to the disorganization of filament structure during fixation (164) have clearly established the presence of thick filaments in vascular and other mammalian smooth muscles under all conditions of contraction, relaxation and stretch (82, 271, 361, 364, 365, 366). Furthermore, the thick filaments form a relatively regular 600 x 800 A° 1 lattice in rabbit portal anterior mesenteric vein preparations and, in the best organized sections, are in the center of rosettes surrounded by thin (50-80 A°) actin filaments (Fig.7). Independent evidence for the existence of organized myosin in mammalian smooth muscle (taenia coli of guinea pig) is provided by the 143-144 A° meridional x-ray spot thought to be generated by the cross bridge (205). Most of our electron microscopic studies, for technical reasons, have demonstrated filament organization in longitudinal muscles of portal vein, taenia coli or vas deferens (367). Thick filaments can also be demonstrated in the large elastic arteries of greater interest to pathologists, such as the main pulmonary artery (80), although the preservation of this material for electron microscopy is technically more difficult. In such smooth muscle fibers of large elastic arteries thick and thin filaments can be found together with elements of rough sarcoplasmic reticulum, suggesting the concomitant contractile and morphogenetic function of these tissues.

RABBIT

GUINEA PIG

There is a third type of filament encountered in vascular smooth muscle that is both structurally and biochemically different from the myofilaments described so far. These intermediate filaments are 100 A° in diameter and in transverse section they often surround the dense bodies. In longitudinal sections, intermediate filaments may be found in bundles over 7μ long within the plane of a given section (364, 365, 368). In some smooth muscle fibers, presumably abnormal or developing, large conglomerations of these intermediate filaments occupy much of the smooth muscle cell (Fig.8), that may contain only a few thin and thick myofilaments (364). The intermediate filaments are highly resistant to damage during fixation or extraction (69, 164, 365). They are composed of proteins other than actin or myosin (272), and are very similar, if not identical, to filaments found in a variety of other tissues

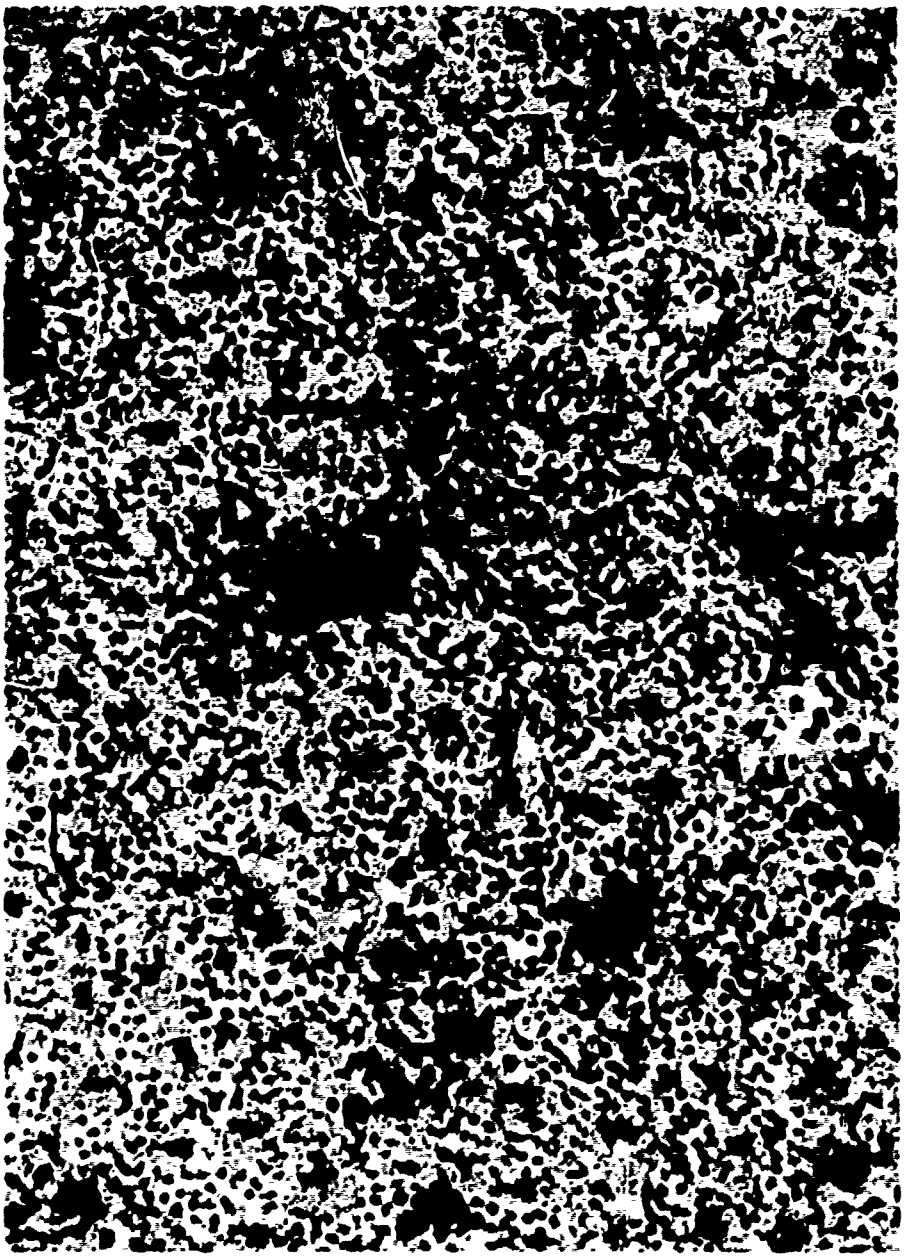

Figure 7: Filament organization in vascular smooth muscle. High magnification transverse section of rabbit portal-anterior mesenteric vein, showing thick (large arrows), thin (small arrows), and intermediate (large arrowheads) filaments. Note the regular spacing of the thick filaments. (Somlyo, A.P., Somlyo, A.V., Devine, C.E. and Rice, R.V.: Nature New Biology 231: 243-246, 1971; Somlyo, A.P., Devine, C.E., Somlyo, A.V. and Rice, R.V.: Phil. Trans. Roy Soc. Lond. B. 265: 223-229, 1973.) Mag. 184,000 x.

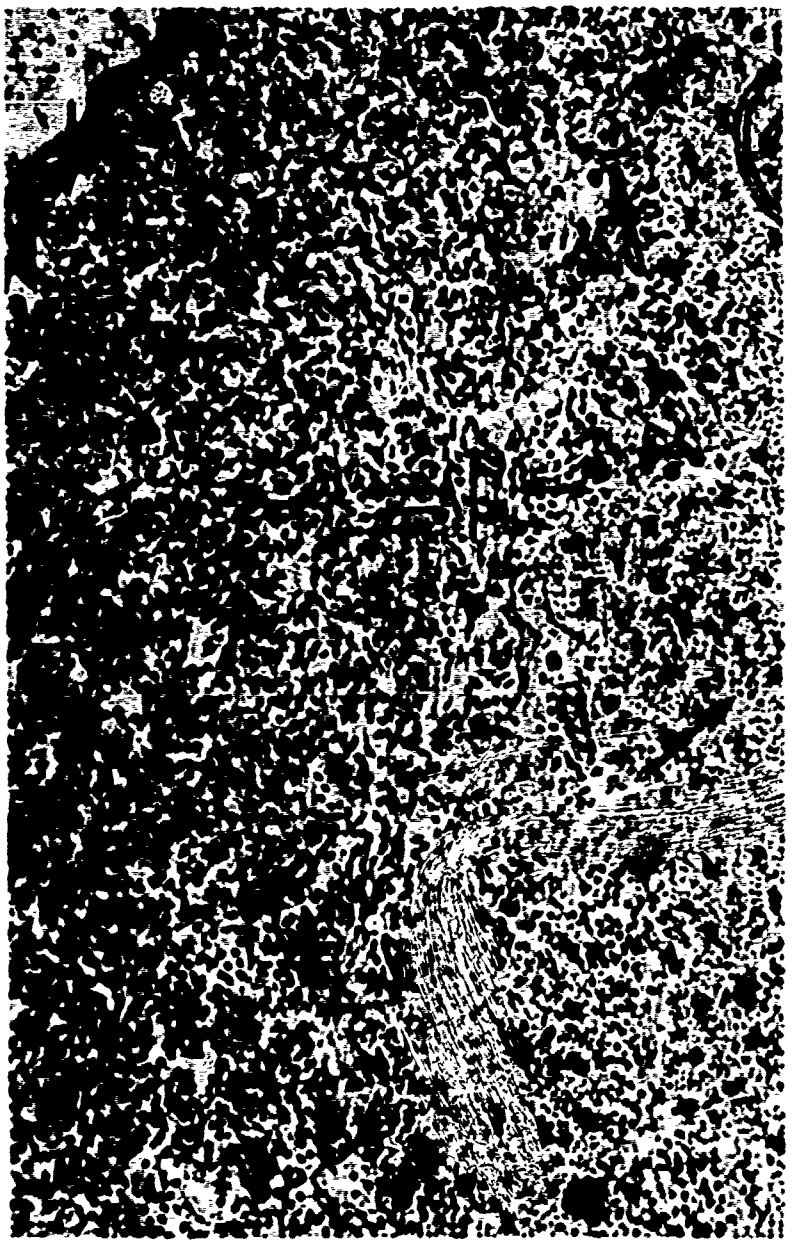

Figure 8: Near transverse section of a smooth muscle fibre containing an abnormally large number of intermediate filaments (small arrows) that occupy much of the central portion of the electron micrograph and are seen in both transverse section and obliquely passing through the plane of section. Comparison with the regions occupied by the thick and thin myofilaments (large arrows) shows the clear difference between these three types of filaments. (Somlyo, A.P., Devine, C.E., Somlyo, A.V. and Rice, R.V., Phil. Trans. Roy. Soc. Lond. B, 265: 223-229, 197. .) Mag. 83,600 x.

(159) and in cultured smooth muscles. It is probable that earlier reports have frequently confused the intermediate with the thick filaments.

The dimensions of the filaments found in portal-anterior mesenteric vein smooth muscle and the ratio of actin/myosin filaments and their separation are shown in Table I. The thin to thick filament ratio is high (15/1), but compatible with biochemical estimates. The center to center, thin to thick filament separation is similar to that found in mammalian striated muscles and compatible with a cross bridge mediated interaction.

The dense areas (dense bodies) on the plasma membrane of smooth muscles are probably analogous to the Z lines of striated muscles, and serve as attachment sites for the actin filaments (364). Other dense bodies, free floating in the cytoplasm, may have a similar function, although it is by no means certain that they represent a homogeneous biochemical or functional entity (368). Identification of these structures by more specific techniques than staining with heavy metals will be required to answer this question.

In conclusion, ultrastructural studies show the presence of a functional sarcoplasmic reticulum in vascular and other smooth muscles. It is suggested that this sarcoplasmic reticulum plays a role, similar to that in striated muscles, in excitation-contraction coupling. Influx from the extracellular compartment is an additional, probably variable source of activator calcium in smooth muscle. Mitochondria also accumulate divalent cations, under some experimental conditions, but the physiological significance of this observation remains to be established. Myosin is organized into thick filaments bearing cross bridges in vascular smooth muscle. The organization of actin and myosin filaments strongly suggests the existence of a sliding filament mechanism contraction in vascular and other mammalian smooth muscles.

TABLE I

FILAMENT DIMENSIONS

Diameter

	$\bar{X} \pm$ S.D.
Thin	64 ± 8 Å (70)
Intermediate	97 ± 10 Å (65)
Thick	155 ± 20 Å (70)

Thin : Thick = 15 : 1

Center thin to center thick distance = 256 ± 59 Å (70)

The number of measurements are given in parenthesis

(Somlyo, A.P., Devine, C.E., Somlyo, A.V. and Rice, R.V.: Phil. Trans. Roy. Soc. Lond. B. 265: 223-229, 1973.)

DR. BECKER: I have a question I would like to address to Dr. Andrew Somlyo and perhaps it comes from my misunderstanding. I thought I heard in your talk that in order to demonstrate the thick filaments the fiber has to be a contracted fiber. Is that, or is that not true?

DR. A.P.SOMLYO: No it is not, but historically it was thought at one time that thick filaments form in smooth muscle just prior to the onset of contraction or, alternatively, that they are only present in smooth muscles that have been stretched. This is no longer considered to be true, and thick filaments have been clearly demonstrated in stretched and unstretched and in contracted and in relaxed smooth muscles.

DR. CHALDAKOV: I should like to present the results of a study with Sp. Nicolov on the fine structure of smooth muscle cells (SMC) in the aorta and pulmonary trunk of growing rabbits.

RABBIT

Newborn, 12-day and 2-month old rabbits were used. Pieces from the thoracic aorta, aortic arch and pulmonary trunk were fixed in glutaraldehyde post-fixed in osmium tetroxide, dehydrated in cold graded alcohols, embedded in Durcupan ACM (Fluka) in flattened capsules. Ultrathin sections, cut with a Reichert OU 2 ultramicrotome were stained with uranyl acetate and lead citrate and viewed through a JEM 7A electron microscope. Other fixations were also used (Luft's ruthenium red, Richardson's KMn04, and block staining in uranyl acetate before dehydration).

Characteristics of Modified Smooth Muscle Cells

Two main types of SMC were found during postnatal development (a) secretory-contractile, and (b) contractile. The secretory-contractile type showed the features of so-called modified (myo-intimal) SMC, namely prominent rough endoplasmatic reticulum and Golgi-complex surrounded by smooth-surfaced and small pinocytotic coated vesicles. Mitochondria, large coated vesicles, subsarcolemmal caveolae intra-cellularis, longitudinally oriented microtubules were numerous but myofilaments and dense bodies were reduced. In the newborn and 12-day old rabbit aorta and pulmonary trunk modified SMC were evident in the media (Fig.9,10), but no subintimal SMC were seen. By two months, however, the subendothelial space was well developed and modified SMC were seen there and in gaps in the internal elastic lamina (Fig.11).

A structural transformation of SMC from secretory-contractile towards contractile type takes place with growth of the rabbit aorta and pulmonary trunk. This is in accordance with Cliff's (66) findings for rat tunica media. The rough endoplasmatic

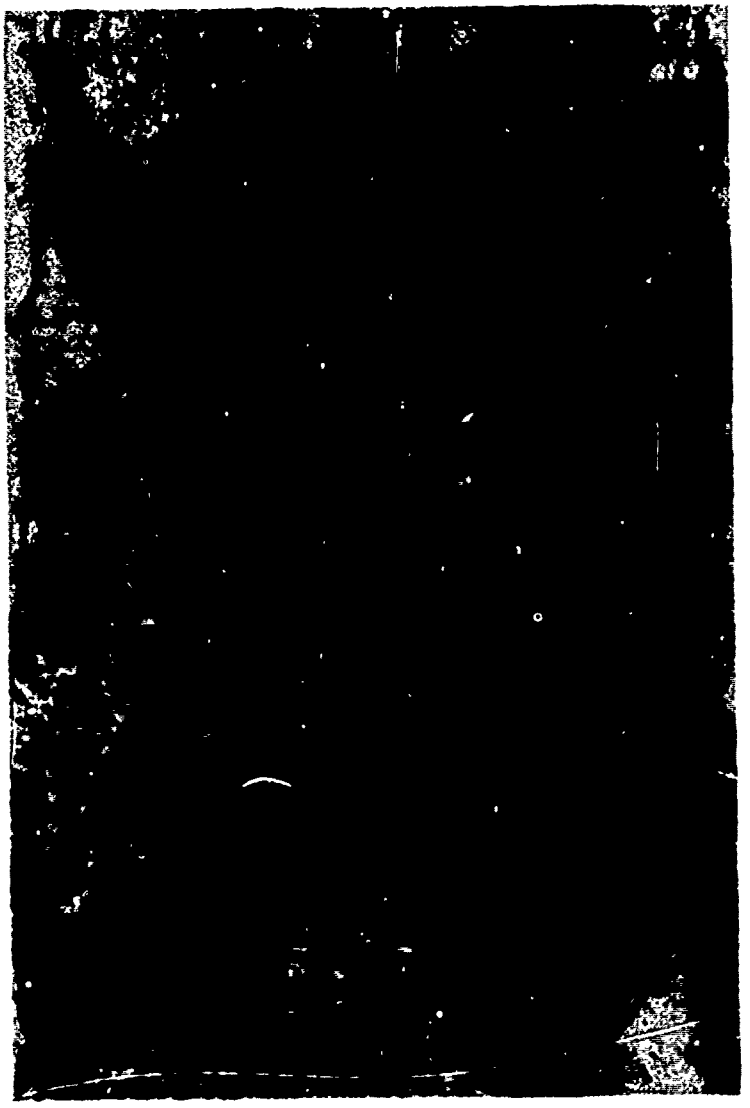

Figure 9: Medial smooth muscle cell in aortic wall of 12-day old rabbit. Rough endoplasmic reticulum and Golgi-complex with small coated vesicles around it are well developed. A large micropinocytotic coated vesicle can be seen. Myofilaments occupy a restricted area beneath sarcolemma. (x 45,000)

Figure 10: Medial smooth muscle cell in aortic wall of 12-day old rabbit. Rough endoplasmic reticulum, Golgi-complex with small coated vesicles as well as subsarcolemmal caveolae intracellularis are prominent. Microtubules, mitochondria, microbody (peroxisome) and basement membrane can be seen. (x 36,000)

Figure 11: Subendothelially disposed smooth muscle cell in pulmonary trunk wall of 2-month old rabbit. Rough endoplasmic reticulum, free ribosomes, Golgi-complex with smooth surfaced and small coated vesicles around it, and mitochondria are prominent. Myofilaments are decreased and localized at the periphery of the cell. Nucleus, microtubules and centriole can be seen. (x 45,000)

reticulum and Golgi-complex, micro-pinocytotic coated vesicles and microtubules become less prominent and give way to myofilaments, attachment devices and subsarcolemmal caveolae intra-cellularis (Fig.12).

It is our impression that modified SMC gain access to the subintimal space through age-induced fenestrations of the internal elastic lamina in agreement with the original description (53). We cannot exclude the possibility that the contractility of these cells may be responsible for their migration into the subendothelial space. Studies with colchicine and other microtubule-destroying agents and with cytochalasine B (microfilament-blocking substance) may shed some light on the question. It is our view that the modified SMC as a secretory-contractile arterial muscle cell may represent a case of "stimulus-secretion coupling" (321), and "excitation-contraction coupling" (359, 369). The proposed cooperation of such "double coupling" phenomena in one cellular type is based on the fine structural findings and the well known dependence of both secretion and contractility on calcium and cyclic 3', 5' - AMP (267). Recently Joo (165) and Masur et al., (212) reported that cyclic AMP increases the formation of micro-pinocytotic vesicles in endothelial cells of brain capillaries and in toad bladder, as well.

We propose the micro-pinocytotic coated vesicles as a new cytological sign of the modified SMC (62).

Two stages in the morphogenesis of large coated vesicles were found: (a) local substructural specialization of sarcolemma followed by, (b) sarcolemma vesiculation and internalization. This is in accordance with Roth and Porter's (320) and Fawcett's (97, 98) results for other cells.

It is well known that coated vesicles are cellular transport devices for selective uptake of proteins (10, 97, 98, 111, 166, 312, 320, 330). Therefore, the smooth muscle coated vesicles may be involved in the uptake and transport of some specific macromolecular substances essential for SMCs function. One may speculate that both basement membrane and sarcolemma may be genetically endowed with properties for special uptake and transport of some macromolecules as implicit in Bennett's theory for pinocytosis (26) and Singer and Nicolson's fluid mosaic model for membrane structure (343). Certainly, the transported proteins may serve as a source of amino acids (lysin-rich and/or proline rich proteins?) for biosynthesis of elastin and/or collagen. Conceivably the coated vesicles of SMC may be involved in Iverson's extraneuronal uptake of circulating catecholamines or they may function as "synthesomes" as suggested by Schjeide and San Lin's studies of oocytes (329).

Figure 12: Pulmonary trunk wall of 2-month old rabbit. Local coated specializations of sarcolemma at contact region of two smooth muscle cells in inner media. (× 45,000)

Certainly our findings are limited at present and our suggestions sound fairly speculative. But they are put forward "by the belief that a specific field of research serves the general cause of science better by asking it an occasional original question" (359).

DR. BURNSTOCK: My particular task is to discuss the innervation of vascular smooth muscle. I will describe the variation in innervation from large elastic arteries, through large muscular arteries, smaller muscular arteries, arterioles, precapillary sphincters to the venous system. There is, of course, a considerable variation in the pattern of innervation in these systems and we really don't know all these variations yet in different species and in different vessels, so I will start by giving you a generalized model of the innervation of smooth muscle (Fig.13) and then go on to describe the variation in different vessel types.

Innervation of Vascular Smooth Muscle

One essential feature of this model is that the effector is not a single smooth muscle cell but rather a bundle of smooth muscle cells in electrical continuity with each other. The second feature which differs from the skeletal neuromuscular system is that the autonomic nerves run very long distances, have varicosities which contain high levels of transmitter and release transmitter 'en passage'. Now in this very generalized model, some of the cells are directly innervated, i.e., they have close (500 A°) neuromuscular junction; these cells have been termed 'directly-innervated cells' and they are directly affected by transmitter released from the nerves. The adjoining cells we have called 'coupled cells' because they are coupled electrotonically to 'directly-innervated cells' by low resistance pathways (nexuses) or gap junctions. Junction potentials can be recorded in them. The junction potentials are very slow so a whole area of a muscle effector bundle becomes depolarized simultaneously and this leads to the initiation of an action potential which then propagates down through the system to activate a third group of muscle cells which we call 'indirectly-coupled' cells, because they are not directly affected by transmitter nor are they directly coupled to the cells which are affected by transmitter. Nevertheless, they are activated when you stimulate the nerves. We shall see that this is very important when considering the nervous control of vascular smooth muscle.

Now we should consider the more specific problem of the innervation of the vascular system, starting with a very large elastic artery. The adrenergic plexus in the aorta is confined to the adventitial medial border. However, there is considerable species variation in density of innervation of large arteries. For example, RAT in the rat aorta few adrenergic nerves can be seen (Fig.14A) but in

Figure 13: Schematic representation of autonomic innervation of smooth muscle. For explanation see text. (Courtesy of Burnstock & Iwayama, Progr. Brain Res. 34, 389, 1971).

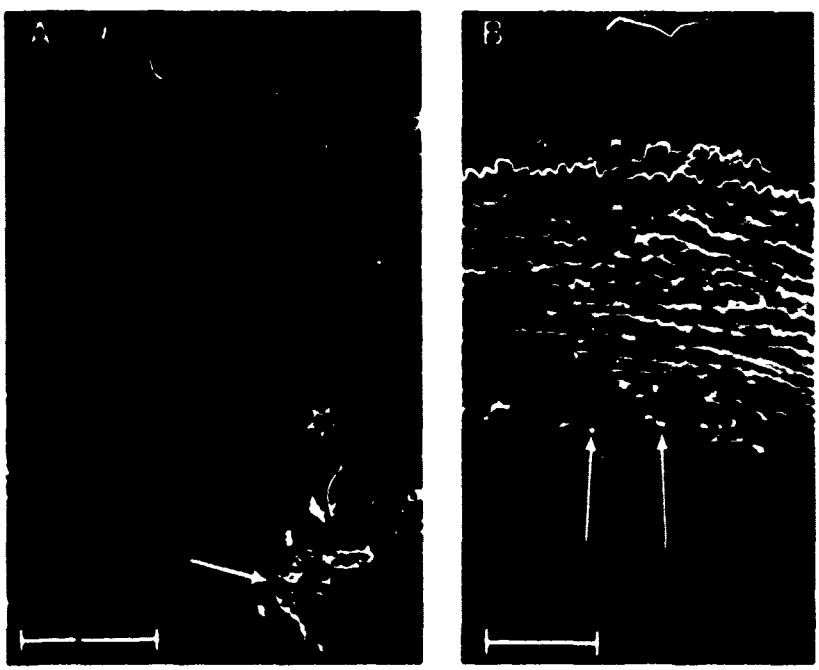

Figure 14: Fluorescence histochemical demonstration of adrenergic nerve fibres supplying arteries.
A. Transverse section through the rat aorta. Note that no fluorescent nerves are present in the adventitia, media or adventitial-medial border, but some nerves (arrows) are associated with small blood vessels in the surrounding tissue. horizontal bar - 100mμ. Note also the autofluorescence in the elastic lumninae of the media.
B. T.S. Rabbit thoracic aorta showing a plexus of adrenergic nerves (arrows) at the adventitial medial border (*) horizontal bar = 100mμ.

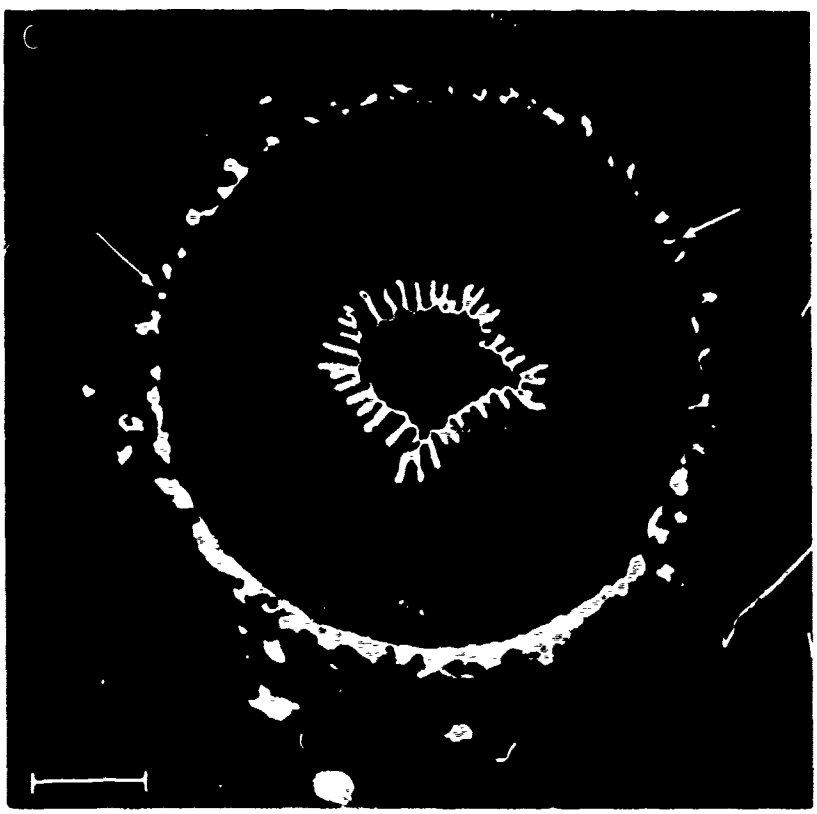

Figure 14 C: T.S. Rabbit ear artery. Note the dense nerve plexus (arrows) at the adventitial-medial border (*). horizontal bar = 50mμ. (A and B. Courtesy of Mary E. Wright, Department of Zoology, University of Melbourne, and C, Burnstock et al., Brit. J. Pharmacol. 46, 243, 1972).

RABBIT the rabbit aorta (Fig.14B) which is commonly used for pharmacological experiments, there is marked variation in adrenergic innervation at different times during development. Sections through the HUMAN human aorta show nexuses between the muscle cells. According to the model presented earlier we consider that only the outer layer of muscle cells next to the perivascular plexus are directly affected by the neuro-transmitter. The other muscle cells are electrically coupled to these and an action potential set up which propagates through to the intimal side of the media. This does not exclude, of course, the very strong evidence for the effect of circulating adrenaline on these muscle cells.

RABBIT If we consider next a muscular artery, such as rabbit ear artery, here again we see the auto fluorescence in the intima, the media completely free of nerves, the adventitia, and a very dense and well defined plexus at the adventitial-medial border (Fig.14C). In general these arteries are more heavily innervated than elastic arteries. Electron microscopy demonstrates that the closest apposition between the nerve and smooth muscle cells is about 800A° (see Fig.15A). Basement membrane is interposed and there may be some postsynaptic specialization with aggregations of plasmalemma vesicles (or caveolae intercellularies). This is in contrast to some visceral smooth muscles, where there is a much closer apposition of nerve and muscle (about 150A°). There is often an elaborate postsynaptic specialization at these junctions with subsynaptic cisterna and an elaborate structure between postsynaptic membranes and the membrane of the cisterna. Now, some muscular arteries, particularly in certain species such as sheep and also man, do not have the nerves confined to the adventitial-medial border. The nerve fibers penetrate at least one-third and nearly halfway down into the media (Fig.15B).

Another feature that we need to look at is the kinds of nerves that innervate arteries (Fig.16). Cholinergic nerves are illustrated here at the adventitial-medial border of a muscular artery and are within a 1000A° of the muscle. They are characterized by a predominance of small (400-600A°) agranular vesicles.

Differentiation of Cholinergic and Adrenergic Innervation

In contrast, adrenergic nerves are characterized by a predominance of small granular vesicles (440-600A°). If the tissue is 'loaded' with 6-hydroxydopamine, this is a marvelous marker for determining whether adrenergic or cholinergic nerves are present. For example, in the rat cerebral artery, some of the vesicles in the nerve profiles illustrated have very dense cores after being loaded with 6-hydroxydopamine; these are adrenergic nerves. Thus, dual innervation of these cerebral arteries by cholinergic and adrenergic nerves is established. Sensory nerve endings in smooth muscle have no vesicles, but they are packed with mitochondria. Traced back in

Figure 15: A. Relation of axons (A) and smooth muscle (M) at the adventitial-medial border of the anterior cerebral artery of the rat. Note the relatively wide junctional cleft of about 800 A. S, Schwann cell.

B. An axon (A) approaching within 1000 A of the surface of a smooth muscle cell (M), deep within the media of the sheep carotid artery. S, Schwann cell. (Courtesy of Burnstock et al., Circul. Res., 26-27, 5, 1970).

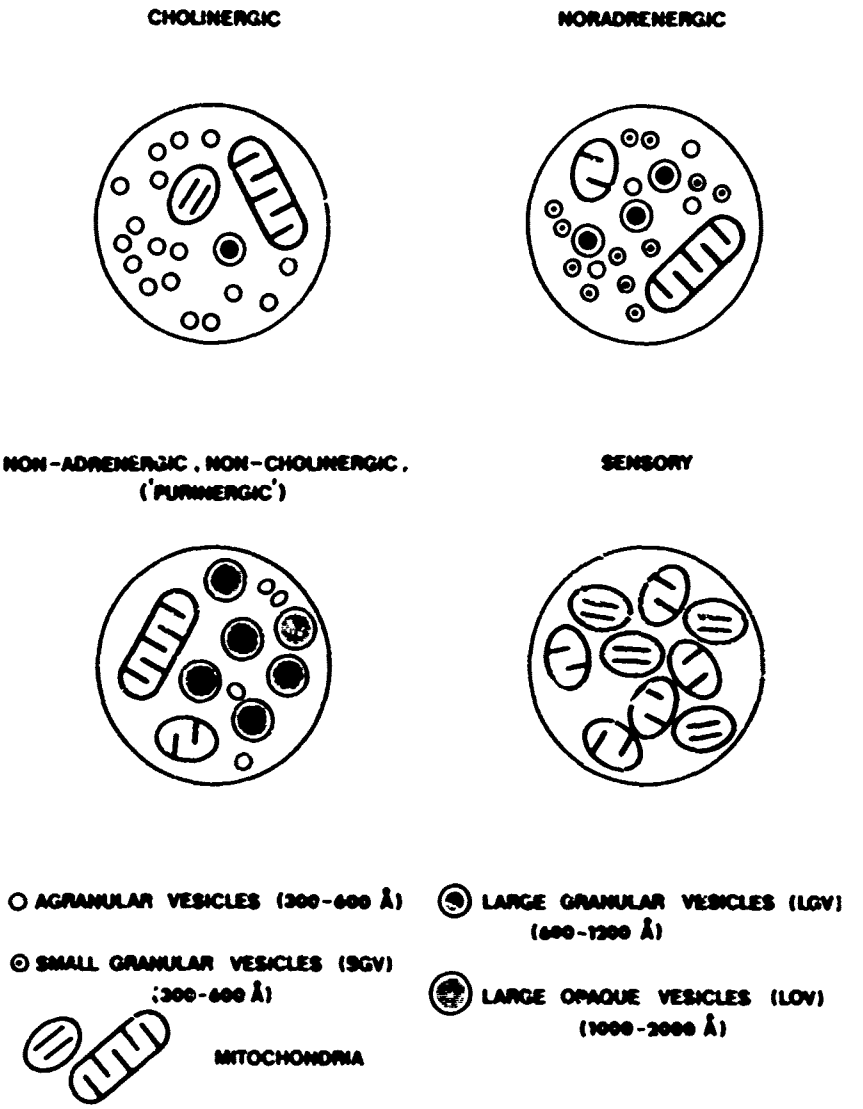

Figure 16: Diagrammatic representation of sections through the terminal varicosities of autonomic nerves. For explanation see text. (Courtesy of Burnstock & Iwayama, Progr. Brain Res. 34, 389, 1971).

serial sections, first they show a Schwann cell investment and eventually they become myelinated fibers in the nerve trunks. The sensory nerves illustrated here in a rat coronary artery sometimes have tiny extra protrusions which lie close to the muscle cell. It is not yet clear whether 'parinergic' nerves, a third type of efferent autonomic nerve found in the gastrointestinal tract and lung, also supply some arteries. RAT

With smaller arteries such as mesenteric arteries there is a fine plexus around the media itself and you can also see larger trunks of nerves which are on their way further down to innervate other regions. Even in the very small muscular arteries, where the nerves are fairly sparse, they are still quite clearly located outside the media at the adventitial-medial border.

Now we must consider the innervation of arterioles. In small arterioles in the heart there is a single layer of smooth muscle cells and few nerves. However, in order to demonstrate the need to examine regional differences in innervation patterns, the scanning electron microscope has been employed to show that at arteriole branching sites in the heart, there are large 'intimal cushions' (Fig.17A).

The smooth muscle is arranged in a complex way in these cushions and they are heavily innervated by at least three different kinds of nerves (Fig.17B). The role of these 'intimal cushions' in coronary activity is not yet clear. Although, as mentioned earlier, the innervation of small coronary arterioles is sparse, precapillary sphincters are heavily innervated by at least three types of nerve.

Intimal Cushions in Coronary Arteries

What about nerves in relation to capillaries?

There has always been a debate about whether they are innervated or not. There are no nerves in relation to the capillary illustrated in the heart. On the other hand, if you look at capillaries in the gut, there are many nerve fibers closely aligned to them. Now it may be that they are just running in a common space between the muscle cells, but we must realize that these smooth muscle cells in the gut, are strongly affected by transmitter released from varicose nerve fibers and they are further away than the capillary. Thus it depends upon whether they are sensitive or not to the transmitter, and you cannot tell from the presence of nerves whether there is functional innervation or not.

In general, veins have a less dense perivascular nerve plexus than arteries. For example in the mesentery illustrated, there is an artery and alongside is a vein with a nerve plexus which is much less dense.

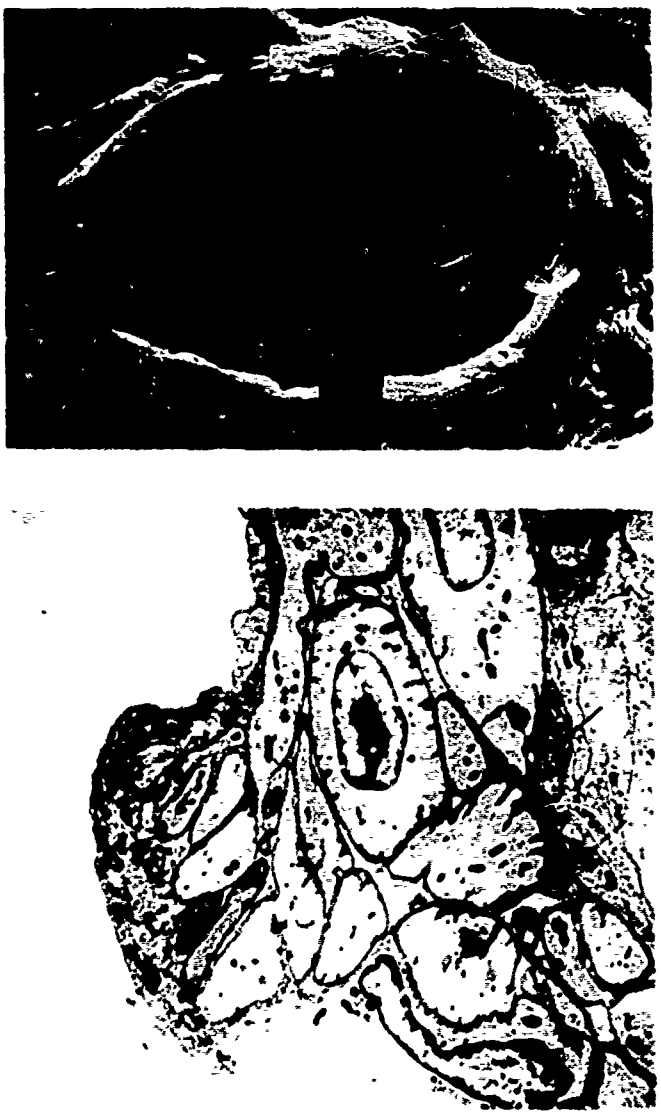

Figure 17: Scanning (A) and Transmission (B) electronmicrographs of an intimal cushion' at the orifice of the rat septal artery at its junction with the left coronary artery.

A. Scanning electronmicrograph demonstrating the well developed ridge of the cushion around the orifice. x 360

B. Transmission electronmicrograph through a ridge showing the complex relationships of smooth muscle cells and the presence of nerves (arrows). (Courtesy of Yohro & Burnstock, Z. Anat. Entwickl.-Gesch. 140, 187. 1973).

Finally, we must not neglect the possibility that extra-adrenal chromaffin cells contribute to the nerve plexuses around blood vessels. These cells contain high levels of catecholamine and they send processes to join the perivascular plexuses. Drugs used to degranulate the nerves do not necessarily affect the chromaffin cells so you need to be very careful about the conclusions from pharmacological experiments under these circumstances. The ultrastructure of chromaffin cells is quite different from adrenergic, cholinergic and sensory nerves; they contain very large granules.

EDITORIAL COMMENT

Eranko (92) has reported on small intensely fluorescent (CIF) cells that elaborate norepinephrine apparently independently of the cardiac innervation. They are not obliterated by treatment of the animal with 6-hydroxydopamine that knocks out sympathetic innervation.

DR. BJORKERUD: It is certainly very interesting that the innervation of the intimal cushions in the coronary arteries has heretofore been overlooked. I think you implied that there might be a mechanism for tangential contraction of the segment. Is that possible since the orientation of the smooth muscle cells in the cushions is mainly longitudinal? Also, do you have any data on the location of nerve endings in cushions in other regions?

Coronary Intimal Smooth Muscle Cushions

DR. BURNSTOCK: First of all, in analyzing the orientation of the muscle fibers in intimal cushions, we find with a combination of scanning and transmission electron microscopy, that, in some coronary arteries at any rate, the intimal ridges are arranged spirally around the orifice. The function of these intimal cushions is not clear. The second question was, where are the nerves located? They are located mostly at the adventitial-medial border, although to some extent they project into the back of the cushion. There appear to be at least three types of nerve involved. Thirdly, your question was, where else have we seen these cushions? We have been looking at the cerebral arteries and in the kidney. We have found them in both these places. It is too soon to say how heavily innervated they are, but we are beginning to look at that now. Do not forget that the precapillary sphincter are also incredibly heavily innervated, even more so than the cushions.

DR. ADAMS: George Koelle, for example, has used the acetyl cholinesterase technique to demonstrate cholinergic nerve fibers.

I should think it would be highly desirable to show adrenergic and cholinergic nerves simultaneously. Is there some problem in smooth muscle in demonstrating acetyl cholinesterase?

DR. BURNSTOCK: There are some problems. In some species,
RAT the rat in particular, adrenergic nerves stain with cholinesterase as well. So you see you have to be very careful if you are looking at rat tissue not to use cholinesterase as a marker for cholinergic nerves.

DR. LINDNER: Did you often find adrenergic or cholinergic nerves around capillaries in this or other tissues? Is it possible that they innervate pericytes?

DR. BURNSTOCK: I really cannot comment on what the proximity of nerves to capillaries means. This sort of evidence is not enough to suggest functional innervation. It would be necessary to inject the transmitters on to the surface of the membrane to gather evidence on specific receptors.

DOG DR. HAUST: I should like to add the dog to the list of species mentioned by Dr. Burnstock as having (arterial) intramedial innervation. We have observed nerve fibers both in the canine carotid and femoral arteries. We have not attempted to identify their type and neither did we have the courage to rush this observation into publication because it was so contrary to conventional teaching (135).

Cytoplasmic Filaments In Relation To Rough Endoplasmic Reticulum

Next, I should like to address myself to the question of the intracytoplasmic filaments of the arterial smooth muscle cells. In addition to the well established two types discussed in detail by Dr. Somlyo, filaments appear in human fetal aorta that are distinct by their spatial association with the profiles of the rough-surfaced endoplasmic reticulum. At this stage, one often observes "open ends" of the profiles and "streaming" from these into the cytoplasm bundles of filaments (136). It is difficult to state whether these filaments are of the "thin" or "thick" variety as they are usually the only filaments present at this stage of development and one is not able to compare their thickness with either of the two varieties. Actual measurements, to my simple mind, are totally unreliable if one wishes to compare the diameters of filaments not in the same but in different tissues.

I would like to ask whether any of the participants had observed similar filaments and how one might interpret this spatial association of filaments and rough-surfaced endoplasmic reticulum.

DR. A. P. SOMLYO: I'd like to talk a bit about the identification of the intermediate filaments. It is quite surprising how in transverse section these filaments really cluster around about 100A°. In other words, in aldehyde-fixed, osmium post-fixed material I think it is reasonable to assume when you see the 100A° filaments that these are, in fact, the intermediate filaments. Other types of filaments are rather sensitive to preparatory procedures. For example, if you swell the muscle, and in our case we have done this with high potassium chloride solutions, or more recently with metabolic inhibitors. In swollen muscle, the thick filaments are very poorly preserved, or not at all. Furthermore, actin filaments, the thin filaments, (50-80A°) are very poorly preserved after primary osmium fixation. So, by using all of these tricks, if you still have a filament that is 100A° and stains rather densely, it is very likely that this is an intermediate filament.

Identification Of Filaments

DR. HAUST: I am wondering, however, whether you have ever observed the phenomenon of spatial association between filaments and rough-surfaced endoplasmic reticulum?

DR. A. P. SOMLYO: We are aided in the identification of the intermediate filaments by two factors: (1) the width of these filaments is relatively constant, clustering about 100A°; (2) the filaments are more stable during preparatory procedures than are the myofilaments, and are preserved in spite of swelling that could tend to destroy the thick filament lattice. Unlike the thin filaments, the intermediate filaments are also well preserved after primary fixation with osmium.

I cannot specifically speak of the close association of the intermediate filaments with the sarcoplasmic reticulum, as we have not looked at this question in detail. In general, we find that such associations are best investigated with stereo electron microscopy, using a tilt stage, to achieve a three-dimensional view and clarify whether associations between different portions of the cell are true contacts or merely represent overlap in the focal plane.

DR. ROSS: I cannot comment on these observations although we have seen such close relationships. I have, however, never seen direct openings of the cisternae of the rough endoplasmic reticulum to the cytoplasmic space. All the studies that I know of (in the literature regarding secretory cells) demonstrate that it is a component of the protein synthetic apparatus that is associated with secretory protein. And by secretory protein, one has to say protein that is sequestered in membrane bounded compartments that is utilized in some way, either intracellularly or extracellularly. For example, lysosomal proteins are secretory

proteins, but they are always bounded by membranes within the cell until the cell secretes them outside, which they sometimes do. Connective tissue proteins are secretory proteins that are sequestered in membrane bounded compartments until they are released outside the cell. So, to my knowledge, all the data that has been accumulated concerning cytoplasmic proteins demonstrates that they are synthesized on ribosomal aggregates that are not attached to membranes. Whereas those that are secreted outside the cell or considered to be secretory proteins are synthesized on aggregates of ribosomes, presumably polysomes, attached to membranes. So I don't think I can give you an answer for the observation, but it certainly does not agree with all of the hard data that is presently available about synthesis of secretory proteins.

DR. ROBERT: Is there any correlation whatsoever in the experiments available, between the morphological signs of innervation and the rate of synthesis of intercellular macromolecules by smooth muscle cells? Of course, for a biochemist it is easier to define the differentiation by relative rates of biosynthesis than on a morphological basis. We can just extend our hypothesis (see page 110) and define differentiation of smooth muscle cells in terms of the ratio of intercellular macromolecules they synthesize. I think you mentioned that innervation had something to do with differentiation. How can that be really proven by morphological observations concerning the rate of innervation and the rate of synthesis of elastin or other intercellular macromolecules?

DR. BURNSTOCK: This is a tremendous question. To me it is really an exciting field, that is, the long term effects of nerves on differentiation and development. I do not think anybody has got terribly far with it yet. My reason for suggesting this in the smooth muscle system is because Julie Chamley in our laboratory has been studying the effect of nerves on smooth muscle jointly in tissue culture. Nerves delay the dedifferentiation of muscle cells grown in tissue culture. You can see in the same field of joint cultures of nerve and muscle, muscle cells which have dedifferentiated and divided which have no nerves on them, while other muscle cells which have formed long-lasting relationships with nerves are still contracting, still have thick and thin filaments and they have not dedifferentiated. At a later stage in the joint cultures those bundles of muscle cells or those monolayers of muscle cells which are heavily innervated by nerves produce nexuses at about twice the rate as those that do not have a relationship with nerves. We do not yet understand the underlying mechanisms.

DR. A. P. SOMLYO: Geoff Burnstock mentioned seeing lanthanum inside the tubules that he considered to be sarcoplasmic reticulum in his preparations. I shall forego the question whether these tubules were longitudinal invaginations, such as found in developing

striated muscle and in some cardiac muscles, and accept for the moment that they were a true sarcoplasmic reticulum. However, it is questionable whether the presence of lanthanum in these tubules reflects the true communication of the sarcoplasmic reticulum with the extracellular space under normal conditions. Sommer and co-workers (373) showed a similar entry of lanthanum into the sarcoplasmic reticulum of cardiac muscle, and took great pains to point out that this was a very rare occurrence and probably represented a preparatory artifact. I think this is a point worth considering. As is also well known to microscopists, maintaining lanthanum in the colloidal state is a rather tricky business, depending upon the control of pH and possibly on a number of other, less well defined factors. Under certain conditions lanthanum may enter the fiber in the ionic form and, once inside, precipitate in the colloidal form. This mechanism, rather than direct communication with the extracellular space, could account for the localization of colloidal lanthanum in the sarcoplasmic reticulum.

DR. BURNSTOCK: We do not know the circumstances when these channels open up, but are currently carrying out experiments to try to find this out. I do not think you can assume that because it occurs in less than 20% of the preparations that it is an artifact.

EDITORIAL NOTE

To help resolve the problem of reliable identification of cells, especially smooth muscle cells and to throw light on problems of cell differentiation and trophic effects, Dr. Burnstock showed a motion picture. The film illustrated the effects of nerves on the development of smooth muscle cells as well as the effect of the muscle cells on the pattern of growth of the nerves.

DR. BURNSTOCK: Two kinds of experiments are depicted in the film. We grow out joint cultures in Rose chambers. Sympathetic ganglia are placed under dialysing cellophane to keep them in position and then we give the nerves a 'choice' between normally densely-innervated tissues like the ventricle, lung or uterus. The nerves appear to grow preferentially to the normally densely-innervated tissue. This is probably because these tissues produce Nerve Growth Factor. In the second kind of experiment we again place the explants of sympathetic ganglia centrally, but this time we enzymatically separate the smooth muscle cells. In this way we can observe the relationship of individual nerves with individual muscle cells under time lapse cinematography. What happens is that nerves palpate any cell in their path for about fifty minutes. Then, if it is a fibroblast, the nerve goes on its way, but if it is a smooth muscle cell it forms a dense, long-lasting and intimate relationship with it. If another nerve comes along

later, it is "rejected." In the case of a non-innervated single smooth muscle cell from the taenia coli, for example, contractions were seen to occur independently on each side of the nucleus at a rate of about three a minute. Sometimes the smooth muscle cells are quite complicated with many processes which contract independently of each other. After they have been in culture for a while they aggregate into muscle effector bundles. Initially they line up but there are no 'nexuses' between them, so they contract independently of each other. Later, when nexuses develop synchronized contractions appear.

Nexuses are the specialized membrane junctions where there is the close relationship between neighboring muscle cells. Nexuses are actually 'gap junctions' and constitute the low resistance pathways allowing electrotonic spread of activity between muscle cells within effector bundles.

The behavior and development of smooth muscle cells in culture depends on (1) the density of cells in culture and (2) whether they are differentiated or not. If they are differentiated cells, they first dedifferentiate and then divide. It is only when they form a monolayer (or confluence) that redifferentiation occurs, and it is only then that nexuses and effector bundles form.

With fibroblasts in the culture with smooth muscle cells it was shown that there was no interaction between sympathetic nerves and fibroblasts.

In a sequence where a nerve is in a 'choice' situation between a beating smooth muscle cell and a fibroblast, it was clearly shown that the nerve terminal "palpates" both cells for a while and then it grows very strongly toward the muscle. As it grows it appears to make the muscle beat faster. Whether this is due to release of the transmitter or whether it is a mechanical effect on the surface is not yet clear.

QUESTION: What was your source of smooth muscle cells?

DR. BURNSTOCK: This is actually a smooth muscle from the vas deferens, but we are now carrying out similar explants with arterial smooth muscle. In general we find arterial muscle harder to culture than the visceral muscles. We have now managed to record from these cells during stimulation of the nerve and have shown that there is a functional relationship. Of course many issues are raised by these experiments but this is not the place to discuss them in any detail.

DR. AVRIL SOMLYO: I plan to briefly review some of the aspects of normal function of vascular smooth muscle with particular

emphasis on the action of drugs and on excitation-contraction coupling. It is well established that there are very marked differences in vascular reactivity and pharmacological behavior in the same vascular bed of different species, and also between different vascular beds within the same species (359). For example, contractile responses to vasopressin, unlike the responses to epinephrine or to angiotensin, differ very markedly at different levels of a canine aorta (Fig.18). The thoracic aorta does not respond to vasopressin at all, but more distal portions of the aorta contract when stimulated with this peptide. Thus the distribution of specific receptors may vary even among different cells within a single blood vessel.

Regulation of Contraction

DOG

In order to have a better understanding of the different contractile effects of drugs, it is necessary to look at some of the electrical events associated with excitation-contraction coupling. There are some vascular smooth muscles that spontaneously generate action potentials which in turn trigger contractions. The portal-anterior mesenteric vein is a good example of a vessel with this type of activity (117, 163, 193, 363, 372). Fig.19 shows a spontaneous tetanus in the rabbit mesenteric vein recorded by extracellular methods. With the second burst of action potentials there is fusion of mechanical activity. Variations in action potential morphology in different vascular smooth muscles are seen when electrical activity is recorded with micro-electrodes (Fig.20). There are differences in the rate of rise of the action potentials in various preparations and also in the time-course and presence or absence of slow wave activity.

RABBIT

When a spike generating type of vascular smooth muscle is stimulated with an excitatory agent such as norepinephrine, there is an increase in action potential frequency and a concentration-dependent depolarization of the tissue (Fig.21). The extent of contraction is proportional to the amount of depolarization. The regulation of contraction by action potentials is an electromechanical coupling mechanism also seen in uterine and intestinal smooth muscle (209, 264). The ionic species involved in carrying the current during this type of electrical activity have not been established, although experiments using voltage clamp techniques (12, 174, 226) as well as experiments involving changes in the ionic environment suggest that both sodium and calcium may be carriers of the inward current during the action potential of uterine smooth muscle.

Spike Generation and Graded Electrical Activity in Smooth Muscle Contraction

Action potentials are not a prerequisite for contraction. If spike-generating rabbit portal vein smooth muscles are exposed to caffeine, their action potentials are abolished: if one stimulates

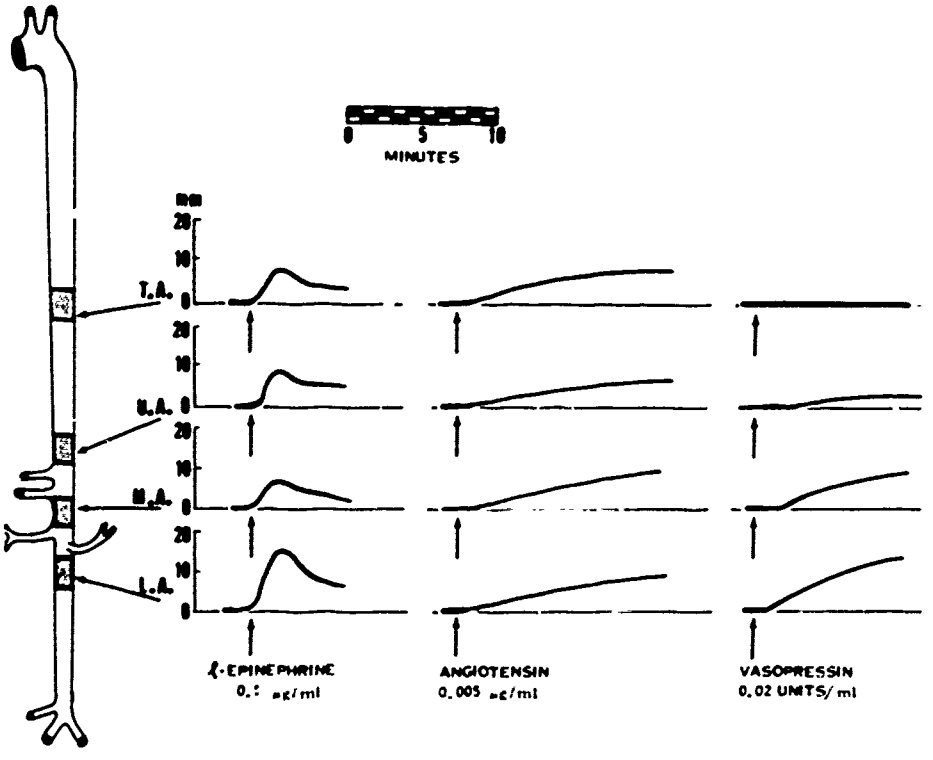

Figure 18: Isotonic responses of the same canine aorta at four levels. T.A.: thoracic aorta. U.A.: abdominal aorta above coeliac axis. M.A.: between cranial mesenteric and renal arteries. L.A.: below renal arteries. The loading tension applied was of the order of in vivo tangential stress. (Somlyo, A.V., Sandberg, R.L. and Somlyo, A.P.: J. Pharmacol. Exp. Ther. 149: 106-112, 1965.)

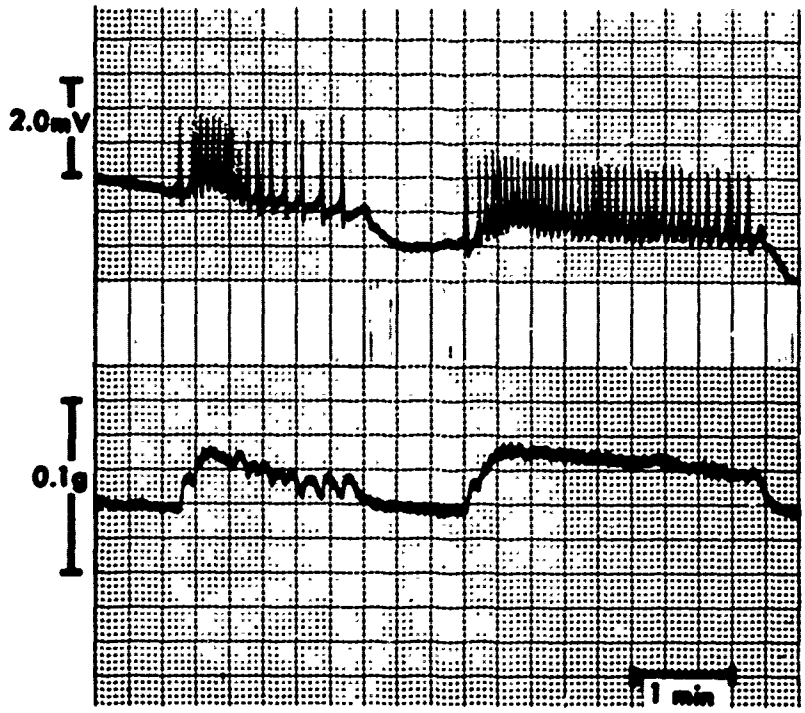

Figure 19: Spontaneous tetanus of rabbit mesenteric vein. Upper record: electrical (sucrose gap). Lower record: isometric tension. The spontaneous contractions are triggered by action potentials arising on the crest of slow waves. During the first contraction, the action potential frequency is too low for complete fusion of the mechanical record, which occurs during the initial part of the second contraction as the result of the increased frequency of action potential discharge. Note that without the electrical record, a tetanus (associated with rapid action potential discharge) and a contracture (sustained depolarization) may be indistinguishable.

Figure 20: Variations in action potential morphology in different vascular smooth muscles. Figure 3a: Slow and fast sweep speed records of intracellular action potentials of guinea-pig portal anterior mesenteric vein. Figure 3b: Slow sweep speed record of initial penetration and a burst of action potentials, and progressively faster sweep speed play backs of portions of the same volley of action potentials. This record was obtained in rabbit main pulmonary artery, where such action potentials are observed extremely rarely (1 out of 500 penetrations), and may represent a fiber type other than the usual, gradedly responsive fiber or, possibly, fibers deteriorated due to injury. 3c: The effect of intracellular polarization on the action potentials in guinea-pig portal-anterior mesenteric vein. Right panel shows a fast sweep speed play back of one of the action potentials in the volley shown on the left during the passing of hyperpolarizing current and one action potential after the hyperpolarizing current was turned off. The second action potential corresponds to the left side (hyperpolarization) of the slow sweep speed record shown in the left panel. Rate of depolarization and spike amplitude are increased by the hyperpolarizing current. In comparing the fast sweep speed record of the portal vein preparation (a and c) with the fast sweep speed record of the main pulmonary artery record (b) note the much faster rate of rise of the action potential in b. (Unpublished observations of Avril V. Somlyo and from Somlyo, A.V., Vinall, P. and Somlyo, A.P.: Microvasc. Res. 1: 354-373, 1969).

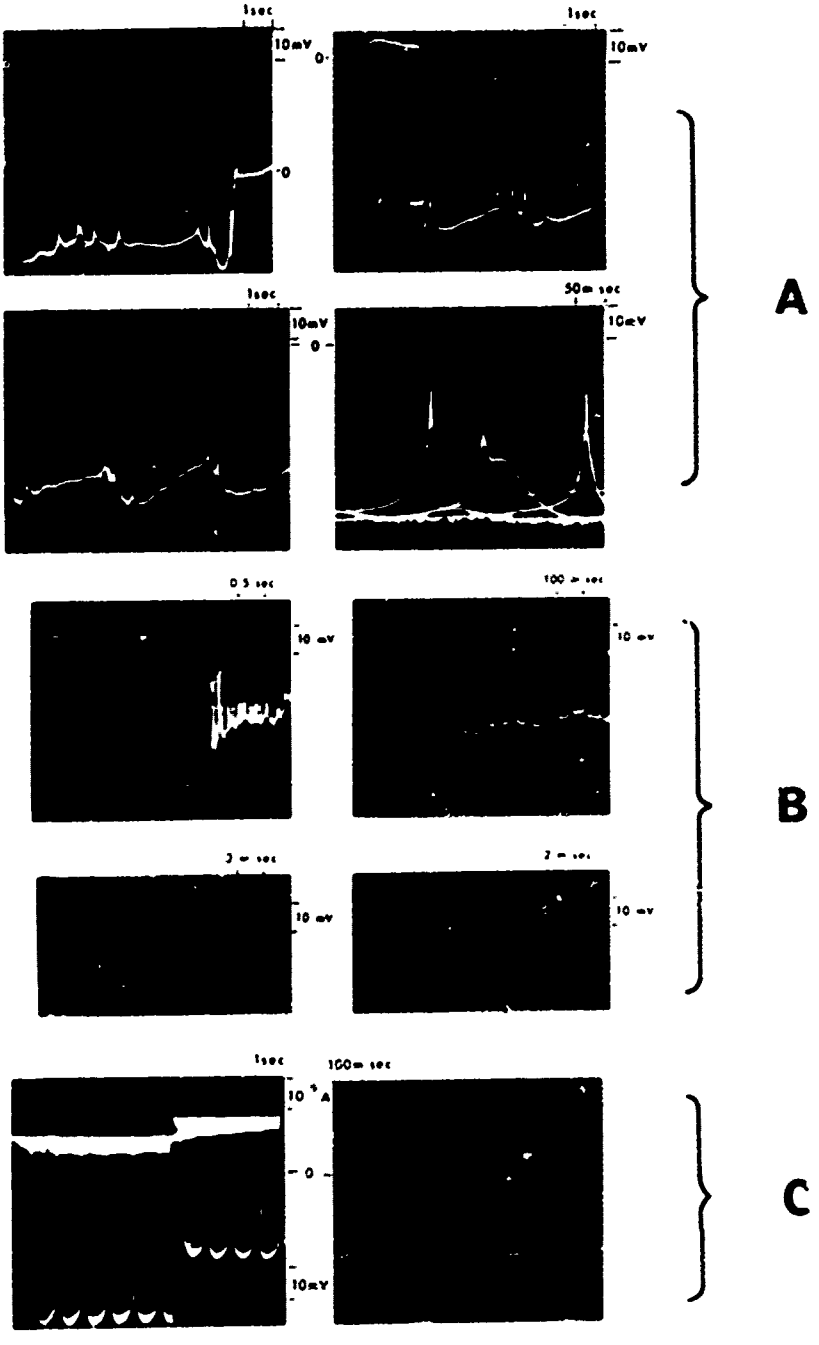
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Figure 20

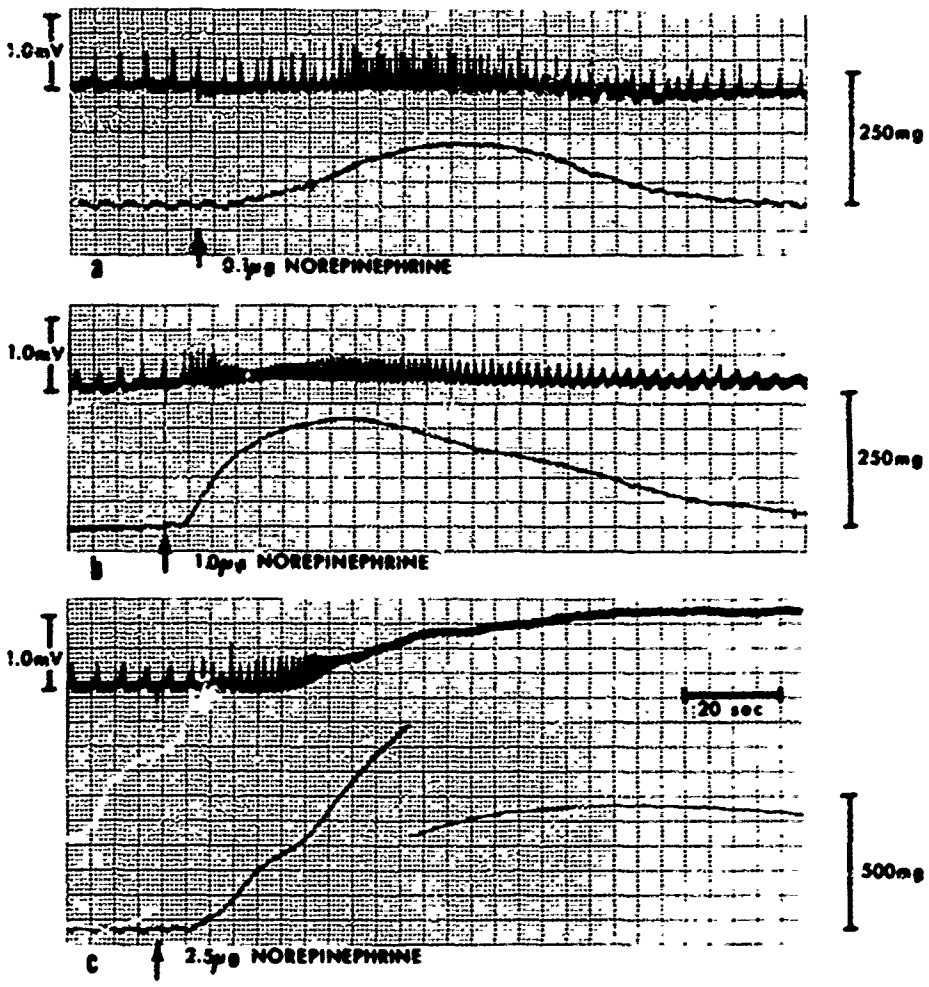


Figure 21: Extracellular recording of electromechanical responses of rabbit portal-anterior mesenteric vein to norepinephrine. Note a and b: the increasing depolarization, frequency of action potential discharge, and contractile response to increasing concentrations of norepinephrine. c, during the maintained phase of the contraction elicited by the largest concentration of norepinephrine, the action potentials have been "wiped out". In the center of the mechanical record, the gain was decreased: see vertical calibration. (Somlyo, A.P. and Somlyo, A.V.: In Airway Dynamics, Ed. by Bouhuys, Charles C. Thomas Publishers, Springfield, Ill. 209-228, 1970.)

such muscles with norepinephrine they will depolarize and contract without generating action potentials (363)

A second type of smooth muscle does not spontaneously generate action potentials and responds to excitatory agents by gradely depolarizing (Fig.22) (130, 146, 363, 372, 390). Norepinephrine, 5-hydroxytryptamine, angiotensin and histamine produce graded depolarization in large multiunit blood vessels such as the aorta and main pulmonary artery.

Smooth muscle exhibits a rather unique ability to respond with unequal contractions to maximal concentrations of various drugs. For example, after stimulation with a maximal concentration of angiotensin, norepinephrine will further contract the same aortic smooth muscle. It is not known whether the underlying mechanisms of these differences are qualitatively or just quantitatively different. The unequal maximal contractile responses persist after depolarization with high potassium solutions (Fig.23) (363), showing, therefore, that the inequalities are not simply due to unequal amounts of membrane depolarization.

Phasic vs. Tonic Contraction

Vascular smooth muscles that exhibit spike electrogenesis respond to high concentrations of potassium with a more phasic contraction than muscles that exhibit graded depolarization and are more tonic (Fig.24). Phasic and tonic muscles also differ in their calcium permeability when depolarized with high K to increase their (low) resting Ca permeability (372). The threshold (Ca) for contraction of depolarized main pulmonary artery is of the order of 10^{-5} M, whereas that for the portal vein is above 10^{-4} M. In this type of experiment the concentration of cytoplasmic calcium that the myofilaments see is membrane limited. When muscles are treated with glycerol, the membranes can be made very leaky and then the minimal calcium concentration necessary for actomyosin ATPase activity and tension can be titrated. Filo, Ruegg and Bohr (100), and Schadler (323) have reported that a calcium concentration of the order of 10^{-7} M is necessary for tension development and for ATPase activity in glycerinated taenia coli; this is comparable to the concentration necessary to activate glycerinated skeletal myofibrils.

Spike electrogenesis and/or graded depolarization precede the rise in intracellular free calcium, the final common pathway of excitation-contraction coupling, leading to tension development. However, Evans, Schild, and Thesleff (93) reported that smooth muscles would still contract in completely depolarized preparations in which the depolarization step in excitation-contraction coupling is eliminated. The unequal maximal contractile effects of different drugs are also maintained after depolarization (363). Drug induced relaxation may also occur in polarized smooth (209) and tonic

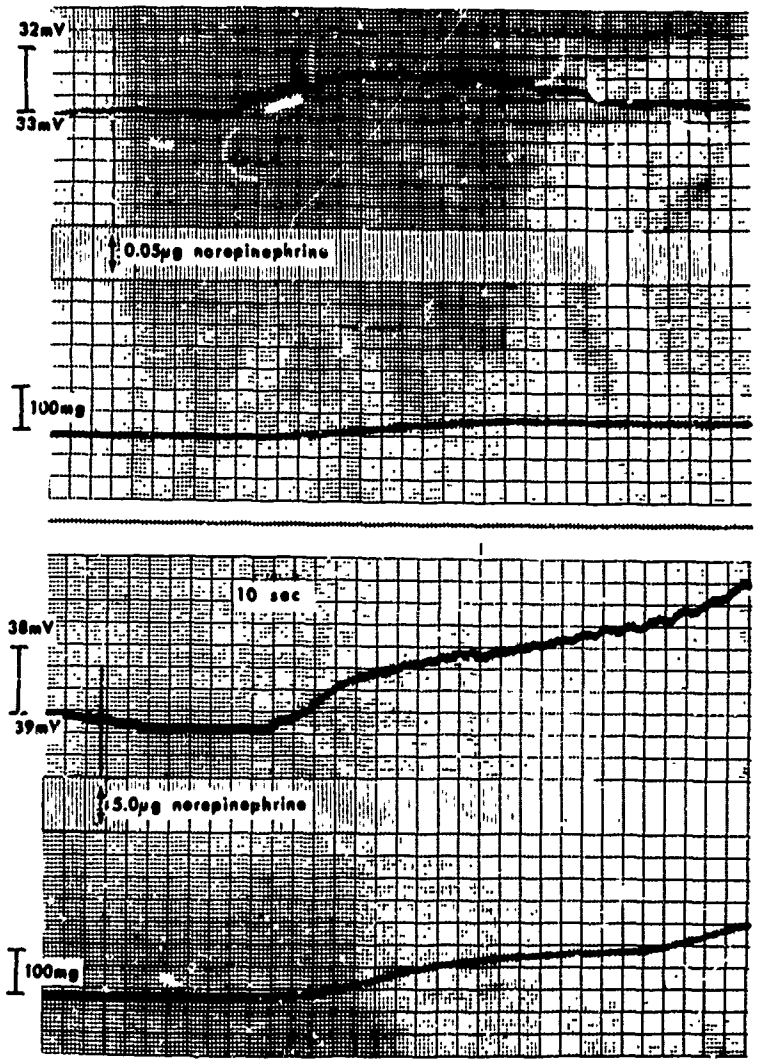


Figure 22: Graded depolarization and contraction of rabbit main pulmonary artery smooth muscle stimulated with norepinephrine. The upper panel shows the response to a low, and the lower panel to a high concentration of the catecholamine. In each panel the upper trace is the electrical (sucrose gap) record and the lower trace shows tension. The depolarization and contraction increase in response to increasing concentrations of norepinephrine and the change in membrane potential preceeds the tension development, ruling out the possibility that depolarization is a junction potential due to movement. The low resting potential shown is indicative of the degree of short circuiting of the sucrose gap in this preparation that, by intracellular measurements, has a normal resting membrane potential of approximately 60 mV. (Modified from Somlyo, A.V. and Somlyo, A.P.: J. Pharmacol. Exp. Ther. 159: 129-145, 1968.)

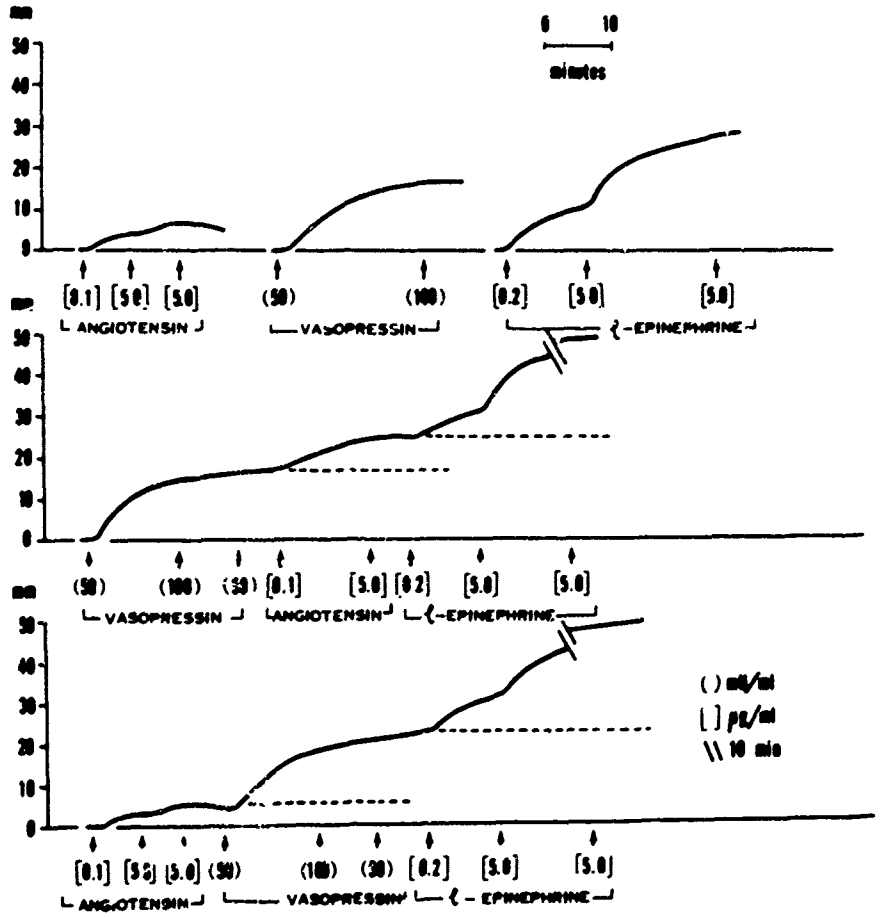


Figure 23: Responses of depolarized canine abdominal aorta to supramaximal drug stimuli. Preparation depolarized with 179.2 mM K (SO_4-Cl) solution prior to addition of drugs, and maintained in depolarizing solution. Upper trace: maximal isotonic responses of single strip, determined individually, to supramaximal concentrations of three agents. Note unequal responses of depolarized v.s.m., similar to those reported by us to occur in polarized state. Middle and lower traces: the effects of the same three agents added consecutively to depolarized preparations. The order of adding angiotensin and vasopressin did not affect the magnitude of the response to either agent. (Somlyo, A.V. and Somlyo, A.P.: J. Pharmacol. Exp. Ther. 159: 129-145, 1968).

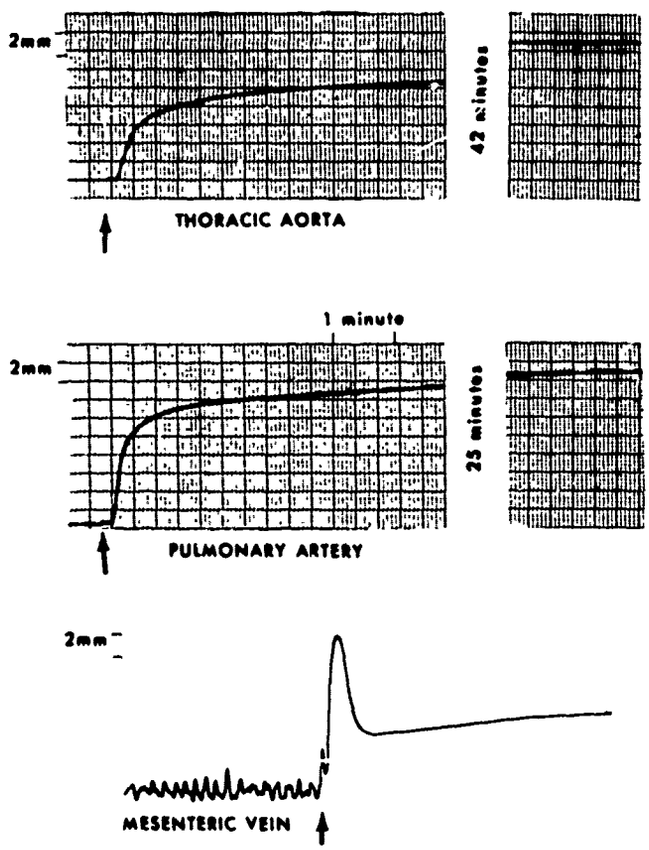


Figure 24: Responses of tonic and phasic vascular smooth muscle to depolarization with high K. Ordinate: total shortening. Length of strips (cm): aorta 2.5; main pulmonary artery 4.2; mesenteric vein 4.1. At the arrow depolarizing solution containing 179.2 mM K and 2.5 mM Ca was added. The K-contractures of the main pulmonary artery and aortic smooth muscle are tonic, while the contracture of the portal mesenteric vein has a large phasic component. Note the spontaneous activity of mesenteric vein prior to depolarization. The rabbit used for this study was reserpinized to eliminate the potential effects of catecholamine release. (Somlyo, A.V., Vinall, P., and Somlyo, A.P.: Microvasc. Res. 1: 354-373, 1969.)

striated muscles without a necessary change in the membrane potential. Pharmacomechanical coupling is a term used to describe the processes through which drugs can affect contraction without a necessary change in membrane potential (363, 371). The relative importance of pharmacomechanical and electromechanical coupling in different vascular smooth muscles and under different experimental conditions may vary. The underlying mechanisms of pharmacomechanical coupling which bring about the rise in intracellular free calcium are not understood.

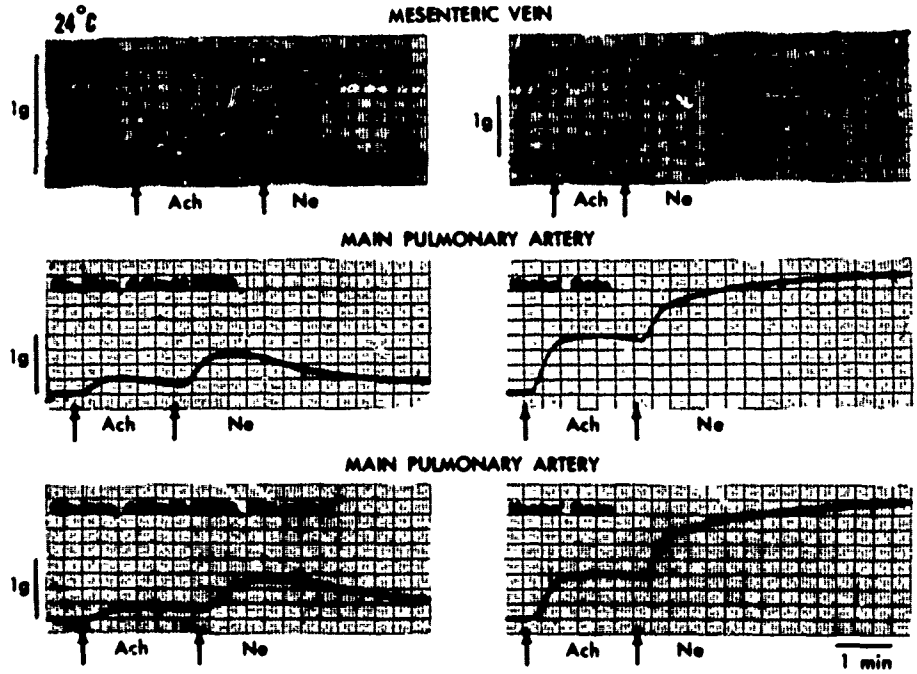
The Relationship of Calcium Concentration and Membrane Potential to Contractile Proteins

Calcium influx from the extracellular space, displacement of bound calcium from the surface membrane or release from intracellular storage sites are possible sources of calcium for the contractile mechanism. Varying degrees of influx of extracellular calcium into vascular smooth muscle are observed under the influence of different excitatory stimuli (123, 370, 397). However, drugs are able to contract some depolarized (and also polarized) smooth muscles even if extracellular calcium is kept below levels that can activate the contractile proteins (48, 80, 358). The relative magnitudes of the drug-induced maximal contractions are also maintained in virtually calcium free solutions (Fig.25). The experiments in Ca-free media are done at room temperature, rather than at 37 degrees C, to retard the loss of calcium from intracellular stores (176, 358).

There are significant differences in the volumes of sarcoplasmic reticulum in different types of smooth muscle and a positive correlation exists between the ability of smooth muscles to contract in the absence of extracellular calcium and the volume of sarcoplasmic reticulum (80, 358).

(For a discussion of vascular smooth muscle ultrastructure including the sarcoplasmic reticulum see A.P.Somlyo, page 1).

The sarcoplasmic reticulum content of the main pulmonary artery and of the aorta is 5 - 7.5%. Such smooth muscles respond with sizeable contraction to drugs (e.g. acetycholine, angiotensin, and norepinephrine) even in the absence of extracellular calcium and also synthesize large amounts of collagen and elastin (314, 363). The latter morphogenetic function may be related to the presence of a well-developed sarcoplasmic reticulum. Other smooth muscles, such as the portal-anterior mesenteric vein and taenia coli normally contain lesser volumes of sarcoplasmic reticulum (2-3% of cytoplasm volume) and unlike the large tonic vessels, contract not at all or only minimally in the absence of extracellular calcium.



Ach=5.0 μg/ml Acetylcholine

Ne=10.0 μg/ml Norepinephrine

Figure 25: Contractile responses of two types of vascular smooth muscle in normal and in Ca-free (4 mM EGTA solution). The contractions in the right-hand column illustrate the responses in normal (Ca-containing solution to supramaximal concentrations of acetylcholine (Ach, 5ug/ml) and to norepinephrine (Ne, 10ug/ml). The left-hand column shows the effect of the same drugs on the same preparations placed in Ca-free Krebs' (two upper records) or Ca-free depolarizing (left lower record) solution. The amplifier gain was increased in the upper left-hand record (see vertical calibration). Note that the contractile response of the portal-anterior mesenteric vein (mesenteric vein) is almost completely abolished in the Ca-free solution, while the main pulmonary artery smooth muscle still develops sizeable contractions in this medium. The unequal maximal contractions produced by the two drugs still persist in the depolarized main pulmonary artery smooth muscle in Ca-free, high K medium (left bottom panel). Note that these experiments were done at room temperature to avoid the loss of intracellular calcium accelerated at high temperatures. (From Devine, C.E., Somlyo, A.V. and Somlyo, A.P.: J. Cell Biol. 52: 690-718, 1972.)

(The accumulation of divalent cations by sarcoplasmic reticulum and mitochondria of smooth muscle are discussed by A. P. Somlyo, page 2.)

Summary: In general there are two major types of vascular smooth muscle: (1) spike generating with relatively phasic K-contractions; (2) gradedly responding with more tonic K-contractions. There are quantitative differences amongst vascular smooth muscles within each category and it is likely that some smooth muscles may normally exhibit properties of both types or may be induced to do so by altering experimental conditions. Pharmacomechanical coupling is a process of excitation-contraction coupling which is not mediated by a change in membrane potential. The relative contributions of electromechanical and pharmacomechanical coupling in normal polarized smooth muscle have not been determined and may vary from one muscle to another. Unequal maximal contractions produced by drugs are usually accompanied by unequal depolarization, however, unequal contractions persist in completely depolarized tissues. The relative volume of sarcoplasmic reticulum varies in functionally different smooth muscles and correlates with their ability to contract in the absence of extracellular calcium. It is probable that both the influx of extracellular calcium and the translocation of intracellular calcium are involved in excitation-contraction coupling and the contribution of these may vary from one blood vessel to another.

Pharmacomechanical Coupling

DR. GOLENHOFEN: During the last twenty years spike potentials have been measured in almost all types of mammalian smooth muscle. This led to the concept that the spike is the only process capable of triggering tension developement under normal conditions. Until recently the relatively few papers which reported normal mechanical activation without spike discharges were not able to convince the majority of smooth muscle physiologists for the following reasons. Spike-free activation was described mainly for tissues such as aortic and arterial smooth muscle (44, 371, 392) where successful intracellular measurements of electrical activity are particularly difficult, and other authors have also described spike-like action potentials in these tissues (31, 177, 178, 218). Furthermore, Somlyo et al (372) themselves sometimes found spike discharges in pulmonary artery. Therefore the interpretation of negative results concerning spike discharges raises special problems: it is difficult to exclude the possibility that spike discharges are present but cannot be detected because of methodological limitations.

Blocking Spike Potentials

A new approach was made possible by using drugs which block the spike potentials of smooth muscle. Verapamil and its methoxy derivative, D 600, were described by Fleckenstein et al (101) as calcium antagonists, which block electromechanical coupling in heart muscle. In smooth muscle these drugs are able to suppress spike discharges (102, 118) as shown in Fig.26. In an isolated portal vein preparation all components of the complex pattern of spontaneous electrical activity disappear after application of verapamil, the potential record becomes a smooth line and the membrane becomes slightly depolarized compared with the maximal potential values during normal spontaneous activity. The suppression of electrical activity is accompanied by a disappearance of the mechanical activity.

Fig.27 shows the reaction of an isolated portal vein on application of noradrenaline (NA), under normal conditions (a) and after application of verapamil (b) and (c) (117). NA produces under normal conditions (Fig.27A) a depolarization and an increase in the frequency of spike discharges. During treatment with verapamil, NA produces a depolarization without spike discharges but still with an increase in tension which was on average 42% of the control reaction. The size of the NA-induced depolarization under verapamil was not significantly different from that under normal conditions.

Parts (a) and (b) of Fig.27 are sections of a continuous intracellular potential recording in one and the same cell. The recovery of the impaled cells after verapamil treatment could also be observed in continuous long-term recordings. We, therefore, have direct evidence that the microelectrode is able to detect spike potentials under verapamil treatment and can therefore conclude that the NA-induced activation of portal vein under these conditions is really spike-free.

Comparable results could be obtained in stomach smooth muscle where acetylcholine is able to produce a spike-free, tonic activation during treatment with verapamil (119). Since, in some preparations, both the spike-free activation mechanism and the spike activation mechanism can similarly be suppressed by lanthanum ions and by calcium depletion, we concluded that the smooth muscle cell membrane has two different calcium activation mechanisms (120).

Calcium Activation Mechanisms

The final proof for the dual nature of calcium activation can be seen from the observation that both mechanisms can be selectively suppressed (43). This is shown for stomach smooth muscle in Fig.28. Under stimulation with acetylcholine (ACh) the antrum preparation shows an increase of the typical phasic,

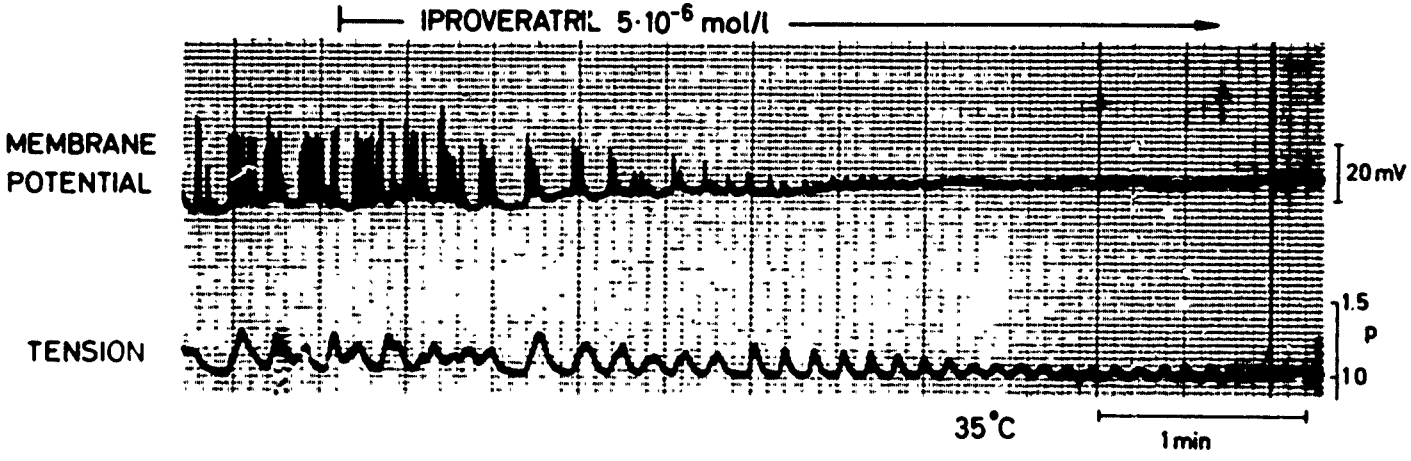


Figure 26: Electrical and mechanical activity of an isolated portal vein of a guinea-pig; spontaneous activity and the effect of verapamil (iproveratril). Membrane potential measured intracellularly with glass microelectrodes. Calibration of tension development in pond.

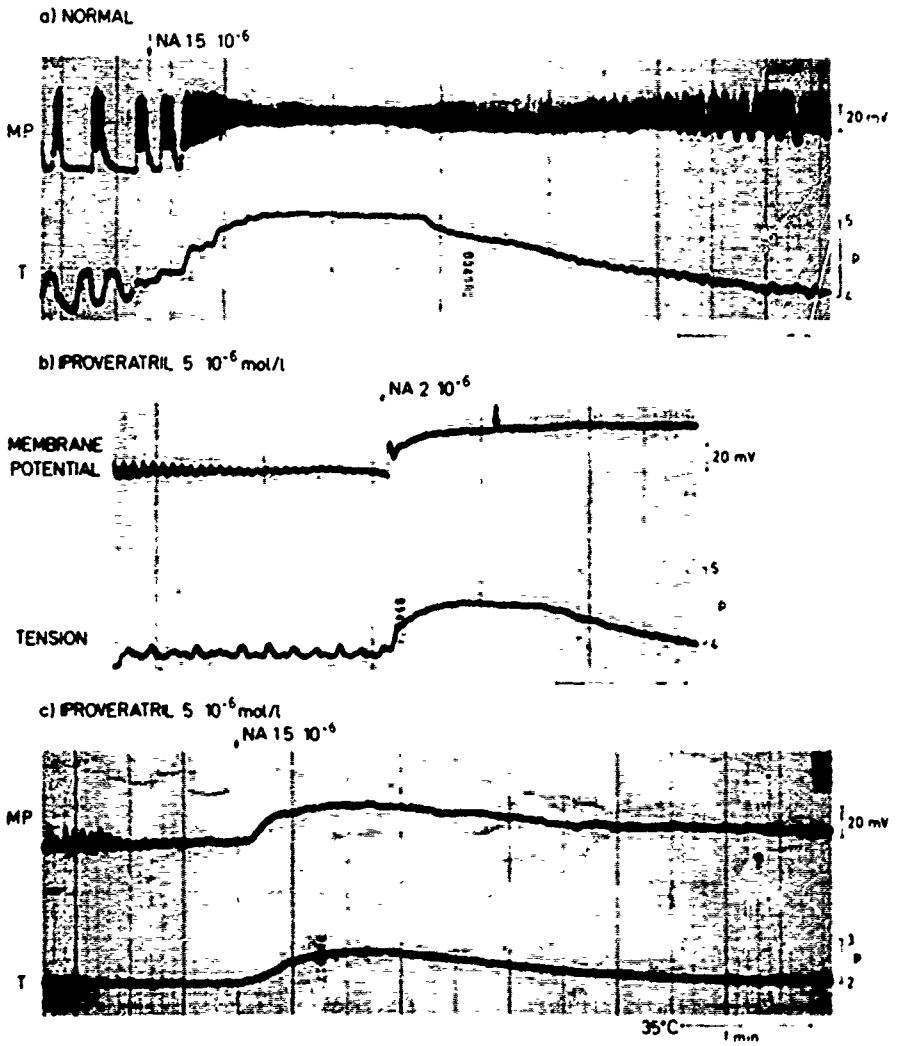


Figure 27: Effect of noradrenaline (NA) application on membrane potential (MP) and tension development (T) of portal vein smooth muscle (guinea-pig). Records (a) and (b) are sections of a continuous potential measurement in the same cell, 5 min excluded. The electrode was accidentally displaced in record (b) at the time indicated by the arrow. Record (c) from another experiment, the electrical recording not disturbed by an electrode displacement. NA was given as a single application to the perfused organ bath; the initial concentration is indicated. (After Golenhofen et al., 1973.)

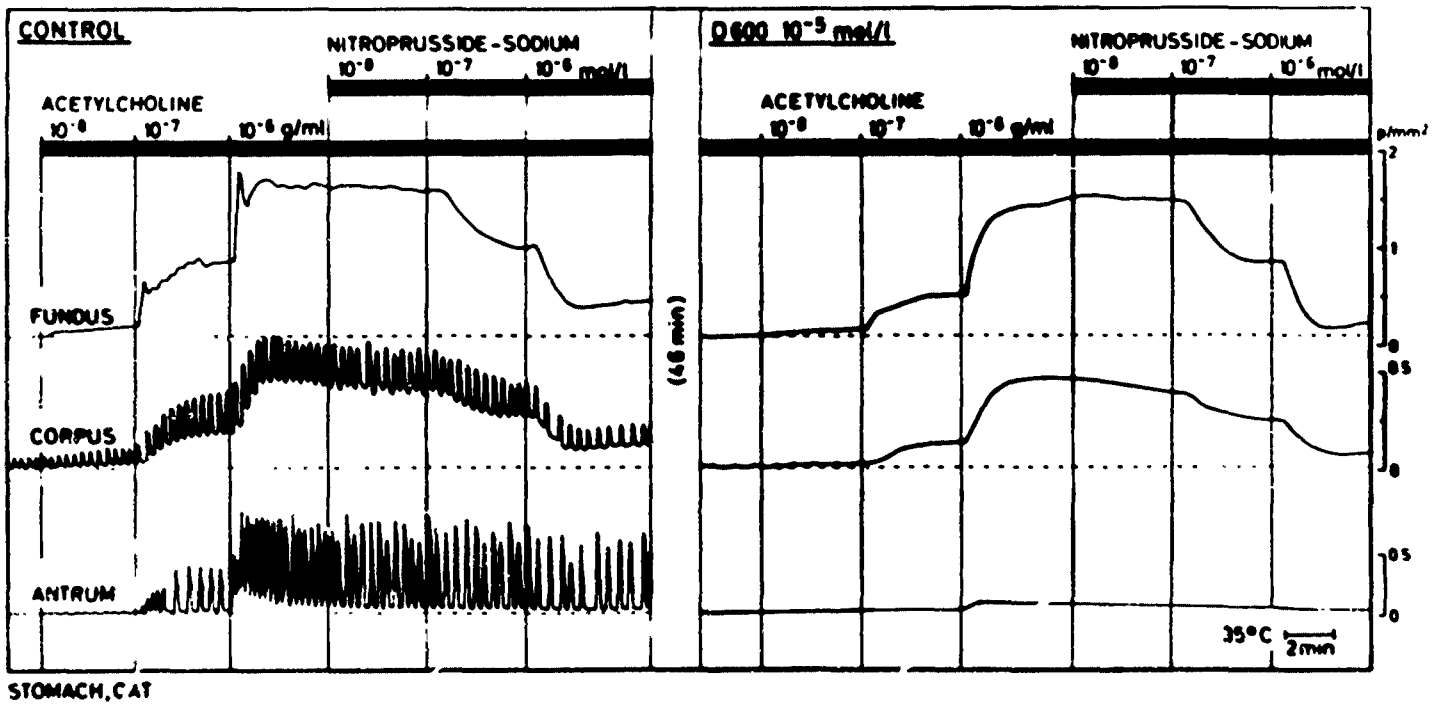


Figure 28: Tension development of circular muscle strips from fundus, corpus and antrum of cat stomach, under normal conditions (left part) and under D 600 10^{-5} mol/l (right part, D 600 applied 15 min before the beginning of the record). (Soev, Golenhofen & Lukanow, unpublished).

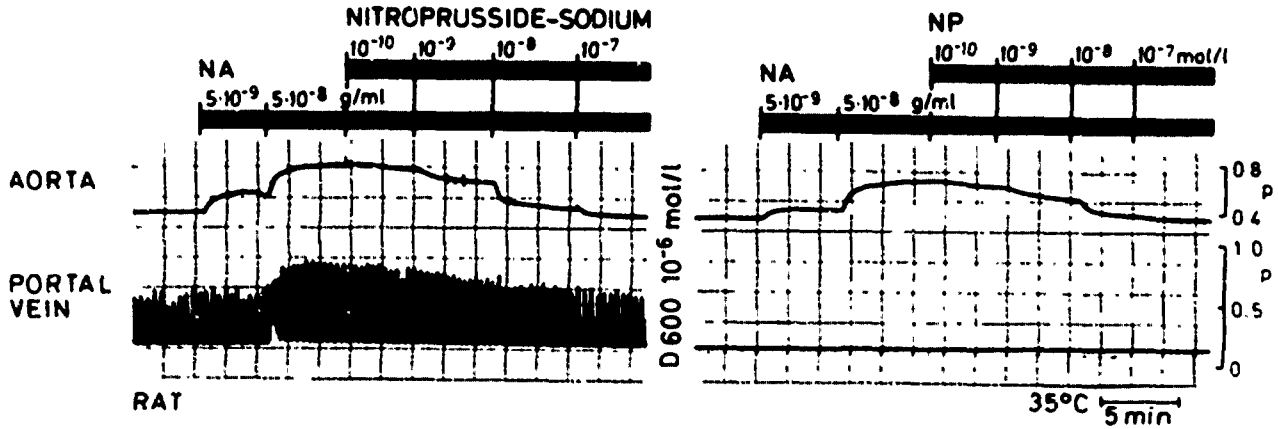


Figure 29: Tension development of a spiral strip of aortic smooth muscle and of an isolated portal vein, both from the rat. Left part: control reaction in normal Krebs solution, application of noradrenaline (NA) and sodium nitroprusside (NP) as indicated. Right part: under treatment with D 600 10^{-6} mol/l, application of the substance 15 min before the record.

peristaltic contractions, while in the fundus strip a more tonic activation is induced, and an intermediate type of reaction occurs in the corpus strip. Sodium nitroprusside (known to block the activation of aortic smooth muscle, (191)) suppresses the tonic reaction in the fundus and the tonic component of the corpus reaction and leaves the phasic contractions of the antrum almost unchanged. D 600 (10^{-5} mol/l), on the other hand, suppresses all phasic components of mechanical activity. After its application, ACh only produces a spike-free, tonic activation which is particularly pronounced in the fundus and nearly absent in the antrum region. This D 600-resistant tonic activation is equally suppressed by sodium nitroprusside (right part of Fig.28) as is the tonic component of the control reaction (left part).

Comparable results can be obtained with vascular smooth muscle as shown in Fig.29 with a spiral aortic strip and an isolated
RAT portal vein (both from the rat). The aorta behaves similarly to stomach fundus, the portal vein similarly to stomach antrum; in the vascular preparations, NA and not ACh must, of course, be used
GUINEA PIG as stimulatory agent. Portal vein of the guinea-pig shows a more intermediate type of reaction comparable to a corpus strip of the stomach, as can be seen in Fig.27.

These results have led to the theory of P- and T-systems for calcium activation in smooth muscle (121) (Fig.30). The P-system, which is blocked by verapamil and related substances, is preferentially used for producing phasic mechanical activity and its activation is usually combined with spike discharges. The T-system is preferentially used for tonic activation and its activation is usually combined with depolarization of the cell membrane, without spike discharges. The basis of this differentiation is a difference in the chemical nature of the calcium activating systems, reflected in the selective antagonistic effects of different substances.

The concept of P- and T-systems allows a better explanation of old observations and opens new aspects for the theory of smooth muscle motility. The description of motoric function of smooth muscle organs has long been dualistic. For example, Cannon (56) distinguished clearly between tone and motility in the stomach. We can now say that this functional dualism is also represented in the differentiation of calcium activation systems in the membrane of smooth muscle cells. P- and T-blocking agents are helpful tools for the further analysis of smooth muscle activation, and the principle of P- and T-blockade will become useful in clinical applications.

DR. KREYE: I should like to comment on the question: "Do there exist two types of smooth muscle cells or two different mechanisms of activation?" Fig.31 shows the relaxant effect of

sodium nitroprusside on various types of smooth muscle. The aorta and trachea (both of which are tonically contracting smooth muscles) are highly sensitive to the relaxing activity of sodium nitroprusside. On the other hand, smooth muscle with phasic responses as in the duodenum and the portal vein is much less sensitive and tends to become tachyphylactic to sodium nitroprusside, or, as in the case of the rat uterus, may be even totally resistant to the action of the drug. These findings apparently support Dr. Golenhofen's assumption that sodium nitroprusside preferentially inhibits the tonic activation mechanism but little or not at all the phasic activation mechanism.

Two Types of Smooth Muscle?

Recently we have tried to provoke phasic contractions in rat aorta. This can be done by cutting the helical strips from rat aortae at a very flat angle; thereby "tight junctions" between the single cells are better preserved and propogation of action potentials becomes possible. In this preparation we do not see any differential effect of sodium nitroprusside on the phasic or the tonic responses. Our observations speak in favor of the existence of two types of smooth muscle cells rather than of two different mechanisms of activation.

RAT

DR. ZEMPLENYI: I would like to address a question to Dr. Avril Somlyo and Dr. Golenhofen concerning some of the electromechanical properties of vascular smooth muscle. We know that in cardiac muscle, which is embryologically and functionally related to vascular smooth muscle, many of the electrical and mechanical properties are associated with the sodium pump and in particular with the activity of an enzyme, the sodium-potassium linked ATPase. Since ouabain is a potent inhibitor of the latter enzyme I wonder whether ouabain and other cardiac glycosides do also alter the electrical and mechanical properties of vascular smooth muscle as studied by Dr. Somlyo.

Sodium Pump in the Control of Muscle Contraction

DR. AVRIL SOMLYO: I am afraid that ouabain is not as useful a tool in vascular smooth muscle as it is in some other systems. Digitalis glycosides and K-free solutions in general do contract and depolarize isolated smooth muscles or perfused vascular beds. Digitalis seems to have both a direct and an indirect neurogenic effect on perfused preparations. Changes in tissue ion contents such as would be expected from blocking the Na pump have been reported in some smooth muscles and not in others. There is also some evidence that ouabain actually affects Na permeability rather than the Na pump. We have recently used ouabain in our laboratory in studies concerning beta adrenergic hyperpolarization, which is K dependent but is not simply due to an increase in K permeability.

Upon addition of ouabain the pulmonary artery resting membrane potential became very unstable, sometimes polarizing and at other times hyperpolarizing and not reaching a steady level for at least two hours.

DR. RODBARD: Once the contractile mechanism is locked, what is the next step toward releasing the contractile element?

DR. AVRIL SOMLYO: Well, this would actually be at the cross bridge-actin site itself. Calcium releases the inhibitory action of troponin allowing the myosin-ADP.P* to interact with actin sites. As the cross bridges turn over ATP is hydrolyzed. The myosin-ADP.P* actin complex is involved in the force generating step.

DR. GOLENHOFEN: Ouabain affects more the slower components of smooth muscle activity and not the spike potentials. For example, in stomach smooth muscle it suppresses the large "slow waves" in the electrical activity. This indicates that an electrogenic sodium pump may be involved in the control of this type of electrical activity.

Membrane Potential Change and Spike Activity

DR. BURNSTOCK: One central question that worries me is the pharmacomechanical coupling idea. I still have some reservations. Does this happen under normal physiological conditions? I agree that you can demonstrate this kind of coupling under certain abnormal conditions. However, this reservation apart, there seemed to me to be some incongruity in your presentation on two particular points.

Avril Somlyo talked in terms of two smooth muscle types. Dr. Golenhofen talked in terms of two smooth muscle mechanisms, presumably occurring in the same muscle type. I would like to know what evidence there is that there are two distinct smooth muscle types and whether there is some agreement on this? The other point which needs clarification is that, whereas Avril Somlyo made it clear that what she meant by pharmacomechanical coupling was that there were no membrane potential changes preceding contraction and in this she presumably included spikes and any slow events that might occur in the membrane, Dr. Golenhofen talked exclusively in terms of no spike activity. These again are very different concepts because, while I do not find it hard to accept the idea of a graded potential change in smooth muscle, I find it very much harder to accept the idea of no membrane potential change at all. I would like clarification on these two important issues.

I will make a comment on a different subject. This is the question that was raised by Andrew Somlyo concerning whether there is any connection of the endoplasmic reticulum to the outside and

this is, after all, a critical question when considering excitation-contraction coupling. Under our experimental conditions there is penetration of lanthanum specifically in the smooth tubular systems, suggesting that under some circumstances at any rate, there might be some connection with the outside. I am well aware of the sorts of problems involved in looking at lanthanum penetration and the dangers that it might be penetrating into the cell in a non-specific way. But it does seem to me that it is very significant that the lanthanum is located on the inside of these membranes and not on the outside, nor is it localized on any other structure.

DR. AVRIL SOMLYO: I classify smooth muscle into two types, on the basis of their electrical properties. One is spike generating while the other produces a gradient response. There are also morphological differences. Muscles that generate action potentials have a small volume of sarcoplasmic reticulum and display phasic potassium contraction while gradiently responding muscles such as found in the large elastic arteries and I think, the anococcygeal muscle and the trachealis have a larger volume of sarcoplasmic reticulum and undergo tonic contraction without normally genera ing action potentials.

DR. A. P. SOMLYO: As Avril Somlyo's discussion and slides illustrated, there are various graduations between action potential generating and gradedly responsive smooth muscles. Furthermore, as the records clearly show, spike generating smooth muscles are also capable of graded depolarization. In these types of smooth muscle both forms of electromechanical coupling (action potential and graded depolarization) co-exist. This behavior, of course, does not differ greatly from striated twitch muscle fibers, in which graded activation can also be demonstrated, if the regenerative action potential is blocked with tetrodotoxin. The difference between Dr. Golenhofen's terminology and ours is one of emphasis: he treats primarily the different types of activation, while we emphasize the fact that in different types of smooth muscle one or the other form of activation may predominate.

Incidentally, I am very much pleased, Geoff, that you no longer find it difficult to accept graded depolarization as a means of activation in smooth muscle, as you were not quite ready to accept this at the 1968 Congress in Washington.

DR. BURNSTOCK: It seems to me that you are saying that there are not two distinct smooth muscle types.

DR. A. P. SOMLYO: The text books are, indeed, going to talk about different types of smooth muscle. As both your laboratory and ours has noted there can be wide variations in the action potential morphologies (e.g. rate of depolarization, etc.) in differ-

ent types of smooth muscle, ranging from the very fast action potential to the "conventional" spike seen in tissues such as the taenia coli and portal vein (see presentation by Avril V. Somlyo). But, allowing for these minor variations and for different degrees of phasic or tonic behavior, there are essentially two major types of smooth muscle: one that responds to excitation normally by generating action potentials, but depolarizes gradedly, when stimulated with an excitatory agent. Examples of the first type are the smooth muscles of the large elastic arteries, and, according to the literature, the rat anococcygeal and canine trachealis smooth
RAT muscles. The second type of smooth muscle has been studied in much
DOG greater detail, and includes intestinal, uterine and portal vein smooth muscle.

DR. GOLENHOFEN: After our first results with verapamil we distinguished between a "spike activation mechanism" (SAM) and a "spike-free activation mechanism"(SFAM). This classification is very similar to Dr. Somlyo's, it only avoids the term "pharmacomechanical coupling" and also the term "nonelectrical activation". The term SFAM therefore does not include any statement about the open question to what extent electrical processes other than spikes or truly nonelectrical processes participate in this type of activation. Our new concept of P- and T-systems has different foundations. It is based on chemical differences in the calcium activating systems, which are reflected in the fact that they can be selectively blocked - comparable to the differentiation of adrenergic receptors into alpha and beta receptors. There is certainly a great over-lap between this classification and Dr. Somlyo's: the "spike-producing type" of smooth muscle operates mainly with the P-system and the "gradedly responsive type" mainly with the T-system. However, the P-T-concept is certainly more than only another terminology. We can, for example, show that in some
GUINEA PIG spike-producing tissues such as guinea-pig portal vein and preparations from the corpus region of the stomach, a T-system is present in addition to a P-system. Other spike-producing types such as taenia coli of the guinea-pig operate with a P-system only. We can also show that the contracture of taenia coli induced by K+ depolarization, which is spike-free, is mediated by a P-system.

Dr. Burnstock's question about the role of the different mechanisms in normal activation can clearly be answered in terms of the P-T-concept. The whole noradrenaline-induced contraction of guinea-pig aorta is mediated by a T-system, it is not reduced by P-blockade (D 600 up to 10^{-5} moi/l), and similar results can be obtained in the gall bladder and in the fundus of the stomach. In some respects the T-system is more important than the P-system, because the P-system in itself appears as rather homogeneous whereas greater differences exist between the T-systems of different organs, which is of particular importance for clinical applications.

DR. KREYE: I have a question for the Somlyo family, the first part of which regards the sources of calcium for excitation-contraction coupling in vascular smooth muscle. It had long been assumed that the calcium necessary for smooth muscle activation comes from the extracellular environment of the cell. More recently, the possible role of intracellular binding sites for calcium, namely the sarcoplasmic reticulum, has been elucidated. Are you prepared to make any decision as to what extent extracellular calcium and calcium released from intracellular sites contribute to the excitation-contraction coupling of vascular smooth muscle under physiological conditions? I think this is primarily a question on the quantity of calcium stored in intracellular binding sites and on the rate of its release into the cytoplasm. Secondly, it has been shown by your group that mitochondria accumulate divalent cations. Is it likely that mitochondrial calcium plays a role in the activation of vascular smooth muscle? Is the rate of calcium extrusion from mitochondria sufficient to contribute to the excitation-contraction coupling?

Extracellular vs. Intracellular Sites of Ca Release

DR. A. P. SOMLYO: It is probable that both intracellular and extracellular calcium contribute to the activation of smooth muscle, but the relative contributions may vary in different types of smooth muscles and with the experimental conditions. Quantitating the different relative contributions from these two sources would be very difficult, except when all the extracellular calcium has been removed with the aid of calcium chelating agents.

Some of our research is obviously motivated by our interest in determining the role of mitochondria in the physiological regulation of intracellular free calcium levels in smooth muscle. However, it has yet to be shown that the apparent affinity constant and the rate of accumulation of calcium by mitochondria from smooth muscle are of the order of magnitude sufficient to reduce the intracellular calcium of vascular smooth muscle sufficiently rapidly to the levels required for relaxation. We hope to test this question directly with the use of electron probe X-Ray microanalysis that has already enabled us to show the net accumulation of divalent cations by mitochondria in smooth muscle.

DR. RODBARD: I would like to discuss some of our findings with respect to the permeability of the arterial wall.

Permeability of the Arterial Wall

We have obtained results which suggest that the arterial wall is much more permeable than would appear from the apparent tightness of its collagenous, elastic and smooth muscle framework. Two discrete pools

appear to enter into the fluids that bathe the smooth muscle cells of the arterial wall. One of these pools originates in the blood that flows in the vessel lumen. This fluid may pass via the vasa vasorum to supply the metabolic needs of the smooth muscle cells. The second pool lies outside of the vascular adventitia, in the trabeculae. The trabeculae serve as avenues through which the arteries, veins, nerves and lymphatics pass, and in which fat cells and other extracapsular cells are found. This conclusion concerning two-fluid mixing is based on a two-injection technique.

1. Ringer's solution containing ferrocyanide ion was infused via the arteries or veins of various tissues, including skeletal muscle, myocardium, lung, kidney and optic nerve. This introduced the ferrocyanide ion into the vasa vasorum and thereby into the outer medial wall of the vessel.

2. Ringer's solution containing ferric ion was then injected into the parenchyma (not intravascularly). This introduced the ferric ion into the trabecular clefts of the tissue (307, 308).

Wherever ferric ion came into contact with ferrocyanide, the insoluble ferri-ferrocyanide (Prussian blue) marked the site with its characteristic deep blue-black precipitate (309). The appearance of this color in the medial wall indicated that the intralumenal fluid containing the ferrocyanide communicated, via the extravascular fluids that contained the ferric ion, with the intramural fluids of the blood vessel.

We have observed this relationship in the arteries that feed skeletal and myocardial muscle bundles, in the small intrarenal arteries adjacent to the glomeruli, in the pulmonary vessels, and in the veins adjacent to the optic nerve. Tangential cuts through blood vessels exhibit precipitation of Prussian blue on the endothelium and in the outer media (Fig. 32). Since our counterstain is nuclear fast red, the blue coloration can be attributed only to ferri-ferrocyanide precipitate. This technique clearly outlines a basket-work arrangement of the fluid pool that surrounds the smooth muscle cells in the arterial wall (Fig. 33).

Arrangement of Vascular Smooth Muscle Cells

The circumferential arrangement of the smooth muscle cells of the inner layers of the vessel withstands the outward push of the blood pressure and the resulting stretching (tensile) forces in the wall. Near the adventitia the smooth muscle cells are aligned with the long axis of the vessel in a manner that can withstand the tendencies of the vessel to be stretched excessively, to elongate, or to buckle. Similar findings have been obtained in the small arteries and veins of the kidney (Fig. 34).

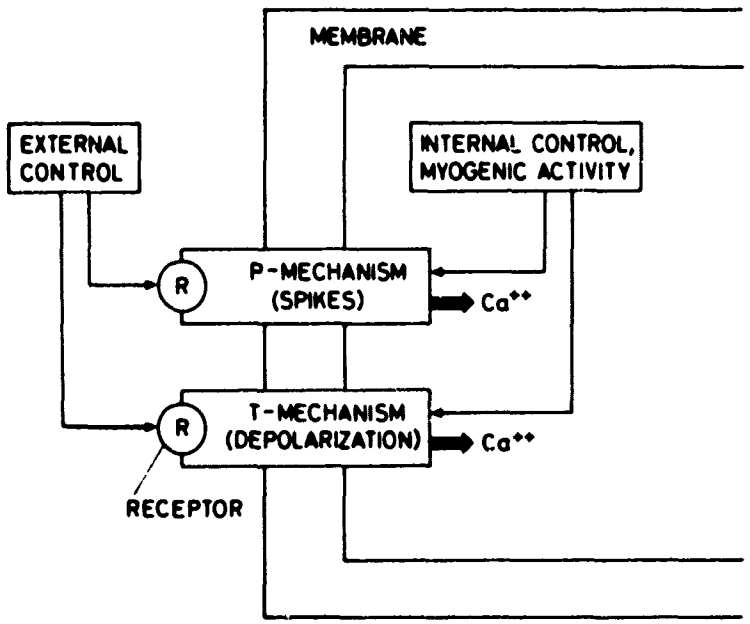


Figure 30: Diagram illustrating the concept of P- and T-systems for calcium activation in smooth muscle.

Organs (Species)	Molarity of Sodium Nitroprusside 10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	Remarks
Aorta (Rat)	(+)	+	++	+++					
Trachea (Guinea pig)		+	++	+++					
Duodenum (Rat)					+	++	++	++	Tachyphylaxis
Portal vein (Rat)				(+)	+	++	+++		Single doses
"				(+)	+	+	+	++	Cumulative doses
Vas deferens (Rat)					–	–	–	++	
Uterus (Rat)					–	–	–	–	
Cardiac auricle (Guinea pig)					–	–	–	–	

Figure 31: Relaxant effects of sodium nitroprusside on different kinds of isolated smooth muscle and cardiac auricle. ((+) = relaxant effect observed in some experiments; + = slight relaxant effect; ++ = intermediate relaxant effect; +++ = total relaxation).

Figure 32: Skeletal Muscle and trabeculae. Ringer's solution containing ferrocyanide ion was perfused through the arterial system. Ringer's containing ferric ion was injected intraparenchymally. Prussian blue was precipitated around the smooth muscle cells of the media of the blood vessel wall (heavy dark lines).

Tissues were stained only with nuclear fast red. Blue color therefore represents only Prussian blue precipitate. Each smooth muscle cell and its nucleus can be identified; the dark lines are the precipitate. x85. 25A Red Vivitar filter.

Figure 33: Tangential section of ophthalmic vein. Blue precipitate is seen as black lines around the smooth muscle cells of the outer media (Bovis). Stain as in Figure 32. × 85

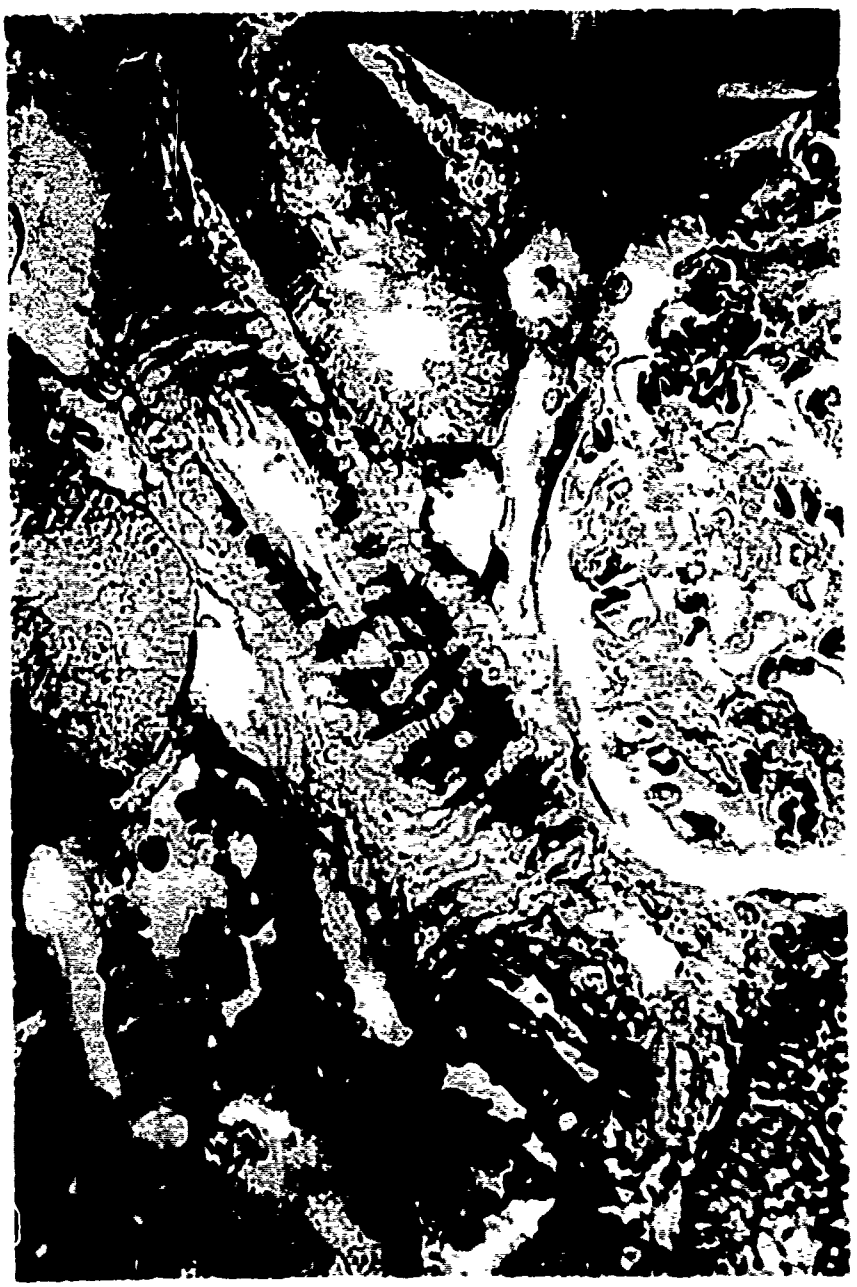


Figure 34: Kidney. Prussian blue around smooth muscle fibers in renal arteriole adjacent to glomerulus. The tangential cut shows radial arrangement of spaces between adjacent smooth muscle cells. Precipitate (dark lines) also appears in the extratubular clefts. x 85.

Under normal circumstances, the direction of flow from the arterial stream may be expected to pass from the lumen through the vasa vasorum to the adventitia. During contraction of smooth muscle, trabecular fluids may be squeezed into the extracellular interstices of the adventitia, thereby reversing the direction of flow. Fluid shifts of this type may transfer information concerning the concentrations of metabolites and other materials in the vessel wall and in the trabeculae, and thereby coordinate and control smooth muscle contractility. Our results indicate a continuous fluid exchange through the apparently tough and tightly organized vessel wall. It is well known that mast cells in the trabecular clefts can release heparin, histamine and serotonin, especially in response to an increased hydration of the tissues (274, 339). Heparin, which originates in the trabeculae, also has a local effect on lipoprotein lipases, apparently at the endothelial level. The possibility that fluids in the trabeculae may enter into the adventitia and thereby come into contact with the smooth muscle cells of the media must therefore be considered. Vasoactive substances derived from mast cells can thereby affect local vasomotion, clotting tendencies and the metabolism of lipids. These considerations must be added to the equation of the known dynamics of the vessel wall, if the potential influences of local factors on vascular regulation are properly to be understood.

Local Chemical Regulation of Vasomotion

I would like also briefly to discuss the sequence of the events that must precede each muscular contraction. If a muscle is to accomplish its function, careful coordination of its contractile activity is necessary. Otherwise, its action and the definite metabolic costs that are incurred, will accomplish nothing. This need for coordination of the contraction process is clearly evident in the uselessness of fibrillation of cardiac or skeletal muscle. Machinery has evolved that ensures proper coordination of the contraction of the units of each muscle. Coordination of contractile energies must also be necessary in smooth muscle fibers.

Electrical and Chemical Coordination of Muscular Contraction

The sequence necessary for such coordination is inadequately appreciated. Several discrete events must transpire before a muscular fiber can contract. The first event is depolarization of the muscle cell membrane. This can be measured with the electrogram. Depolarization may be viewed as equivalent to pressing a "power on" button. The system is thereby armed for contraction, but contraction does not yet take place. Thus, there is no immediate temporal relationship between the action potential and the ultimate mobilization of the tensile forces of the affected contractile elements. This is clearly evident in the myocardium,

in which measurable shortening does not usually begin until relatively long after much of the depolarization process has been completed. Only then does the force of myocardial contraction take effect. This force increases during another long interval, for as much as 300 msec. (310). Similar lags between depolarization and contraction are measurable in skeletal and in smooth muscle.

Depolarization increases the permeability of the membrane of the muscle fiber. Potassium ions then diffuse out of the fibers, while sodium ions diffuse into the fibers. Calcium ions, previously bound to the proteins of the membrane, are also released and these diffuse or possibly are pumped through the transverse tubules toward the Z band. Near the Z band, the calcium ions chelate troponin, a myocardial protein. Troponin functions as a safety-lock which in its unbound state inhibits contraction. Chelation of the troponin by calcium removes this safety lock. Contraction may then take place, provided one more important step is completed. That step is the mechanical application of a pulling force on the muscle fiber.

Evidence for this pulling force may be seen in the phenomenon of latency relaxation (325). Within a millisecond or two after depolarization, the load acts to stretch the muscle fibers. Only after this "latency relaxation" has taken place can the tensile force of the muscle come into motion.

Our studies on muscle pain (311) suggest that the pulling force of the load fragments a portion of a molecule of the contractile equipment. This may be viewed as equivalent to applying a small force to the trigger of a mouse-trap. Only then can the energy of the system, previously stored in the pre-loaded spring, be released to accelerate the tensile forces of the muscle and to produce muscular shortening.

Coordination of the process of contraction therefore requires a sequence of events that includes: (1) depolarization; (2) release of calcium; (3) binding of troponin; and (4) a stretching force that finally triggers the release of the energy of the contractile machinery.

Our findings that the smooth muscle cells of the arterial wall appear to be bathed in fluids that are derived from the blood plasma and from the extra-vascular fluids of the interstitium, suggest that this arrangement may also contribute to the coordination of the contraction of vascular muscle fibers.

DR. ROSS: The experiments I shall present have been conducted in collaboration with Dr. John Glomset and Ms. Beverly Kariya. Dr. Lawrence Harker also collaborated in some. I would like to take

you through the growth of arterial smooth muscle, not in vivo but in vitro; describe the cell culture system we have developed and how we use it with particular emphasis on the growth properties of these cells in culture. I will discuss their requirements for growth and how we can modify their growth response. When we get into a discussion of metabolism this afternoon, we will talk about some of the many things these talented cells can make.

The Growth of Arterial Smooth Muscle Cells in Tissue Culture

It is important to recognize the limitations of the cell culture system in terms of relating anything one observes in vitro to what happens in vivo. Nevertheless, if one can find reproducible circumstances where one can establish criteria for cell performance, in vitro, in terms of cell physiological responses, that are akin to what one observes in vivo, then one begins to have some confidence that the manipulations one can exert under controlled circumstances in vitro may provide some insight into how these cells actually respond in the animal. We are particularly interested in the proliferative response of these cells to growth factors.

First we had to convince ourselves that arterial smooth muscle can be grown in homogeneous culture; second, that the cells retain the phenotypic appearance of smooth muscle cells from the donor; and, third, that they will do many if not all, the things in vitro that they do in vivo.

We hoped to go one step further and gain insight into how we could BETTER understand the response of the cells by developing a chemically defined medium. Then we would be able to add various substances and be able to interpret, hopefully, what we are doing to the cells. I think we are well along this road as you will see from our observations. There are many problems left.

First let us begin by showing how these cells appear in culture and how we get the cultures going. Everything I am going to show you this morning with the exception of a few micrographs deals with the primate Macacca Nemestrina, the pig-tailed monkey. The University of Washington fortunately had a breeding colony so that we are able to breed the monkeys. Thus we know a great deal about their genetics.

MONKEY

In our initial cell culture studies we used a culture medium that has the black magic of serum added to it. We thought it would be proper if we could use serum from the same genus of primate so we could systematically alter the properties of the medium. Thus, in theory, the cells would see in culture at least a large proportion of the same substances they see in vivo.

Requirements for Growth

Our cells are derived from explants of the thoracic aorta of this primate. We remove the inner one-third of the media, which is easily done using a dissecting microscope. These segments are cut into small cubes approximately one millimeter square. Large numbers of these explants are placed in Falcon flasks. Each flask may contain from fifty to one hundred explants in a medium containing up to twenty per cent homologous serum. This medium is a modified form of the Dulbecco-Vogt modification of Eagle's medium containing five, ten or twenty per cent homologous primate serum.

Within two or three days the cells begin to grow out from the explants. Their outgrowth is quite variable. If one looks within a given flask one will find after several days that perhaps 20% of the explants have cells growing out, and after seven days as many as 70% may have cellular outgrowth. No matter what we do, about ten to twenty per cent of the explants never display outgrowth.

It takes perhaps a total of two to three weeks before the growth points will become confluent. The cultures are then trypsinized -- the explants are trypsinized away. We carry them through one trypsinization which represents three to five cell generations. The doubling time for these cells is apprximately 36 hours. We start to use the cells for our studies after the second trypsinization.

We have karyotyped the cells and about 80 to 90 per cent of the cells are diploid and 10 to 20 per cent of them are tetraploid. The cells will generally survive for fifty cell generations before we run into difficulties and they begin to die. Therefore we must periodically go through the process of setting up new cultures. All of the cells are derived from one to two year old monkeys.

All of the experiments I am going to describe were performed with smooth muscle cells obtained between the second and seventh trypsinization. We do not use the cells after that because they begin to react oddly in fashions we don't completely understand. The cells demonstrate changes in culture that are not reproducible after that point in time, therefore we don't use them.

To prepare the cells for electron microscopy we fix them in situ, embed them in situ, and invert beam capsules over them filled with resin and polymerize the whole thing. As soon as we take them out of the oven we pop the capsules out of the dish so that the cells are on the flat end of each capsule. Then we can easily stain them with a number of stains, visualize them in the dissecting scope, pick out the area we want and them trim the block so we obtain a section tangential to the surface of the dish. We can turn the block ninety degrees and obtain a transverse section. As

you will see in the light micrographs the cells have interesting growth properties in that they grow in hills and valleys in culture. The hills may have as many as fifteen cell layers and between each layer there are extra-cellular products. In the valleys there may be no cells or there may be a single cell. Fig.35 shows these cells containing dense bodies between bundles of myofilaments in low magnification. In Fig.36 one of these cells is shown in higher magnification illustrating that the mitochondria are often bifurcated or trifurcated.

Ultra structural Characteristics

The bundles of myofilaments can be seen to run in numerous directions in culture. The cells take different shapes depending on what is present in the medium. If one should have serum in the medium as the cells grow out these cells become rather long and ribbon-shaped. In the presence of no serum or one per cent serum, when they reach stationary growth the cells are long and irregularly shaped.

The filaments described by Andrew Somlyo can be seen together with microtubules (mt) as well as aggregates of ribosomes. At higher magnification one can see sixty angstrom filaments that Somlyo described. In some regions all three sizes of filaments (60 angstrom, 100 angstrom, 150 angstrom) are visible. These cells also make junctional contacts in the forms of gap junctions. They are not "tight junctions" because there is a twenty angstrom gap between the external leaflets of the two unit membranes. In some tissues in vivo these gap junctions have been shown to be associated with electrotonic couplings between the cells. We have no evidence for this in our in vitro system, but there may be evidence in other systems.

In addition, I would point our some other material outside the cells that appears morphologically like basement membrane. Therefore you can see that phenotypically, the cells retain the appearance of well developed, differentiated smooth muscle cells in culture. If one examines them during the very early stages of growth (Wight, who works in our laboratory, has done this with smooth muscle cells from the pigeon) one can see that smooth muscle cells from the monkey already contain numerous myofilaments. There are areas in their cytoplasm that don't contain filaments and as the cells go through logarithmic growth, fairly rapid changes occur in their phenotypic appearance. It takes a couple of days in culture before they appear like fully developed smooth muscle. We wanted to begin to look at factors that influence the growth of these cells in culture. To do this we modified a system that Robert Holley has been using in

BIRD
MONKEY

Modifiers of Cell Growth

Figure 35: This micrograph demonstrates a cross-sectional view of a 6 week culture of smooth muscle cells. The bottommost cell rests on a carbon film. Between three of the layers of cells, microfibrils similar in appearance to elastic fiber microfibrils can be seen. Material reminiscent of basement membrane (arrows) is also visible around many of the cells. Mag x 18,000

Figure 36: Electron micrograph demonstrating the typical appearance of a macaque aortic medial smooth muscle cell after several generations of growth in culture. The cells were fixed in situ, embedded, and sectioned parallel to the plane of the surface of the culture dish so that an en face view of the cell is seen. The cytoplasm is abundant with myofilaments and dense bodies (arrow). Microtubules (mt) and mitochondria (m) too are visible.

his laboratory at the Salk Institute. In essence what he did was to take fibroblasts in culture and put them in a medium that provided minimal growth. After the cells had reached stationary phase after minimal growth, he examined the effects of adding various serum factors, to test the potential proliferative properties of these factors in the cultures.

In the design of our studies, the cells are placed in a particular medium containing 1% pooled primate serum. They are then grown to stationary phase. At this point, if one finds a proper additive the cells will return to logarithmic growth. This is a very sensitive method to monitor the effects of the addition of various substances or factors which may eventually lead us, we hope, to the development of a defined medium. In the case of primate smooth muscle cells we begin with approximately 10^5 cells in a 35 mm petri dish. The cells are grown in medium containing 1% pooled homologous monkey serum. One usually observes a drop in cell number in the first 24 hours, due to plating efficiency. Then they grow logarithmically for from two to four days and then become essentially stationary.

At this point, we set up a growth experiment with perhaps a hundred dishes. Each dish will contain an essentially identical number of cells ($\pm$ 5%) to which the various factors to be tested can be added. Proper instruments to plate out the cells permit this accuracy.

One of four groups of cells were grown in zero per cent serum for the entire experiment. Another group of cells were grown in 10% serum initially. Fig. 37 shows that they grow logarithmically quite a while before they become stationary. A third group of cells grown in 4% serum failed to reach the same level as those grown in 10% serum when they became stationary. The fourth group was grown in 1% serum until they became stationary.

We then changed the medium to 10% serum and at that point the cells took off again (Fig. 37). It has been known for many years that there are many factors present in serum that are stimulatory to the growth of these cells, or if you wish to look at it from another view, that are able to control the growth of cells.

One of the things we have done was to fractionate serum constituents in a way that has not been done for fibroblasts. We separated the lipoproteins by differentially floating them out and then fractionated the LDL from the HDL in a potassium bromide gradient solution to a maximum density of 1.25 grams per ml. at the bottom of this tube there is a non lipoprotein containing fraction of serum constituents that contains the remaining serum proteins and fatty acids. This experiment contained two control groups,

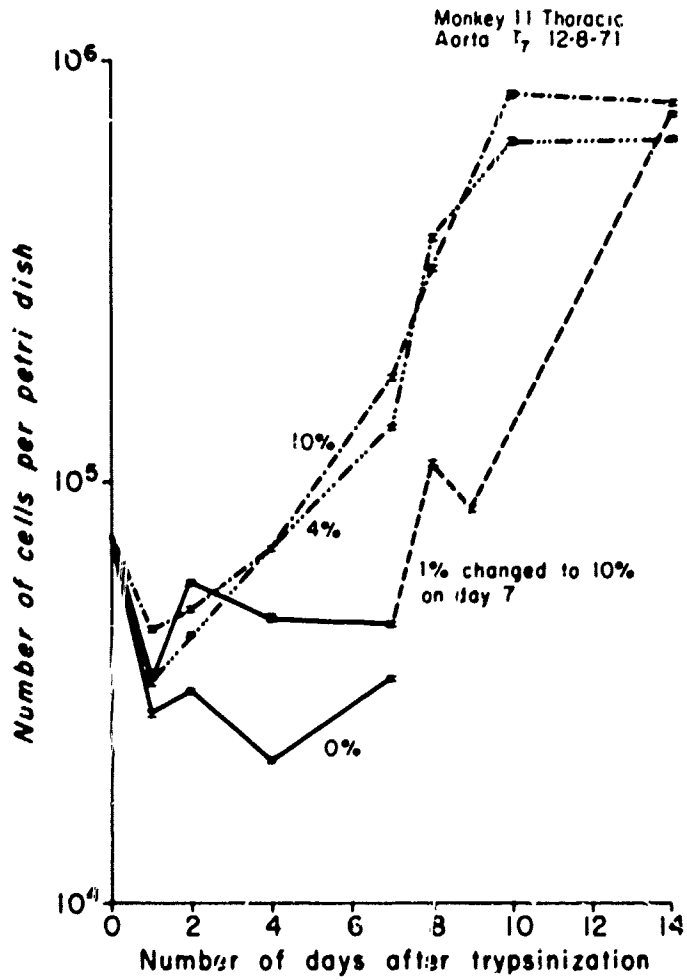


Figure 37: Replication of smooth muscle cells in tissue culture in various concentrations of serum

zero per cent serum and 5% serum which grows logarithmically for this period of time.

Effects of Lipoproteins and Serum Concentrations in Medium

We found, interestingly enough, (Fig.38) that a number of serum constituents were stimulatory to growth. In particular, we found that when we added low density lipoproteins (on a 5% cholesterol equivalent basis) together with the 1.25 bottom (on the same basis) that we obtain growth equivalent to that seen in 5% whole serum. In contrast, high density lipoproteins (HDL) were not stimulatory when compared with LDL. The 1.25 bottom is also stimulatory but again not to the same degree as LDL. 10% LDL has the same effect as 5% LDL, however albumin will not act as a carrier for the lipoprotein. There is something in the 1.25 bottom that we derive from the serum that is important and it is not albumin.

When the cells are grown in 1% serum they are very large and are irregularly shaped. If one looks at these cells with the electron microscope they have a fair amount of rough endoplasmic reticulum and a number of autophagic vacules. They contain a full complement of myofilaments that are visible if one sections the cells in the appropriate plane.

When the cells are grown in zero per cent serum they appear similar to those grown in 1% serum except that there appears to be a perinuclear halo around them when viewed in phase optics. When the cells are grown in medium containing 5% serum, they grow in the form of hills and valleys. The hills may contain as many as ten or fifteen cell layers. In the presence of 5% serum, the cells contain an extensive rough endoplasmic reticulum, bundles of myofilaments, mitochondria and autophagic vacules or secondary lysosomes.

When the cells are grown in 5% LDL plus 1.25 bottom, they contain lipid droplets of which we saw very few in the other experiments. The lipid droplets are usually associated with aggregates of glycogen. The HDL showed one marked change that the LDL did not show. This is the presence of many large autophagic vacules and secondary lysosomes that form in the presence of HDL that we have not seen to the same extent as when the cells are grown in the presence of LDL. This is interesting and may be potentially important, because in long term studies with HDL the cells begin to appear sick. Even though it appears that HDL may be stimulatory to growth it may also have a secondary effect on the cells that is deleterious. Interestingly enough there are many one hundred angstrom filaments in the cells that we do not believe to be contractile. When the cells are grown in the presence of 1.25 bottom alone they contain relatively few lipid droplets but demonstrate a fairly extensive development of their rough endoplasmic reticulum.

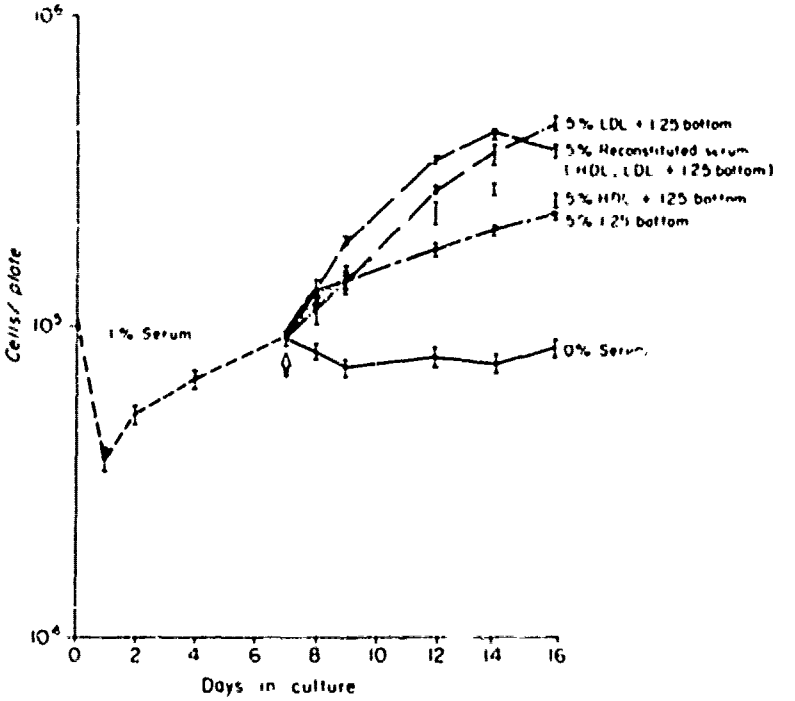


Figure 38: Response of arterial smooth muscle to serum fractions. Equal numbers (10^5) of smooth muscle cells were added to a large series of petri dishes and incubated in a modified Dulbecco Vogt modification of Eagle's medium containing 1 percent serum pooled from several Macaca nemestrina. After 7 days (arrow), the dishes were separated into five groups to be further incubated. One group was incubated in serum-free medium. The remaining groups were incubated in media containing: dialyzed protein of density greater than 1.25 g/ml from the equivalent of 5 percent serum, this protein fraction contained very little high density lipoprotein (HDL) or low density lipoprotein (LDL); proteins of density greater than 1.25 g/ml plus HDL (154 nmole of cholesterol per milliliter of medium); proteins of density greater than 1.25 g/ml plus LDL (154 nmole per milliliter of medium; and reconstituted serum containing proteins of density greater than 1.25 g/ml plus HDL (77 nmole of HDL cholesterol per milliliter of medium) plus LDL (77 nmole of LDL cholesterol per milliliter of medium). The pooled primate serum used as a source of lipoprotein in these experiments contained 154 nmole of lipoprotein in 5 percent whole serum. This experiment demonstrates that both serum lipoprotein and proteins of density greater than 1.25 g/ml stimulate smooth muscle cell proliferation in vitro and that LDL is at least as effective as a combination of LDL and HDL. Thus, these observations support the concept that endothelial injury in vivo could promote smooth muscle cell proliferation by increasing the concentration of plasma proteins in the extracellular fluid of the vessel wall and points to the potential importance of plasma LDL in this response. Vertical bars represent standard error of the mean.

Thus, in summary, with LDL we see the largest accumulation of lipid droplets and glycogen, together with small numbers of autophagic vacules. With HDL there are large accumulations of autophagic vacules and secondary lysosomes and relatively little lipid accumulation, whereas with the 1.25 bottom, the cells do not look too terribly different from those grown in the presence of 1% serum.

Importance of Serum Source to Growth

Since our monkey colony is limited and the number of monkeys available to us for bleeding is limited, we found that we could only tap small numbers of animals for the pools of serum we needed for our experiments. Hence we decided to become modern and use the technique of plasmapheresis to obtain a larger amount of serum. When we made serum from this monkey plasma, by addition of calcium by dialysis against Ringer's solution with calcium or by adding calcium back directly and then grew the cells in plasma-derived serum versus whole blood serum, we saw a number of interesting results. We were able consistently to observe that plasma-derived serum would not support the growth of our smooth muscle cells to the extent that whole blood serum would.

CHICKEN

Balk at Rockefeller University (20) had published observations of chicken fibroblasts grown in the presence of chicken serum vs. chicken plasma derived serum. In these studies he showed that the chicken fibroblasts would not grow in plasma-derived serum. He was more interested in the role of calcium in his plasma-serum and so he emphasized that point instead of the fact that his cells did not do well in plasma at all. But if one examines his growth curves he observed growth differences similar to those observed by us.

Repeatedly and characteristically we found that when the cells were grown in the presence of 5% plasma derived serum they were irregular in shape as contrasted with their appearance in 5% whole blood serum. In whole blood serum, as Dr. Burnstock has already described, they are long and ribbon or spindle shaped, but in plasma serum they are not.

Since plasma serum was not as good as whole blood serum for growth, we decided to try and determine which constituents were important. So we performed a mix-match experiment in which we mixed low density lipoproteins from plasma serum with carrier proteins from blood serum and with the carrier proteins from plasma serum and vice versa (Fig.39). It did not matter where the lipoproteins were from as long as the carrier protein was derived from whole blood serum, the cells grew very well. When the carrier protein was derived from plasma serum the cells did not proliferate at all. And as it turns out even the cells in the 1.25 bottom did better if the 1.25 bottom came from blood serum rather than the plasma serum.

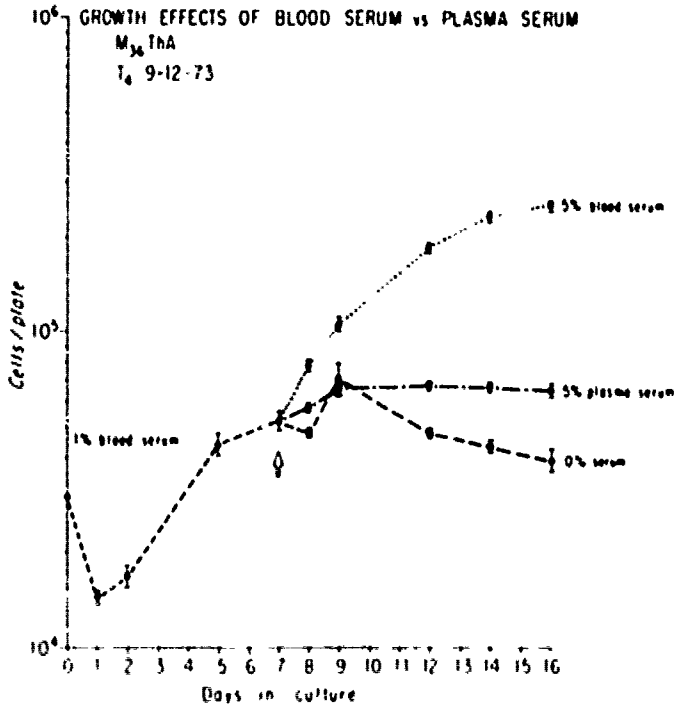


Figure 38: Response of arterial smooth muscle in cell culture to blood-serum vs. plasma-serum. Equal numbers ($3x10^4$) SMC were added to a large series of petri dishes and incubated in medium containing 1% pooled serum from several Macaca nemestrina. After seven days (arrow) the dishes were separated into 3 groups. One group was incubated in serum-free medium. The other two groups were incubated in medium containing either 5% dialyzed whole blood serum or 5% dialyzed plasma serum. This experiment demonstrates that 5% dialyzed plasma serum had little to no proliferative effect when compared with dialyzed blood serum.

Platelet Factor

Examining the potential source of the carrier protein derived from blood serum to determine what was absent in the carrier protein from plasma serum led us to look at platelets. We decided to examine platelets because of a number of other observations that were seemingly unrelated, two of which we were able to find in the literature. One of these were observations made by Hirsch (147) of the Rockefeller University in 1960, which he published in the Journal of Experimental Medicine. He demonstrated that plasma serum lacked a cytotoxic factor effective against several gram positive organisms that was present in blood serum. He found it was derived from platelets. In the same journal Wexler et al. (415) looked at factors chemotactic for cells that were absent in plasma serum but present in blood serum. They demonstrated that this factor was also platelet derived.

We took the plasma and recombined the platelets (on a quantitative basis equivalent to the number of platelets that would have been present in 5% serum) together with calcium to pull down the fibrinogen and produced a platelet rich plasma serum. We then exposed the cells to several different media, all derived from the same pool of blood. These included 5% blood serum and zero per cent serum as our two controls. The experimental groups consisted of (1) 5% platelet free plasma serum which was recalcified by dialysis against Ringer's with appropriate calcium, and (2) 5% platelet rich plasma serum, as noted above. Under these circumstances, in the presence of 5% platelet-rich plasma serum the cells grew logarithmically in a fashion similar to that seen with 5% blood serum. Most exciting of all, we were able to completely restore the proliferative activity of plasma serum by having platelets present at the time we made the serum (Fig.40).

We then asked whether the proliferative activity was due to the platelet release phenomenon. We took an equivalent number of platelets present in 5% serum and combined them with purified thrombin obtained from a colleague, Dr. Earl Davie, thereby aggregating the platelets so that they would release their granule constituents. We then spun down the platelets at high speed and, on a quantitative basis, added the supernatant back to the plasma serum. This permitted us to ask whether the proliferative cofactor(s) was present in the platelet granules or not. We were able to restore approximately seventy per cent of the proliferative activity to plasma serum by adding supernatant derived from the platelet release reaction.

We are excited about these observations from several points of view. One is the obvious relation to atherosclerosis. It is

conceivable that in the genesis of this lesion that if there is endothelial injury and if platelets adhere to sites of injury and locally interact with vessel wall constituents they may potentially be able to release their factors locally at the site of injury which could then interact with plasma constituents such as LDL causing the smooth muscle cells to proliferate.

Second, there may be a fundamental biological principle. It is clear from numerous experiments in many laboratories that investigators have been looking for serum factors responsible for cell proliferation in culture. If we can reproduce the activity of whole serum with low density lipoproteins and a given platelet factor that we can isolate and identify, then we will be on the verge of understanding not only the factors that control proliferation of these cells in culture but we will be able to control this growth in known ways. We think this reaction is probably not too different from the response one sees in a healing wound. In this situation, many differerent serum constituents are released into the wound followed by proliferation of fibroblasts. These factors we have been examining may also be responsible for not only the proliferation of these cells, but for their migratory activity as well. We hope to eventually develop a defined medium in which we can grow the cells so that we can begin to examine some of their other properties in culture.

DR. ROBERTSON: I would like to make a short comment on Dr. Ross's statement that platelets are required for the growth of endothelial cells in culture. In fact, it is possible to culture endothelial cells without adding platelets. We have been able to grow smooth muscle cells without adding platelets. I wonder if he has done any studies regarding growth factors in platelets. Could serotonin be one of the factors? Furthermore, is it possible that other factors like histamines or any of the hormones that platelets are able to carry so well may be indispensable for the survival of these smooth muscle cells in culture?

Platelet Factors as Growth Stimulators of Smooth Muscle

DR. ROSS: Have I missed something in the literature you published in relation to platelets, because I don't remember that? At any rate, to answer your question, initial experiments suggest that our platelet factors are not dialysable, and this would remove from consideration a 5-hydroxytryptamine as well as histamine as being the factor that we are studying. This does not rule out the possibility that either one of these substances may be important in proliferative response but they are not the key to the in vitro growth response. It appears to be a protein, or proteins, of reasonably high molecular weight. We are now attempting chromatographic separ-

ation and purification. May I also clarify my reference to "plasma serum". What I mean is recalcified plasma. It is serum derived from plasma instead of the serum ordinarily derived from whole blood.

DR. DAOUD: In the arteriosclerotic lesion we usually find in addition to smooth muscle cells some cells which are much less differentiated. Some of them look like fibroblasts, some look like primitive cells. I wonder if the speakers can talk about the origins of these cells; whether they derive from smooth muscle cells or have some other origin. In our system which is a bit different from Dr. Ross's system, we start with mature smooth muscle cells. About four days after labelling a culture, an electron microscopic autoradiograph shows the labelled cells and also shows that the majority of these cells synthesize DNA and are of the type listed as differentiated. At about four days of growth the majority of cells have no filaments and some of them look like fibroblasts. Later on these cells have tremendous amounts of endoplasmic reticulum and some of them have partial basal membrane. About two weeks later you start to see the filaments and by the end of the study the cells have quite a bit of myofilament. Would anyone care to comment about the origin of these immature cells?

DR. BURNSTOCK: We have been looking very carefully at the structure and function of cultured smooth muscle over several years now and have come to the conclusion that first of all it matters a great deal whether you are using undifferentiated cells or differentiated cells in your experiments.

Differentiation and Dedifferentiation of Cells in Culture. Trophic Effects of Nerves

The first thing that enzymatically-separated undifferentiated muscle cells do in culture is to divide; then they go on dividing until they form a confluence. They are then able to differentiate; they then begin to form muscle effector bundles with nexuses between them. In differentiating cells there are extense areas with granular endoplasmic reticulum, while in dedifferentiating cells, there are often many lysosomes, clumping of myofilaments.

Now if you grow differentiated cells in culture the first thing they do is dedifferentiate. Sometimes it takes eleven days or more, depending on the system. Once they dedifferentiate then they divide and then they go through the whole sequence of events described earlier for undifferentiated cells. We think that we have evidence that this is the pattern that goes on during normal development, in wound-healing and in transplants.

Incidentally, it is very fascinating that if you grow these cultures with nerves, the nerves delay the dedifferentiation process, while at a later stage they accelerate the formation of muscle

bundles and nexuses. I think that these findings mean that one has to take into consideration the influence of nerves on smooth muscle morphogenesis.

DR. ROSS: I think one has to be very cautious in using terms like "modulation" and "differentiation" or using criteria like rough endoplasmic reticulum as a sign of differentiation. Most cells, given the proper opportunity and the environment, can do most things. I think that what we are really talking about are phenotypic characteristics that have been described and physiologic characteristics that are well known. What we have to try to do is to relate these to what we see. It is also important to emphasize that a cell culture system and an explant system are totally different and really cannot be compared.

DR. A. P. SOMLYO: The Seattle group have shown that in media of the mature pigeon aorta there are two kinds of cells. In mammals, PIGEON however, there are only smooth muscle cells. Do you recognize two major cell types in culture, or do we still have to do the experiments?

DR. ROSS: All avian arteries have two cell types, fibroblasts and smooth muscle cells. Mammalian systems only have smooth muscle cells in the media. Benditt and his group have demonstrated that point in the chicken. Wight studied the development of avian arterial cells in culture and demonstrated both fibroblasts and smooth muscle cells. That is why one has to be cautious in using avian systems because they clearly are different from the mammalian. BIRD

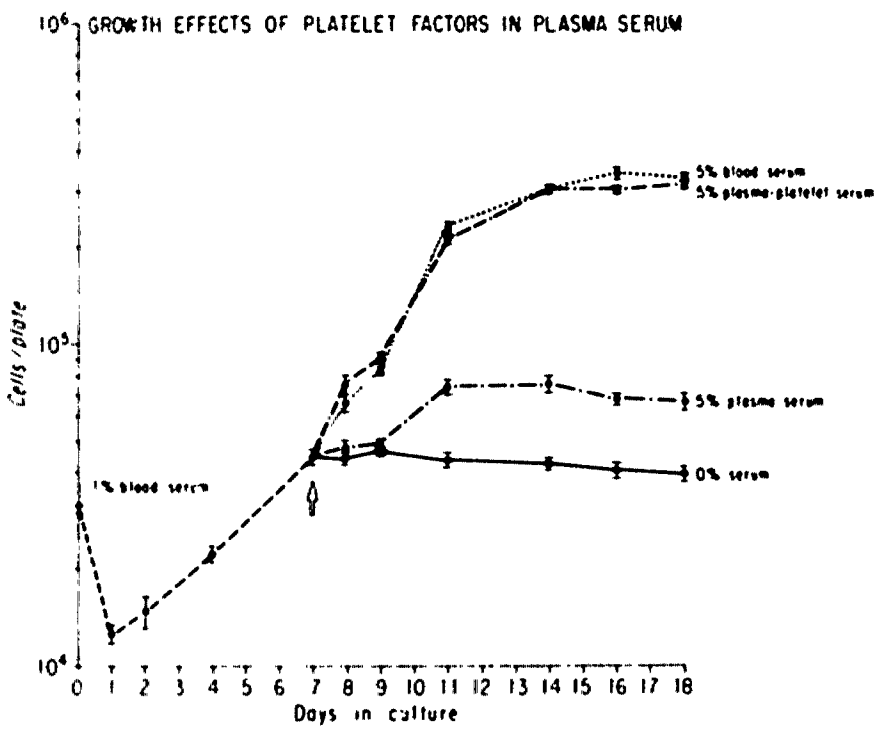


Figure 40: Response of arterial smooth muscle to platelet factors in plasma serum. Equal numbers ($3x10^4$) of primate arterial smooth muscle cells were added to a large series of 35 mm petri dishes and incubated in medium containing 1% serum pooled from several Macaca nemestrina. After 7 days (arrow) the dishes were separated into four groups. One group was incubated in serum-free medium. The remaining groups were incubated in medium containing. 5% dialyzed serum from whole blood containing 3.95×10^8 platelets/ml; 5% dialyzed plasma serum which had been exposed during the process of recalcification and serum formation to an equivalent number of platelets, derived from the same pool of blood; 5% dialyzed plasma serum in which no platelets were present during the process of serum formation. This experiment demonstrates that 5% dialyzed plasma serum has little or no proliferative effect unless allowed to clot in the presence of platelets.

Chapter 2
METABOLIC CHARACTERISTICS OF SMOOTH MUSCLE INCLUDING SYNTHETIC AND SECRETORY FUNCTIONS AND THE CHEMISTRY OF CONTRACTION

LIPID METABOLISM OF THE ARTERIAL SMOOTH MUSCLE CELLS

Dr. Yechezkel Stein and Dr. Olga Stein

I would like to confine my talk to certain aspects of lipid metabolism and shall try to emphasize the correlation of structure and function. The subject of lipid metabolism will be supplemented further by Drs. Adams, Bowyer, Day, Zemplenyi, Kritchevsky and others. Other subjects, such as synthesis and secretion of collagen and elastin, will be covered by Drs. Ross and Robert, and Dr. Ruegg will tell us about the contractile protein of the smooth muscle cells.

Table II lists a number of approaches that have been utilized in the investigation of lipid metabolism of arterial smooth muscle cell. Studies have been made on the whole artery in vivo, on an isolated perfused artery and on preparations of tissue slices and homogenates as well as isolated intact cells and smooth muscle cells in culture.

An enormous body of results has accumulated from these studies, but it is still very difficult to bring these data to a common denominator. To give a few examples:

1. The intact artery in vivo is subjected to control by hormones, neurotransmitters, mechanical factors, etc., which are lacking in all the other approaches.

2. The intact artery, slices or homogenates contain not only smooth muscle cells, but also endothelium and extracellular material.

3. Isolated cells are mostly devoid of extracellular components and are thereby altered by treatment with proteolytic enzymes.

4. Cells in culture, derived from adult arteries undergo changes when released from normal inhibitory constraints and thus certain processes not expressed in the whole animal might become prominent.

TABLE II

LIPID METABOLISM OF ARTERIAL SMOOTH MUSCLE CELLS HAS BEEN STUDIED IN

1. THE WHOLE ARTERY IN VIVO
2. ISOLATED PERFUSED ARTERY
3. TISSUE SLICES AND HOMOGENATES
4. ISOLATED SMC
5. PURE CULTURES OF SMC

TABLE III

Incorporation of Acetate-1-^{14}C into Long-Chain Fatty Acids by Cellular Fractions from Normal Rabbit Aorta

Fraction	Acetate incorporation
Mitochondria	71 2
Microsomes	12 3
Supernatant fraction	16 4

I shall try to cover two main aspects of lipid metabolism, i.e., synthesis and degradation and shall attempt to compare results obtained by the different approaches.

Lipid Synthesis by Arterial Tissue

Almost a quarter of a century has already passed from the time when Chernick et al (64) first demonstrated the incorporation of labeled acetate into fatty acids in a homogenate of rat aorta. It took many more years and much refinement of techniques to analyze more closely the meaning of this finding. The main question centered around the problems: is the entire fatty acid synthesized de novo or is acetate used only or mainly for chain elongation of pre-existing fatty acids? At first it seemed that controversial data were obtained, but later some of the differences were resolved by being attributable to differences in methodology. Thus Wbereat (420) has shown that in mitochondria isolated from rabbit aortic homogenates the main reaction is that of chain elongation (Table III). However, de novo synthesis through the malonyl CoA pathway has been demonstrated in the high speed supernatant of squirrel monkey aortic homogenates by Howard (152). Using perfused pigeon aorta St. Clair et al (376) were able to show that in the intact aortic tissue, palmitic acid can be derived from de novo synthesis while stearic and oleic acids were derived primarily by chain elongation and desaturation.

Capacity of Arterial Smooth Muscle Cells to Synthesize Lipid

I would like to turn next to the synthesis of complex lipids and remind all of us of the pioneering work in this field done by Zilversmit (431, 432) who was the first to demonstrate synthesis of phospholipids in the intact aortic wall in vivo using ^{32}P as substrate. Later on, segments of both normal and atherosclerotic aortae were shown to utilize labeled acetate, ^{32}P and choline for phospholipid synthesis (236). I shall avoid any further mention of atherosclerosis, in accordance with the directives received from the Chairman to refrain from atherosclerosis and to remain within the realm of the metabolic function of the <u>normal</u> smooth muscle cell.

The study of complex lipid formation in aortae of various species has been a very fruitful one, as evidenced by the vast body of information from which I would like to draw just a few examples. One question asked was what kind of complex lipid is synthesized predominantly by the normal aortic smooth muscle cell when fatty acids are presented in the form of a fatty acid-albumin complex at physiological concentration. Studies with aortic slices from different mammals have shown that the main lipid class synthesized from fatty acid are phospholipids (Table IV). However,

TABLE IV

Fatty acid incorporated into lipids of aortic slices of rabbits, dogs, rats and baboons

Species	Fatty acid incorporated (mμmoles/100 mg dry, defatted tissue)	
	Neutral lipids	Phospholipids
	Mean ± S. E.	
Rabbit (5)	14.6 ± 5.0	51.9 ± 8.4
Dog (5)	12.4 ± 4.3	17.4 ± 2.9
Rat (4)	15.1 ± 2.8	20.4 ± 3.8
Baboon (2)	5 7	11.1

Stein and Stein, 1962. J. Atheroscl. Res., 2: 400

TABLE V

INCORPORATION OF 1-^{14}C LINOLEIC ACID INTO NEUTRAL LIPID FRACTIONS OF AORTIC SLICES

SPECIES	FATTY ACID INCORPORATED INTO NEUTRAL LIPIDS n MOLES 100 mg DRY WT	DISTRIBUTION OF RADIOACTIVITY %		
		CHOLESTEROL ESTER	TRIGLYCERIDE	DIGLYCERIDE
RABBIT	14. 6	10	78	12
RAT	15 1	4	87	9
DOG	12 4	2	94	4
BABOON	5. 5	5	91	4

ADAPTED FROM STEIN & STEIN 1962

fatty acid is incorporated also into triglyceride and to a small extent into cholesterol ester (385) (Table V). It seems of interest to try to compare the contribution of fatty acid derived from de novo synthesis to that supplied in the form of preformed fatty acids towards complex lipid synthesis. Howard (152) has made some calculations using his data on acetate incorporation into fatty acids and our data on fatty acid incorporation into aortic slices and has come to the conclusion that about a half to one-fifth of the fatty acid used for esterification could be derived from de novo synthesis. His calculations were derived from the contribution of each subcellular fraction, which has been incubated at optimal concentrations of acetate and other co-factors and extrapolated to the whole tissue. However, in the study of St. Clair et al (376) in which acetate was added during perfusion of an intact pigeon aorta - the rate of incorporation seems to be one-fifth to one-tenth of that obtained by Howard (152) in isolated subcellular fractions. I bring up this point to further illustrate the problems created by the different methodologies. Even though open to criticism, study of enzymic pathways is still best accomplished in cell free preparations. Phospholipid and neutral lipid formation was shown to proceed through the α-glycerophosphate pathway (Table VI). The possibility that an alternative pathway of phospholipid synthesis may be operative in the aortic tissue was first considered on the basis of results obtained with aortic slices (385). In the absence of glucose, dog aortic slices incorporated only minimal amounts of (1-^{14}C)-linoleic acid into neutral lipids, whereas the phospholipids became labeled quite extensively. With the use of homogenates it was found that α-glycerophosphate was indispensable for triglyceride but not for phospholipid synthesis. Since most of the labeled phospholipid formed, in the absence of added precursor, was lecithin, it was suspected that endogenous lysolecithin might serve as precursor. Indeed, when lysolecithin was used as acceptor instead of α-glycerophosphate labeled fatty acid became incorporated into lecithin by an acylation reaction, which was ATP and CoA dependent (Table VII). The presence of this pathway in an intact dog carotid artery could be further confirmed using labeled lysolecithin as substrate (88). Another precursor of phospholipids, which labels lecithin predominantly, is choline. Owing to the very high specific activity of this precursor, it became possible to localize the site of phospholipid synthesis to aortic smooth muscle cells, using radioautography at light and electron microscope level (379).

Another controversial problem in the study of lipid metabolism in the aorta was the question of cholesterol synthesis. Siperstein et al (344) were among the first to demonstrate that in the rabbit and chicken aorta labeled acetate is incor-

Aortic Synthesis of Cholesterol

TABLE VI

Incorporation of [1-^{14}C] linoleic acid into lipids of aortic homogenates with α-glycerophosphate as fatty acid acceptor

Conditions of incubation	Fatty acid incorporated (μmoles/mg protein)			
	Dog		Rabbit	
	Neutral lipids	Phospholipids	Neutral lipids	Phospholipids
Complete system	500	900	550	1200
ATP omitted	30	130	10	110
CoA omitted	40	220	10	130

Stein et al., Biochim Biophys. Acta, 1962, 70, 33.

TABLE VII

Incorporation of [1-^{14}C] linoleic acid into lipids of aortic homogenates, with lysolecithin as fatty acid acceptor

Conditions of incubation	Fatty acid incorporated (μmoles/mg protein)			
	Dog		Rabbit	
	Neutral lipids	Phospholipids	Neutral lipids	Phospholipids
Complete system with lysolecithin	40	3000	20	4700
ATP omitted	10	230	10	300
CoA omitted	30	470	10	530

Stein et al. Biochim Biophys Acta, 1962, 70, 33

TABLE VIII

Species	Age	Sphingomyelin μg P/mg DNA	Sphingomyelin hydrolyzed μmoles/mg DNA/hr
Rat	1 month	4.3 ± 0.3	0.32 ± 0.04
	18 - 24 months	13.1 ± 0.8	0.41 ± 0.03
Rabbit	1 month	8.9 ± 0.5	0.09 ± 0.01
	18 - 24 months	26.9 ± 1.4	0.15 ± 0.03
Human	0 - 19 years	12.5 ± 1.0	0.13 ± 0.01
	70 - 97 years	62.7 ± 3.3	0.05 ± 0.01

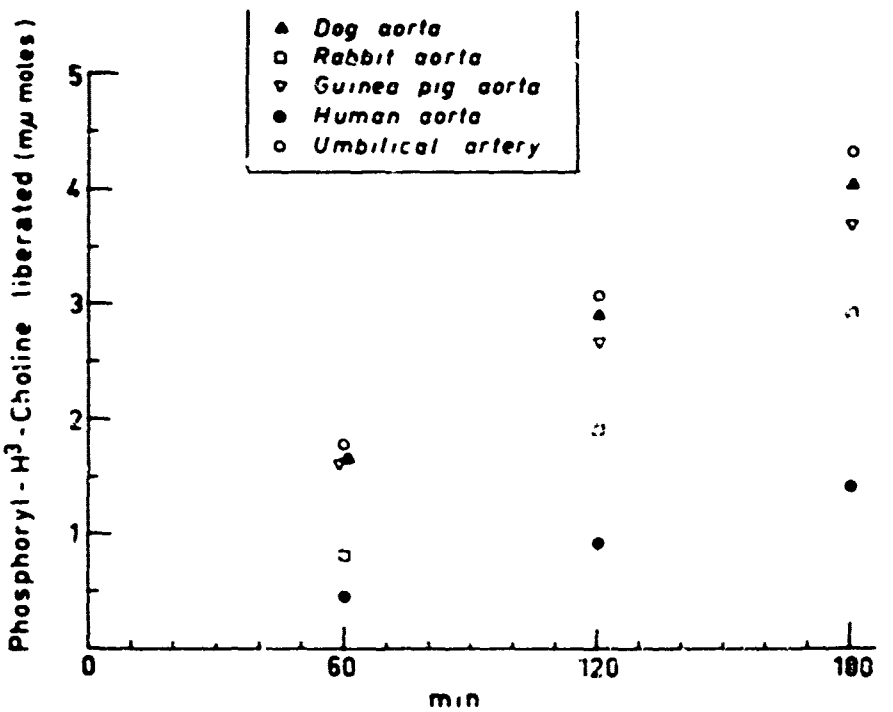


Figure 41: Hydrolysis of Sphingomyelin with time of incubation. From Rachmilewitz et al 1967.

porated into digitonine precipitable material which at that time was taken as evidence for cholesterol synthesis. However, Schwenk and Werthessen (337) showed that further purification of the digitonine precipitable labeled material through a dibromide step resulted in the loss of most of the radioactivity. They coined a term of the "high counting companions" of cholesterol which were further analyzed by St. Clair et al (377) in perfused arteries using ^{14}C mevalonate as substrate. They were able to show that the labeled peak which comigrated with authentic cholesterol on alumina columns contained only a small fraction of labeled material which comigrated with authentic cholesterol on florisil columns. In addition, when the so called "cholesterol" peak from the alumina column was subjected to bromination only 1.6% of the label was recovered as the dibromide, i.e. authentic cholesterol. These findings seem to indicate that the synthetic capacity of the normal aortic smooth muscle cell for cholesterol is at best quite low.

Changes in Smooth Muscle Membranes with Age

RAT
RABBIT
HUMAN

The smooth muscle cells of the aorta do not have a fixed lipid composition throughout their life span and I would like to focus briefly on those changes which occur in the normal aortic cells with age. One such change is the rise of PL/DNA ratio, which is quite steep early in life and continues at different rates in various species. In the rat, rabbit and in the human this increase of cellular phospholipid is accounted for to a great extent by the increase in sphingomyelin and by a less prominent increase in lecithin. During that time there is also a rise in cellular cholesterol. Since phospholipids and cholesterol are components of cellular membranes, these biochemical changes have also their ultrastructural counterpart. In cells derived from 4 week old rats the rough endoplasmic reticulum is quite prominent, while the outer contour of the cell is still quite smooth. The opposite is true in cells derived from one year old rats in which little rough endoplasmic reticulum remains and the plasma membrane is very prominent. This change in membrane population is expressed in an enrichment in plasma membrane relative to endoplasmic reticulum. The rise in 5'-nucleotidase activity indicates also that an absolute increase in plasma membrane had occurred (380).

Enzymes in Phospholipid Synthesis

During the elucidation of the enzymic pathways active in synthesis of phospholipids, we have noted that addition of lecithin to aortic homogenates stimulated greatly the incorporation of linoleic acid into phospholipids (386). Since this stimulation was ATP and CoA dependent it became evident that the active acceptor must be lysolecithin, formed by hydrolysis of lecithin. This reaction occurred at pH 7.4, a finding which indicated that the phospholipase involved

does not belong to the lysosomal family. Subsequently, Patelski et al (249) showed that fatty acids are liberated after incubation of pig aortic extracts with lecithin. The enzyme activity in this process was assumed to be a phospholipase A on the basis of a relative thermostability, though it was innibited by Ca^{2+} and had a narrow pH optimum at 8.0. In our studies, the phospholipase activity of the arterial homogenates was stimulated by Ca^{2+} and sodium deoxycholate. The pH optimum (7.9 - 8.6) of the arterial enzyme coincided with that described for pancreatic phospholipase A. The enzymic preparation was found to release preferentially the fatty acid from the 2 position of lecithin and hence the arterial enzyme could be designated as a phospholipase A_2 (249). The arterial enzyme did also hydrolyze phosphatidylethanolamine to lysophosphatidylethanolamine and fatty acid in analogy to the phospholipase A from pancreas and liver. The other enzymic activity investigated was that of lysolecithinase, which was demonstrated in the arterial homogenates when sodium deoxycholate was omitted from the incubation mixture. The presence of sphingomyelin cholinephosphohydrolase activity was established in homogenates of aortae of rats, rabbits, dogs, guinea pigs and of human umbilical arteries (266). The products of hydrolysis were identified as ceramide and phosphorylcholine. The enzymic activity was found to be dependent on the presence of Triton, had a pH optimum of 5.1 and remained in the supernatant of homogenates prepared in 0.4% Triton even after centrifugation for 1 h at 100,000 xg. The arterial enzyme hydrolyzed both endogenous and exogenous sphingomyelin to the same extent. The species investigated varied in the sphingomyelin cholinephosphohydrolase activity of their arteries (relative to the DNA content) in the ascending order: rabbit > guinea pig > dog > human umbilical artery > rat (Fig.41). In contradistinction to the other phospholipases, the sphingomyelinase seems to be a lysosomal enzyme and I shall discuss later the significance of the different localizations of these enzymes in the overall metabolism of membrane phospholipids. I allow myself to dwell somewhat longer on the subject of aortic phospholipases and not to mention the other lipid hydrolases, as Drs. Bowyer, Day and Zemplenyi will discuss more fully cholesterol esterases and lipases.

PIG

RAT
RABBIT
DOG
GUINEA PIG
HUMAN

As I have pointed out previously, the phospholipid composition of mammalian aorta undergoes a change with age. In our studies we have determined the activity of various phospholipases as related to the cellular content of the artery and hence we may speak about the activity of phospholipases in aortic smooth muscle cells. In all the three species examined, rats, rabbits and humans (Fig.42), there was a marked increase in the activity of lecithinase, while sphingomyelinase activity either did not change or even diminished with age (89). Since the main phospholipid which accumulates in the artery with age is sphingomyelin, it seems of interest to

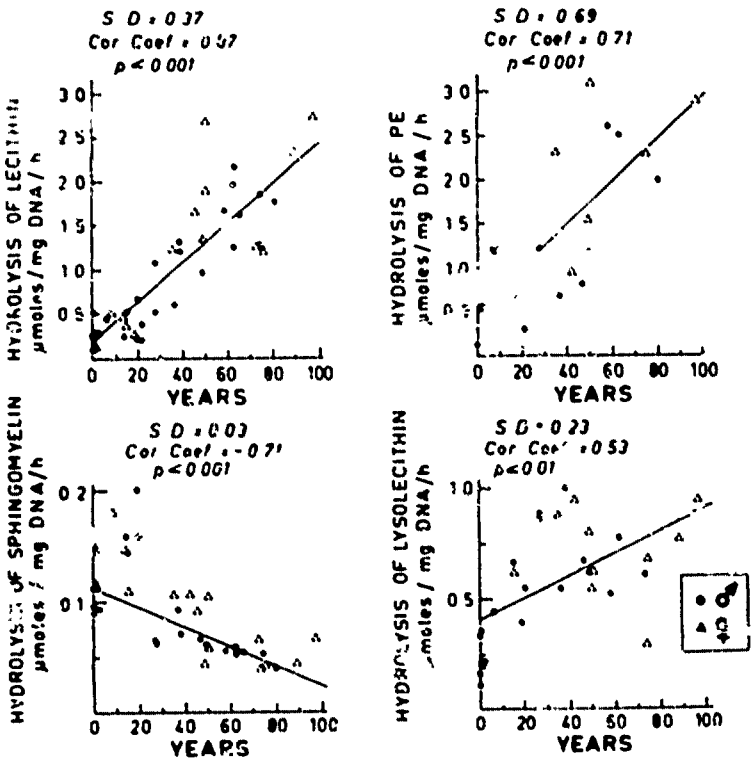


Figure 42: Regression analysis of relation between the activity of aortic phospholipases and age. From Eisenberg et al 1969.

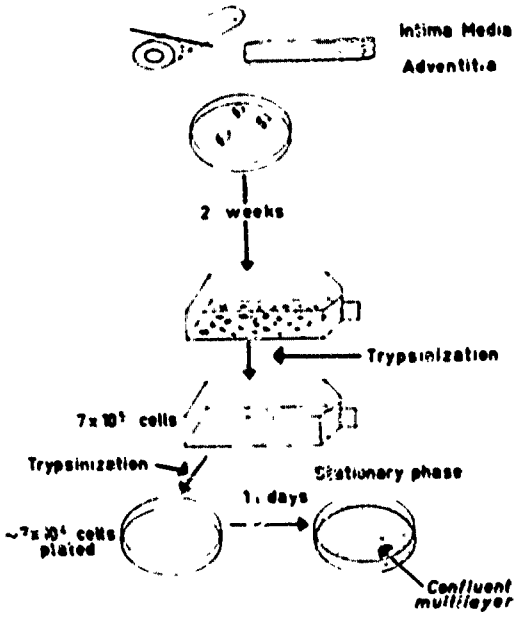


Figure 43

correlate the activity of this enzyme as determined in vitro with the sphingomyelin which accumulates in the artery with age (Table VIII). The data suggest that the aortic phospholipases participate in the regulation of phospholipid composition of aortic smooth muscle cells, as the activity of the enzyme was inversely proportional to the degree of sphingomyelin accumulation.

Phospholipid Metabolism Studied in Arterial Smooth Muscle Culture

The data I have just discussed were obtained from the study of the smooth muscle cells in the whole artery (composition) or in homogenates (enzymic activity) and so suffer from the drawbacks of each respective methodology as I have pointed out in the introduction. Therefore, we have tried to complement some of these findings with a different approach, i.e., by studying the smooth muscle cells in culture. It was very fortunate that Dr. E. Bierman decided to spend his sabbatical leave in Jerusalem and has brought with him all the know-how concerning the culturing of aortic smooth muscle cells, which has accumulated in the laboratory of Dr. Ross in Seattle. For those of you who may not be quite familiar with the technique, I would like to describe briefly how we have adapted the method of Ross (315) to culture smooth muscle cells from rat aorta (Fig.43). The aorta is removed under sterile conditions, cleaned from extraneous fat, and small rings are cut and opened to expose the luminal surface. Using fine tweezers, the intima and the inner half of the media are peeled from the outer portion and the strips thus obtained are cut into small cubes which are placed in culture flasks. Cells being to grow out of the explants in about two weeks and when they fill the flask they are trypsinized and seeded into another flask. When they reach confluency they are trypsinized again and plated in Petri dishes, where they reach the stationary phase at 14-16 days and form a confluent multilayer. In most of the experiments I shall describe, we have used cells obtained from 3 month old rats, but we have also successfully grown cells from a 17 month old rat and their growth characteristics were similar (Fig.44). The morphology of the aortic cells has been examined and it can be seen that they grow in a multilayer and following longer periods of culture in the same Petri dish they form a structure resembling a wall of an artery, complete with extracellular material (Fig.45). The ultrastructural characteristics of the cells, such as an abundance of myofilaments, the presence of dense bodies, plasmalemmal vesicles, as well as the fact that they elaborate extracellular material resembling elastin, permit us to classify them as smooth muscle cells. These cells contain variable amounts of mitochondria, secondary lysosomes and lipid droplets (Fig.46). Those cells which had been in the culture dish for 45 days contained less rough endoplasmic reticulum and had a more distorted outer contour than cells cultured for 14 days or less.

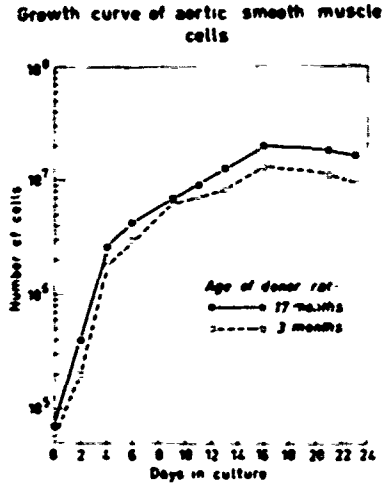


Figure 44

Figure 45: Electron micrograph of a multilayer of aortic smooth muscle cells grown for 45 days in a Petri dish. x 8.500

Figure 46: Electron micrograph of aortic smooth muscle cells cultured for 16 days. The membrane at lower left corner represents the bottom layer. Elastin like material is seen in the extracellular space. x 35.000

We have used these cultured cells in order to study the metabolism of phospholipids. The cells take up labeled glycerol (Fig. 47) or choline (Fig.48) and the incorporation of these precursors into lipids is linear with time for at least 6 hours. In order to study the turnover of lecithin and sphingomyelin, the following experimental design was used, which is summarized in this scheme (Fig.49). After a 2 hour pulse with labeled glycerol and choline, the medium was removed and the cell layer was washed repeatedly. Both the wash and the chase were carried out in the presence of carrier choline and glycerol (3.3 mM) in order to minimize reutilization of the precursors. Following exposure to both glycerol and choline, the specific activity of lecithin isolated from the cells declined at quite a similar rate. These findings provide additional evidence as to the activity of phospholipases in the intact cell. In addition, the rather similar fall in specific activity of lecithin labeled with choline and glycerol indicates that the turnover measured is of the entire molecule. These findings also show that base exchange does not have a prominent role in this system. Comparison of the $t_{\frac{1}{2}}$ of glycerol labeled lecithin in culture of two ages (14 and 45 days) has shown a somewhat slower turnover in the younger cultures, suggesting a higher activity of phospholipases in the older cultures (Fig.50). The initial amount of labeled sphingomyelin was too low to permit an accurate determination of a change in specific activity. However, one can obtain an indirect evidence as to the difference in turnover rates of the two phospholipids by comparing the % distribution of label among the phospholipids during the chase. The rise in the per cent radioactivity in sphingomyelin, relative to lecithin indicates a slower turnover of the first (Table IX). The presence of labeled lysolecithin, after exposure of the cells to ^{14}C-choline is another indication of the activity of phospholipase A_2 in the intact cell. If one accepts that the sphingomyelinase is a lysosomal enzyme, while the phospholipase A is a non-lysosomal "membrane bound" enzyme, then one might envisage that the latter participates in the turnover of membrane molecules, while the former is more active in the degradation of the membrane proper, which is a much slower process.

These cells served for yet another type of investigation, which chronologically preceded the one I just mentioned, namely the question of metabolism of serum lipoproteins. The transport of serum lipoproteins in the aorta will be discussed in another session, and I would just like to point out that evidence has accumulated that aortic smooth muscle cells do come in contact with serum lipoproteins such as LDL and HDL (381). For obvious reasons, it is quite difficult to study uptake and metabolism of such particles in the intact animal, and in the perfused aorta long term studies are not feasible. The smooth muscle cells in culture present a model system to investigate these problems.

Incorporation of ^{14}C-Choline into lipids by aortic smooth muscle cells in culture

Figure 48

Incorporation of ^{3}H-Glycerol into lipids by aortic smooth muscle cells in culture

Figure 47

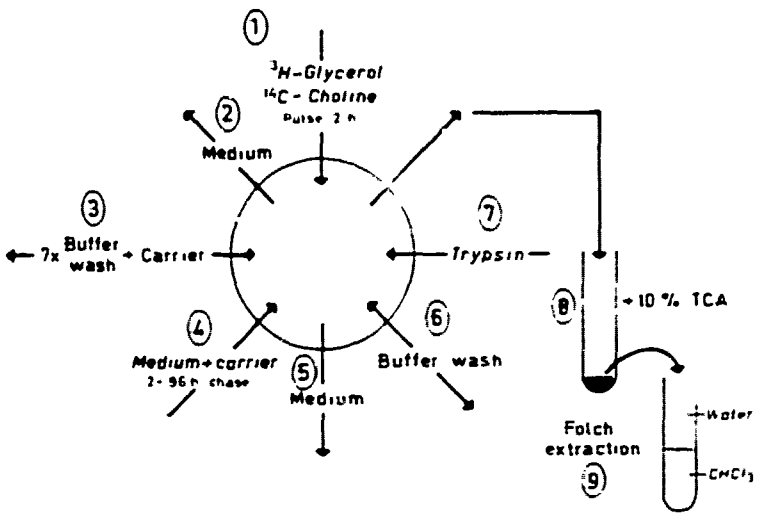


Figure 49: Scheme of a pulse chase experiment with choline and glycerol.

Turnover of ^{3}H-Glycerol labeled lecithin in aortic smooth muscle cells 14 or 45 days in culture

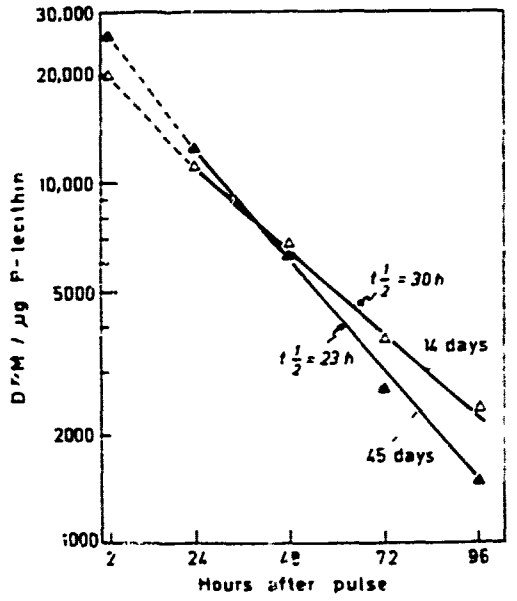


Figure 50

TABLE IX

DISTRIBUTION OF LABEL IN PHOSPHOLIPIDS OF CULTURED AORTIC SMOOTH MUSCLE CELLS, PULSED WITH ^{14}C-CHOLINE FOR 2 h AND CHASED FOR 24 – 96 h.

PULSE h	CHASE h	LL	SP	LE	PE
		% OF RADIOACTIVITY			
2	0	6.2	6.1	56.2	0
2	24	6.9	13.4	75.9	0
2	48	10.5	21.1	66.3	0
2	96	15.4	36.1	46.6	0

LL = LYSOLECITHIN, SP = SPHINGOMYELIN, LE = LECITHIN, PE = PHOSPHATIDYL-ETHANOLAMINE.

The data I will now present were obtained in collaboration with Dr. E. Bierman during his stay in Jerusalem. The two lipoprotein classes studied, VLDL and HDL, are shown here in a negatively stained preparation to remind you of the size difference between them (Fig.51). The uptake of the lipoproteins was studied in the manner presented here in a schematic form (Fig.52). Care was taken to eliminate adsorbed label by repeated washing which was quite effective in order to measure cellular uptake. With the help of radioautography it was possible to show that after incubation with both lipoproteins, VLDL and HDL, the radioactivity taken up was associated with the cells (Fig.53). The persistence of the radioautographic reaction over cells which had undergone trypsinization (Fig.54) prior to fixation and embedding, indicated that interiorization of the labeled lipoproteins had occurred (32).

The fate of the ingested lipoprotein was studied in pulse-chase experiments (Fig.55) with and without replating, using HDL as substrate. During incubation with unlabeled medium there was release of TCA precipitable radioactivity into the medium, which occurred also following trypsinization after pulse labeling and replating of the cells in a new culture dish. This indicated that a portion of the lipoprotein released into the medium could have been derived from particles adsorbed to the plasma membrane, and that some had come most probably from lipoprotein not accessible to trypsin action. A similar phenomenon was described also by Schmidtke and Unanue (331) who studied uptake of iodinated albumin by macrophages. The aortic smooth muscle cells, which had been pulsed for 24 hours released about a third of cellular label into the chase medium, of which 75% were TCA precipitable.

In a series of experiments, using both non-replated and replated cells, it was found that less than 10% of ingested HDL - protein had been catabolized during 48 hours. This value is much lower than those reported for the catabolism of albumin, hemoglobin or peroxidase (86, 87, 388) by cultured macrophages in which the $t_{\frac{1}{2}}$ of the ingested molecules was about 20 - 30 hours. The low rate of intracellular breakdown of HDL - protein is reflected also by the ultrastructural localization of the label in the aortic cells. Most of the radioautographic reaction even though intracellular was seen over the cytoplasm of the cell, and many grains were seen in the vicinity of the cell surface. Only few grains were encountered over structures resembling secondary lysosomes.

The present findings seem to indicate that aortic smooth muscle cells have a limited ability to catabolize ingested lipoproteins but possess a mechanism for elimination of such molecules by regurgitation. The low catabolic rate may be due to the relative paucity of lysosomal enzymes in this type of cell or be an attribute of a specific lipoprotein. These findings bring up an interesting

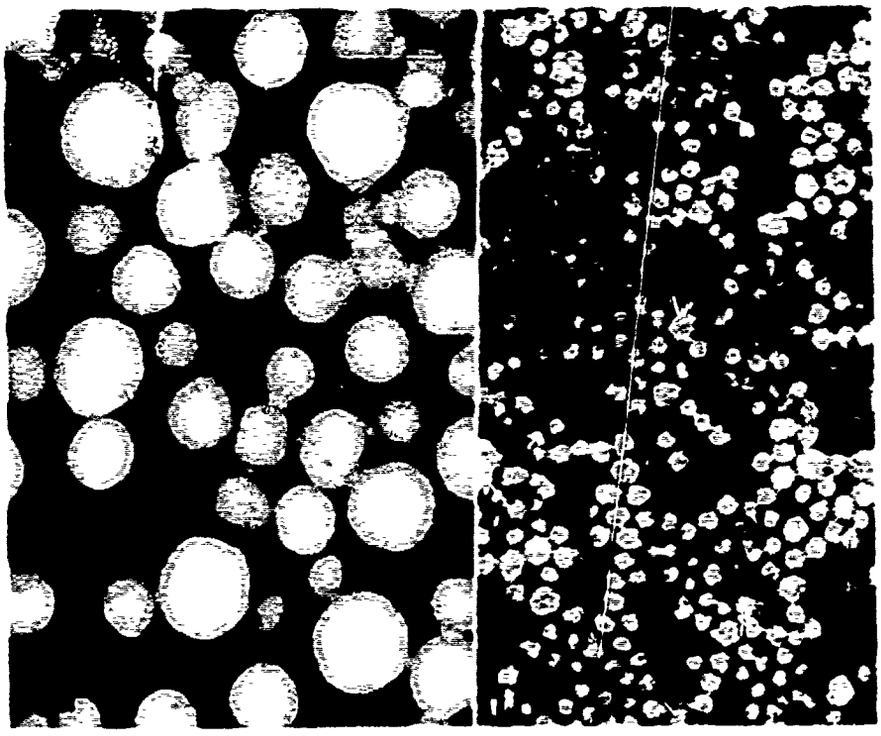


Figure 51 a and b: Negatively stained preparations of VLDL (a) and HDL (b). x 210.000

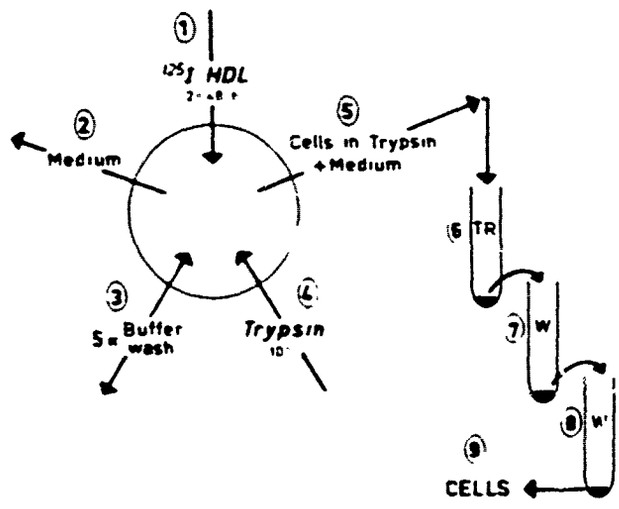
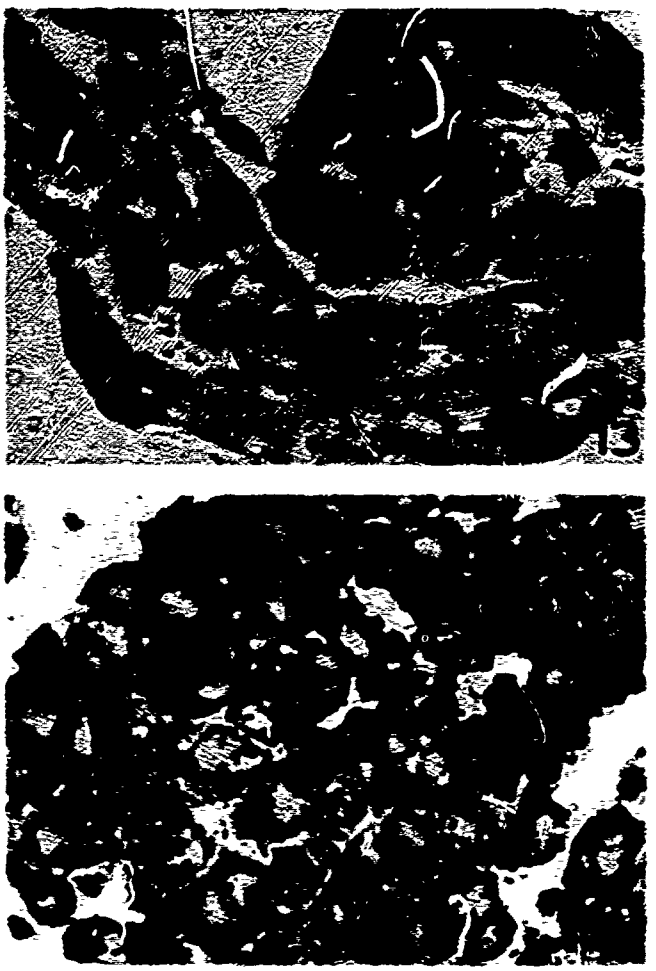


Figure 52: Scheme of pulse labeling with ^{125}I-HDL and subsequent treatment of the cells.



Figures 53 and 54: Light microscope radioautographs of aortic smooth muscle cells incubated for 48 h with ^{125}I-HDL. The cells in Fig. 54 were trypsinized prior to fixation and preparation of radioautography. x 1170

possibility, namely that during the intracellular "roundtrip" the lipoprotein divests itself of some of its cholesterol. This could provide a possible pathway for the progressive accretion of cholesterol in aortic smooth muscle cell known to occur in both humans and other species with age (90).

DR. ROSS: In normal muscular arteries and in the aorta of the monkey the intima is a very thin layer. The endothelial cells are closely adherent to the internal elastic lamina. The media in this muscular artery consists solely of smooth muscle cells between which are collagen fibers, glycosaminoglycan and small elastic fibers. One can see numerous extracellular 100-120 A° microfibrils. These microfibrils were described by a number of people, including Dr. Haust (137) who described them in the aorta several years ago. We have observed them in the development of elastic fibers and now know that elastic fibers consist of two proteins, and that the microfibrilar protein is one of these and elastin is the other. The microfibrils are a glycoprotein that is a ubiquitous component of the connective tissue. If one adds ascorbic acid to the culture one sees not only bundles of these microfibrils, but one can also see banded collagen fibrils as well. A great deal is now known about collagen and in particular about aorta collagen, largely from the work of Miller (222) and his group at the University of Alabama. The collagen present in tendons and in the dermis, now called "Collagen Vulgaris" consists of 2 α 1 and 1 α 2 chain to make up the collagen macromolecule. We know that the collagen molecule is a three stranded chain consisting of these three strands wound about each other in a right handed helix. The α1 chain is interesting in terms of its amino acid sequence because it was subsequently shown by Miller that cartilage collagen has two kinds of collagen, one the vulgaris type and the other contains three identical $(\alpha 1)_2$. The α1 chain of skin is homologous with that of cartilage. It has amino acid substitutions which presumably leads to the formation of a different kind of collagen fibril, and therefore a different kind of connective tissue matrix. Miller (222) and Trelstad (395) have subsequently discovered that in fetal skin and in the aorta there is a third type of collagen which we shall call $(\alpha 1)_3$, which is different from the other two α1 chains.

MONKEY

The Structures of Collagen and Elastin

We now have evidence that these same monkey smooth muscle cells in culture form soluble elastin based on the following kinds of evidence: they incorporate tritiated lysine into a precursor which will migrate electrophoretically with purified soluble elastin. This labelled protein serves as a substrate for the enzyme lysyl-oxidase, which is responsible for the cross linking of the monomers of elastin from the soluble form to the insoluble form. We can also demonstrate autoradiographically that these cells will incorporate lysine into precursors. We have also been able to isolate large

TABLE X

Incorporation of ^{35}S-sulfate and ^{3}H-acetate into non-dialyzable, TCA soluble material by primate arterial smooth muscle cells *in vitro*.

^{35}S		^{3}H	
Medium	Cell Layer	Medium	Cell Layer
117,412*	3,843	36,597	4,511
89,349	5,698	31,210	11,139
86,267	4,303	237,181	29,188

* All values are specific activities DPM/10^6 cells.

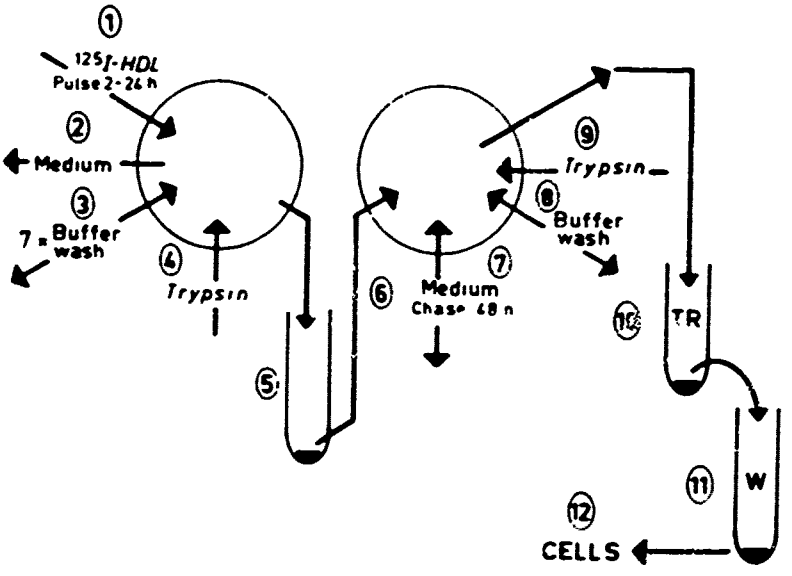


Figure 55: Scheme of pulse chase experiment with ^{125}I-HDL with replating of the culture of cells after pulse.

quantities of the microfibrilar protein from cultures and identify them in terms of their amino acid composition which is identical with the amino acid composition of the microfibrilar protein that one can isolate from intact elastic fibers in vivo. We are now systematically pursuing the mechanisms that control elastin synthesis and microfibril synthesis in culture by these cells, in a similar fashion to the collagen studies. I would like to ask Dr. Thomas Wight if he would say a few words about the synthesis of proteoglycans by these cells in culture as well.

The Synthesis & Secretion of Glycosaminoglycans by Primate Arterial Smooth Muscle Cells in vitro

DR. WIGHT: Recent in vivo and in vitro experiments in our laboratory (315, 316) have demonstrated that arterial smooth muscle cells are capable of synthesizing and secreting collagen and elastic fiber protein, two of the three major connective tissue matrix components present in blood vessel walls. This study is designed to illustrate that these cells also synthesize and secrete glycosaminoglycans, the third major connective tissue matrix component. The three basic questions of the study are:

1. Do primate arterial smooth muscle cells synthesize and secrete glycosaminoglycans in vitro?

2. If so, what types are made and in what amounts?

3. At what stage in their growth phase do these cells engage in the synthesis of these macromolecules?

Arterial smooth muscle cell cultures from the sub-human primate Macaca nemestrina, were established as previously described (317). At saturation density, cultures were double labeled with ^{35}S-sulfate and ^{3}H-acetate (20uCi per flask) for 24 hours and the glycosaminoglycans were extracted from medium and cell layer by the procedure of Nameroff and Holtzer (235). The majority of activity was present in the non-dialyzable, TCA soluble material isolated from the medium (90%) with less activity associated with similarly isolated material from the cell layer (10%) (Table X).

To obtain an estimate of the types of glycosaminoglycans being synthesized, the isotopically labeled extracted material was subjected to glycosaminoglycan specific enzymes (324, 394) and the digestion products were separated by gel filtration on Sephadex G-25. By determining the percentage of the sample degraded in each enzyme digest (leech hyaluronidase, testicular hyaluronidase, and chondroitinase ABC), it is possible to obtain an estimate of the types of glycosaminoglycans present in the sample (84). Results indicate that little or no degradation occurred (0-5%) when the

^{3}H-acetate labeled samples were incubated with leech hyaluronidase, indicating only trace amounts of hyaluronic acid present (Table XI). Slight degradation occurred (10-12%) when ^{35}S-sulfated labeled samples were incubated with testicular hyaluronidase, an enzyme specific for chondroitin sulfate A and C (Table XI). Extensive degradation (80-90%) occurred when parallel ^{35}S-labeled samples were incubated with chondroitinase ABC (Table XI). By subtracting the amount of activity retarded in the testicular hyaluronidase digests from the amount of activity retarded in the chondroitinase ABC digests, it is possible to obtain and estimate of the amount of dermatan sulfate present since both chondroitinase ABC and testicular hyaluronidase degrade chondroitin sulfates A and C but only chondroitinase ABC degrades dermatan sulfate (chondroitin sulfate B). Both medium and cell layer gave similar elution profiles for each enzyme digest. Using this assay system, the following estimates of the types of glycosaminoglycans synthesized and secreted by primate arterial smooth muscle cell in vitro are: hyaluronic acid (0-5%): chondroitin sulfate A/C (10-20%): dermatan sulfate (60-80%): other sulfated glycosaminoglycans (10% or less).

Cellulose acetate electrophoresis (235) of isotopically labeled samples confirmed the enzyme digestion assays by demonstrating that the major peak of activity corresponded to the dermatan sulfate standard with lesser peaks associated with the chondroitin sulfate C standard and a fraction which migrated behind dermatan sulfate (Table XII).

In order to determine at what stage in the growth phase these cells are most active in the synthesis of glycosaminoglycans, arterial smooth muscle cells were grown in various concentrations of serum, as previously described by Dr. Ross, and labeled with ^{35}S-sulfate during log and stationary phases of growth. Subsequently, the glycosaminoglycans were extracted from the cell layer and medium as previously described. The specific activity of isotopically labeled glycosaminoglycans present in the medium of stationary cultures was consistently higher than the specific activities of comparable cultures in logarithmic growth (Table XIII).

In conclusion, primate arterial smooth muscle cells synthesize and secrete glycosaminoglycans in vitro. Under our conditions of culture, these cells synthesize and secrete primarily dermatan sulfate with smaller amounts of chondroitin sulfate A and C and trace amounts of hyaluronic acid. Both cell layer and medium gave similar results. Dividing and nondividing smooth muscle cells are capable of synthesizing sulfated glycosaminoglycans although nondividing cells are more active in the synthesis of these macromolecules.

DR. LINDNER: Dr. Ross spoke of collagen synthesis in smooth muscle cells culture, especially the two enzymes responsible for

TABLE XI

	Void Volume	Retarded	Total	% Retarded
Control	7293*	426	7719	6%
Leech hyaluronidase**	7930	646	8576	8%
Control	3225	0	3225	0%
Testicular hyaluronidase	2972	324	3296	10%
Control	3740	205	3945	5%
Chondroitinase ABC	504	6816	7320	93%

* DPM

** Leech hyaluronidase digests were done on ^{3}H-acetate labelled material. Other digests were done on ^{35}S-sulfate labelled material.

TABLE XII

Fraction # *	Cell Layer	Medium
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6 (HA)	0	0
7	45**	20
8 (DS)	95	130
9 (CSC)	20	30
10	0	0
11	0	0
12	0	0

* Fractions were obtained by slicing the cellulose acetate strips into 0.5cm segments after electrophoresis.

HA = hyaluronic acid standard; DS = dermatan sulfate standard; CSC = chondroitin sulfate C standard.

** DPM

TABLE XIII

Incorporation of ^{35}S-sulfate into non-dialyzable, TCA soluble material by primate arterial smooth muscle cells during stationary and logarithmic growth in vitro.

Days in Culture	Stationary Culture (1% Serum)		Logarithmic Culture (5% Serum)	
	Cell Number	Specific Activity*	Cell Number	Specific Activity
7	$3.1x10^5$	354	--------	----
9	$1.4x10^5$	1037	$4.5x10^5$	231
13	$1.3x10^5$	1350	$9.0x10^5$	200
16	$3.3x10^5$	454	$14.8x10^5$	185

* Specific activity = CPM/10^5 Cells present in the medium after a 24 hour labelling period.

the hydroxylation of collagen, protocollagen-prolin and protocollagen-lysin-hydroxylase (Fig.56). The enzyme which is responsible for the splitting up of the additional peptides is procollagen-peptidase. Absence of this enzyme in the body is lethal and if it is lacking only in the skin we have the typical picture of dermatosparaxis. Collagen-peptidase works extracellularly, while the hydroxylases mentioned work intracellularly (Insufficiently hydroxylated collagen cannot be extruded) (Fig.56).

Nowadays more attention is given to the breakdown of collagen than to the breakdown of proteoglycans (without any real justification, and occasionally for methodical reasons). Enzymes which breakdown native collagen under physiological conditions are termed specific collagenases. Their specificity is directed primarily at the amino acid sequences from which the apolar regions of the native collagen fibrils are formed (Fig.57).

These specific collagenases have only been demonstrated within the last eight years in humans and other mammals, and more recently in rheumatoid synovitis, inflammatory granulation tissue, leucocytes, etc. Together with our study groups (and particularly with Grasedyck) Strauch and Gries are now carrying out routine determinations of collagenolytic activity with a synthetic PBZ-substrate which is cleft between the amino acids leucine and glycine by the collagen peptidase present in the tissue studied. This results in liberation of a fragment of water-soluble polypeptide, which contains the chromophoric group. Fig.58 illustrates determination of collagen peptidase activity in various organs of the rabbit in comparison with those obtained in serum. Ideal conditions for PBZ-substrate-cleaving enzyme activity appear to be a pH of 7.2-8.0.

Fig.58 also shows quite clearly that of all the organs listed the aorta has the highest content of PBZ-substrate-cleaving activity; this is also considerable in other organs with smooth muscle cells, for example, the esophagus. This means (in the light of the corresponding results in studies of the metabolic rate of collagen) that organs with untreated musculature - such as the aorta, the esophagus, the intestine, etc. - can have a high rate of collagen metabolism, even with a normally low total collagen content. In addition, organs consisting primarily or completely of smooth muscle cells, like the aorta and higher arteries, are capable of synthesizing and breaking down collagen.

Fig.59 represents a schematic review of the data on catabolism in connective tissue cells smooth muscle cells. The increasing number of electron microscopic studies of lysosomes (which have been released from the cells as well as extracellularly sited lysosomes) demonstrate that lysosomes are important not only for the

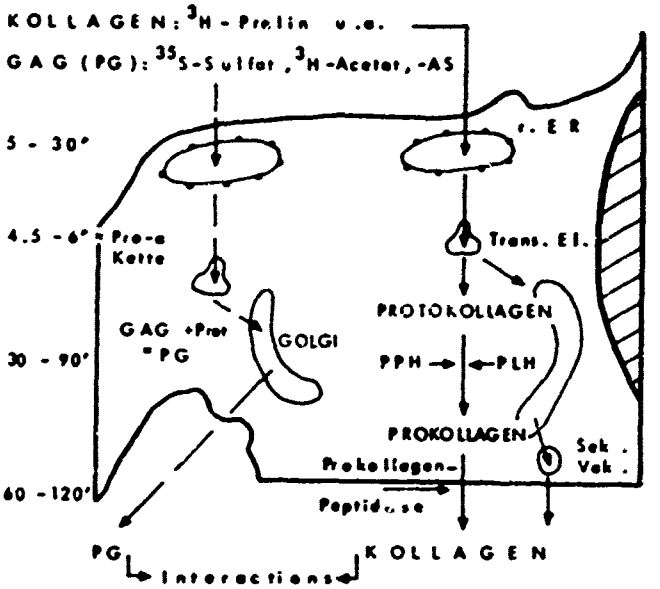


Figure 56: A schema (in German) for the process of collagen synthesis by osteoblasts.

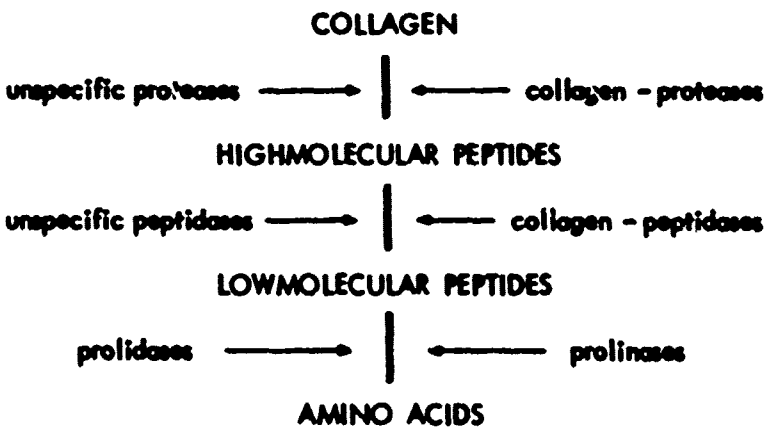


Figure 57: Schema of collagen breakdown with the main responsible enzymes.

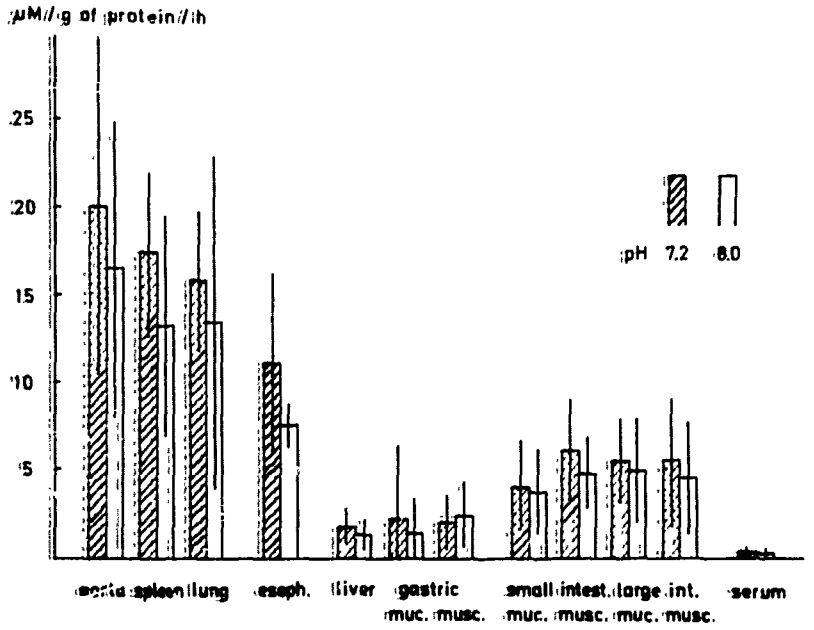
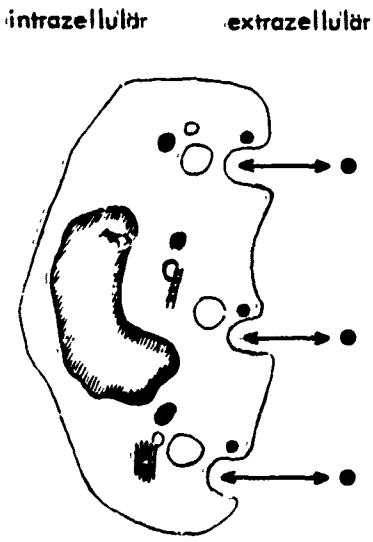


Figure 58: Collagen peptidase activity of several rabbit organs compared with that of serum.



GAG-PROTEIN (PG)

1. Depolymerisierung

   GAG-(MPS-) asen
   Proteasen

2. Glykosidasen

   (ß-Glukuron., Iduronid., Hexosamin.)
   Peptidasen

KOLLAGEN

1. Depolymerisierg.(Denat.)
2. Kollagenproteasen
   Kollagenpeptidasen
3. unspez. Proteasen
   unspezif. Peptidasen

Figure 59: Schema (in German) of 1. extra - and 2. intracellular catabolic processes of connective tissue cells.

extracellular breakdown of collagen and fibrin but also for breakdown of proteoglycans as well; corresponding enzymes have already been demonstrated. Breakdown of proteoglycans in groundsubstance is achieved by a continuation of depolymerization of these macromolecules. This takes place within the framework of what are known as "de-mixing processes", together with an enzymatic activity of the corresponding glycosidases and protease on the component parts of these proteoglycans (see schematic review in Fig.59). The data assembled to date on the breakdown of collagen are presented in Fig.59 (see also Fig.59 and the relevant text). The arrows (Fig. 59) in both directions are quite clearly indicative of the facts presented and show that the breakdown commences primarily extracellularly and is continued, secondarily, intracellularly. The arrows pointing from the intracellular areas into the extracellular ones represent the release of hydrolase-containing lysosomes into the extracellular regions for the catabolic processes. Arrows pointing toward the cell represent the uptake of breakdown products from the extracellular areas into the cell, with continuation of further breakdown processes and with occasional proof of fragments of the material which is being broken down (particularly collagen and fibrin). The schema applies to vascular (and other) smooth muscle cells as well as any connective tissue cells engaged in the synthesis and breakdown processes of GAG and collagen.

DR. ROBERT: I should like to offer a bit of speculation. Fig.60 shows a primitive mesenchymal cell which can give rise during histogenesis either to something which looks like cartilage or cornea or the smooth muscle cells or "mediacytes" if I may use this expression, which gives rise then to the aorta (280).

Glycoprotein Biosynthesis

Fig.61 shows a highly simplified speculative theory of differentiation (281). Really what happens to our specialized cells during histogenesis and also what happens when our cells age (I feel encouraged by Dr. Stein's presentation to mention aging studies also) is that we have a sort of regulatory mechanism built in somehow in the cells which enables these four taps (Fig.61) to prescribe a rate of flow of the four major types of macromolecules of the intercellular matrix: collagen, proteoglycans, elastin and structural glycoproteins. The structural glycoproteins compose the microfibrils of elastic tissue. We isolated these glycoproteins about 13 years ago with Dr. Dische (282) from many different tissues. Recently we realized that they appear to compose the "microfibrils" of elastic tissues (171, 279) (Fig.62).

We heard from the presentation of Dr. Somlyo that a clear discrimination can be made between such intermediate-sized microfilaments (we think are composed of structural glycoproteins), the

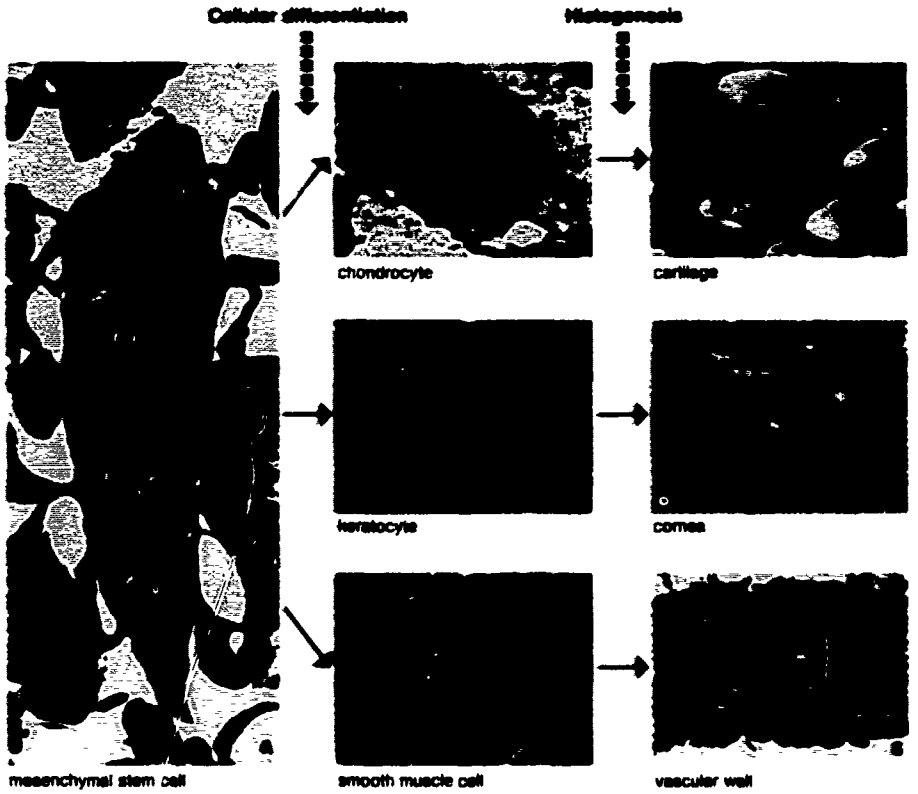


Figure 69: Schematic representation of the differentiation of mesenchymal tissues. Appearance during ontogenesis of differentiated cells synthesizing, in various ratios, the macromolecules of the intercellular matrix. This phenomenon leads to the formation of differentiated connective tissues such as cartilage, blood vessels, dermis, tendons, etc. C, F and G: optical microscope, low magnification; A, B, D and E: electron microscope, low magnification (from ref 280).

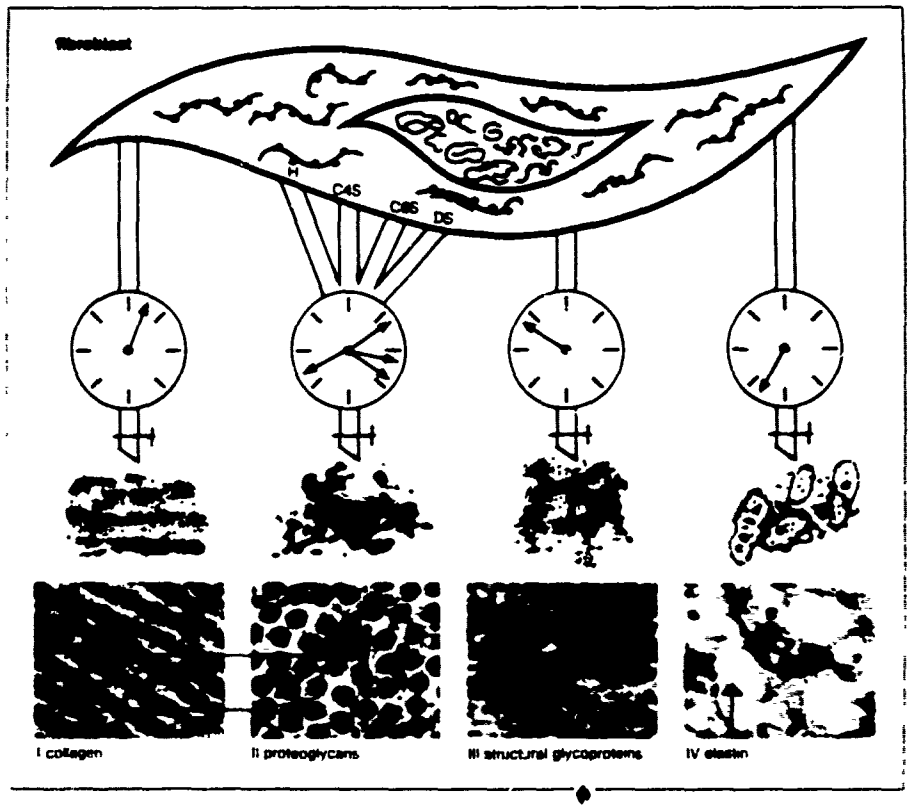


Figure 61: Schematic representation of the hypothesis assuming that differentiation of mesenchymal tissues is obtained by the regulation of the rate of biosynthesis of the four major classes of intercellular macromolecules: collagen, elastin, proteoglycans and structural glycoproteins. The fibroblast is represented as equipped with four taps, the flow from each being regulated by a separate chronometer (in reality it would be necessary to conceive many more, for the different types of proteoglycans of which four types are represented: H = hyaluronate; C4S = chondroitin-4 sulphate; C6S = chondroitin-6 sulphate; DS = dermatan sulphate). The kinetics of operation of the four chronometers determine the proportions in which the four types of macromolecules are synthesized as a function of age. I and II: electron microscope, fairly high magnification; IV: electron microscope, high magnification; III: electron microscope, very high magnification. (from ref 281).

Figure 62: "Microfibrillar" appearance of structural glycoprotein preparations obtained from pig aorta (uranyl acetate-Pb x 315,000) (from ref 171).

smaller actin filaments and the big myosin filaments. All three types of filaments are present in smooth muscle cells but only the structural glycoprotein-microfibers can be found in the intercellular matrix. The biosynthesis of elastin involves a coordinated regulation of the synthesis of structural glycoproteins and of proelastin (171, 283).

Uptake of Precursors as a Function of Age

We have used several different approaches to study the biosynthesis as a function of age, and state of differentiation of elastin. One of these approaches was the organ culture of newborn or fetal rabbit aortas and adult aortas. Aortas were maintained in MEM medium with 10% calf serum from 24 hours to 6 days in the presence of radioactive tracers, usually ^{14}C-lysine (283). After washing, the aortas were extracted by using one of the several so-called "chemical dissection" methods we devised. Thereby all the macromolecules of the intercellular matrix are present allowing one to determine the distribution of the label in the aorta (283) (Fig.63).

We determined the quantity of these macromolecular fractions in newborn and adult aortas and calculated the change with age of these fractions. The calcium chloride extract which contains the diffusible macromolecules increases by about 24% between the newborn and the adult. The collagenase extract (which is about 12% in hydroxyproline) by 57%. The urea-mercaptoethanol-extract which contains the structural glycoprotein fraction increases by 47%. Elastin (which was solubilized by a method using potassium hydroxide in ethanol) increases by 400%. Thus we conclude that the main activity of the young aorta would be elastin synthesis. When we used a ^{14}C-lysine to trace this biosynthesis a great difference in the rate of its incorporation appears between the young and the adult aortas (Fig.64). The drop of specific activity with age is not uniform over the different fractions (243).

Biosynthesis of Elastin

Now let us focus on elastin. It is currently admitted that elastin has no turnover in the adult animal. We came to the conclusion (243, 283) that adult aorta is capable of synthesizing elastin. In order to substantiate this claim we wanted to really get out the label not only from isolated and purified elastin but also from the cross links themselves. We devised with Dr. and Mrs. Moczar a high voltage electrophoretic procedure to isolate lysinonorleucine and the desmosines (227). After 24 hours of culture we were able to isolate lysinonorleucine which was labelled in the young but still unlabelled in the adult aorta (243). At this stage desmosine was not yet labelled. After six days we could isolate labelled desmosine from both the young and the adult aorta also (Table XIV).

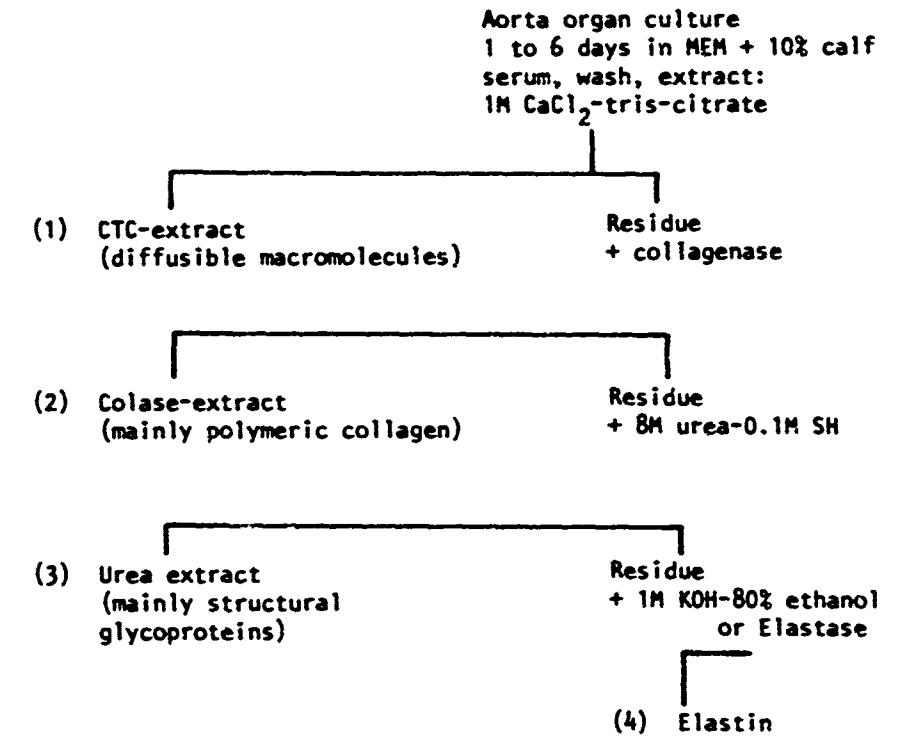


Figure 53: Chemical dissection procedure used to analyse the incorporation of labelled precursor in the macromolecules of the aorta.

TABLE XIV

Incorporation of U-^{14}C lysine in lysine and desmosine residues of elastin in organ cultures of young (400 g) and adult (2 kg) rabbit aortas.

cpm/mg residue

	3 days		6 days	
	young	adult	young	adult
Lysine	220,000	49,000	90,000	55,000
Desmosine	3,700	0	10,300	3,700

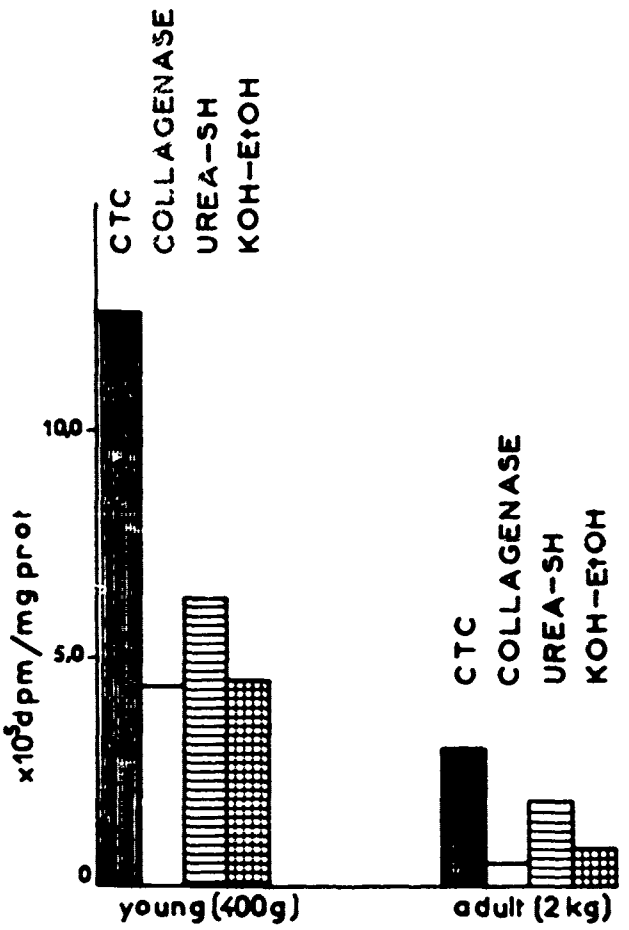


Figure 64: Histogram representing the specific radioactivity of the macromolecular fractions of the new born and adult rabbit aorta after 24h organ culture in the presence of ^{14}C-lysine. The aorta extracts were obtained as indicated on Fig. 63.

Similar results were obtained by incubating rabbit aorta slices in Krebs-Ringer-PO_4 medium for 4 hours with ^{14}C lysine or ^{14}C galactose, followed by "chemical dissection", and determination of distribution of radioactivity, either from the fractions or from isolated molecules (hexoses or amino acids) (283).

^{14}C galactose is incorporated during a four hour incubation period in all three major glycopeptides isolated (glycopeptides from proteoglycans, glycoproteins and collagen). A very strong incorporation in the structural glycoprotein fraction was observed and also a weaker one in collagen (Fig.65).

One way of characterizing the state of differentiation (as well as its changes in pathological conditions) of different connective tissues is to compare the rate of incorporation of labelled precursors into the isolated glycopeptides of glycosaminoglycans, structural glycoproteins and of collagen (228, 229, 283). After hydrolysis of the labelled glycopeptides, separated by gel filtration (see Fig.65), galactose, glucose and mannose could be isolated, quantitated and their specific activity individually determined. We found that the highest specific activity was present in galactose but glucose was also labelled. So really a rabbit aorta can do a great deal in four hours with these sugars. Again the ratio of labelling of the monosaccharides turned out to be different for instance from cornea to aorta (229, 283). In the conditions used (4 hours incubation at 37 degrees) calf cornea could convert ^{14}C galactose to ^{14}C mannose; pig aorta did not.

Similar experiments were carried out with ^{14}C lysine as a tracer and this aminoacid was also significantly incorporated in all macromolecular fractions of the rabbit aorta during a four hour incubation period (283). Here again the strongest incorporation was obtained in the structural glycoprotein fraction. A lower but significant incorporation was also observed in the polymeric collagen and elastin fractions. All these experiments confirm the capacity of rabbit aorta explants to carry out actively the biosynthesis of all the macromolecular components of the intercellular matrix. For most components this biosynthesis is relatively rapid and can be observed after four hours of incubation. Elastin is actively synthesized in aorta organ culture and crosslink formation could be detected by the isolation of labelled lysinonorleucine and desmosine not only in young but also in adult aortas. This shows that elastin neosynthesis is not definitively repressed or inhibited in the adult organism (243, 283). The rapid incorporation of label in the urea-soluble glycoproteins and the coordinated synthesis of structural glycoprotein-microfibrils and proelastin for polymeric elastin biosynthesis is confirmed by these experiments.

To summarize to this point: The properties of structural

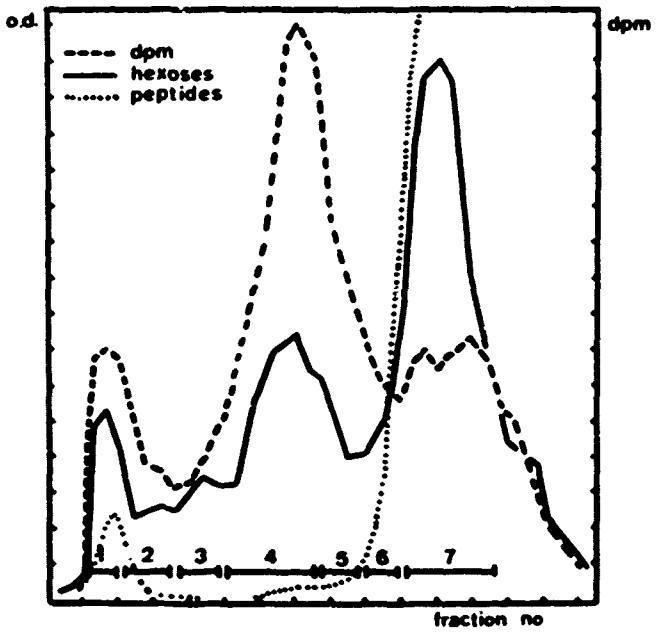


Figure 65: Elution diagram of the collagenase and pronase hydrolysate of the insoluble stroma (extracted with M $CaCl_2$) of the media of pig aorta incubated with ^{14}C-galactose. Sephadex G-50 medium column (4.8 x 800 cm for 2.6 g hydrolysate). Elution with 0.1 M acetic acid at 1 ml/3 min; ——, orcinol (carbohydrates); ——, radioactivity dpm in fraction; (from ref 283).

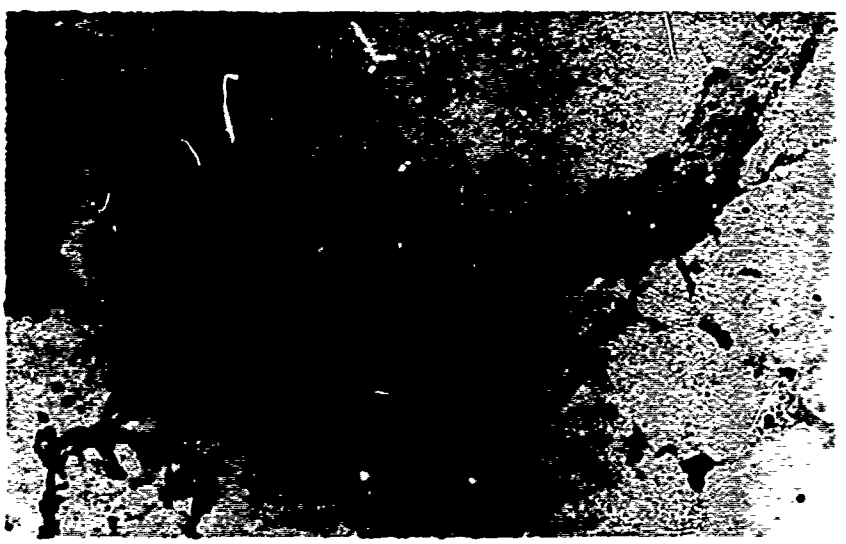


Figure 66: Elastin from porcine aorta stained with ruthenium red (x 13,000) (picture of Dr. Kadar) (see ref 171).

glycoproteins of aorta were described (228, 284). They have a characteristic amino acid composition, are rich in aspartic and glutamic acids as well as in alanine and other aliphatic amino acids. They easily form aggregates by disulfur and hydrophobic interaction. Their amino acid composition shows a striking homology to "transplantation antigens" (285). Purified preparations of structural glycoproteins were obtained from human, porcine or rabbit aorta and also from polymeric elastin preparations (228, 279, 284). Under the electron microscope these glycoproteins present typical "microfibrillar" aggregates, composed of globular subunits with an average diameter of 120 A° (Fig.62) (171).

The following experiments were carried out in collaboration with Dr. Kadar from the University of Budapest (171). The first preparation was obtained (Fig.62) from the aorta and another (Fig.66) from purified elastin. It is interesting to notice that they react with ruthenium red. This is again a warning to those who think that ruthenium red is a stain for polysaccharides only. This glycoprotein is not very rich in sugar components ($\pm$ 5%) (228, 284), but is a very acidic protein (286). Fig.66 shows elastin stained with ruthenium red: you may see the nice microfibrillar glycoproteins taking the stain.

Fig.67 presents a summary of the proposed mechanisms of elastic tissue biosynthesis from two components (275, 287): 1. the structural glycoprotein-microfibrillar component, and 2. the translucent polymerized elastin composed of tropoelastin monomers linked together by desmosin cross links (248). The glycoprotein-microfibrils can be extracted with 8M-urea-0.1 M mercaptoethanol (279) then we obtain the microfibrillar structures (171). Now we can stain with phosphotungstic acid the residual elastin which reveals a fine structure composed of globular subunits. The question arises whether or not this be polymerized elastic units.

This has still to be confirmed. All these experiments support, however, our original proposition (171, 243, 279, 283) attributing to the structural glycoprotein-microfibrils the role of orienting scaffolding onto which the proelastin molecules can aggregate through electrostatic interactions (between the positively charged lysine-residues of proelastin and the negatively charged glycoproteins). The crosslinking process, initiated by lysinoxydase could then take place in an oriented, sterically determined fashion ("vectorial synthesis") (275, 287). The regulatory mechanisms responsible for this coordinated biosynthetic activity must reside somehow in the smooth muscle cell genome and can be considered as a direct consequence of its "differentiation" to aorta "mediacytes". Pathological disturbances of this regulatory process might well be involved in the structural alterations observed in the intercellular matrix of arteriosclerotic aortas.

DR. ADAMS: I would like to ask Dr. Robert if there are any sulphated glycoglycans in the structure of collagen because although he showed that the proteoglycans are present there in the structure, there are sulphated gags formed during synthesis of collagen.

DR. ROBERT: I did not go into detailed descriptions of structural glycoproteins but at least the one we isolated from cornea does have some sulphate but it has no detectable glycosamino-glycan-sulphate, no uronic acid so it is not really a GAG (282).

DR. ADAMS: I am sorry, but I am not sure you answered my question. I said in the structure of collagen or elastin, but not just in the aortic wall.

DR. ROBERT: Oh, here we face a very sophisticated semantic question. Collagen is coded by a structural gene and it cannot have GAG-s in it naturally. Elastin, we have just discussed the problem, is a composite thing composed of at least two types of proteins: proelastin which is not a glycoprotein and the structural glycoprotein-microfibrils. But in this picture we do not have, and we do not need, the GAG-s. These are separate macromolecular components of the aortic stroma. I do not know again if we get through this time?

DR. LINDNER: Synthesis of sulphated glycosaminoglycans (GAG) or proteoglycans starts before collagen synthesis in embryonic or post-embryonic connective tissue. The location of sulphated GAG-synthesis (labeled with the most suitable precursor: ^{35}S-sulphate) are shown in examples of light microscopic radiograms of smooth muscle cells with the same shapes etc. which you have seen in culture yesterday. The same is true for smooth muscle cells of human biopsy and autopsy materials. This incorporation and synthesis pattern and course of sulphated GAG is just, the same as in other connective tissue cells, shown by examples, of cartilage cells: semithinslide autoradiogram with labeled Golgi field - 30 minutes after in vivo incorporation of ^{35}S-sulphate. It is possible to count the number of silver grains (especially by the automatic translucent photometric analysis) and therewith the synthesis level in cells - and here in organelles also. The Golgi field is important for any mesenchymal as well as epithelial synthesis of acid and neutral polysaccharides. This concentration of silver grains in the Golgi apparatus is demonstrable also in smooth muscle cells and shown by examples of electronmicroscopic radiograms in epithelial cells also which are involved in the synthesis of acid and neutral polysaccharides besides other syntheses potentials: examples of this Golgi field labeled with some silver grains and then formation of premucous and mucous vesicles labeled

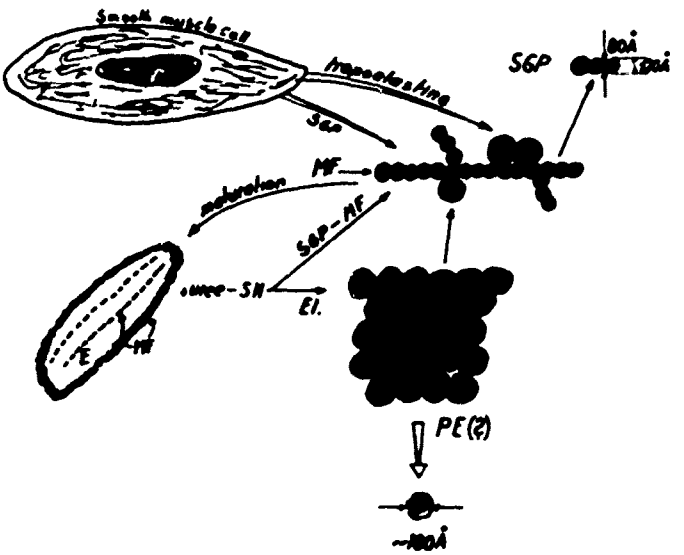


Figure 67: Schematic representation of the theory of elastogenesis: the smooth muscle cell produces the "microfibrillar" glycoprotein-scaffolding (MF) onto which the proelastin subunits aggregate to form the elastic lamellae (E). The "microfibrils" are composed of structural glycoproteins (SGP) extractable in 8M urea-0.1M mercaptoethanol leaving a polymeric elastin core (PE) (modified from ref 171).

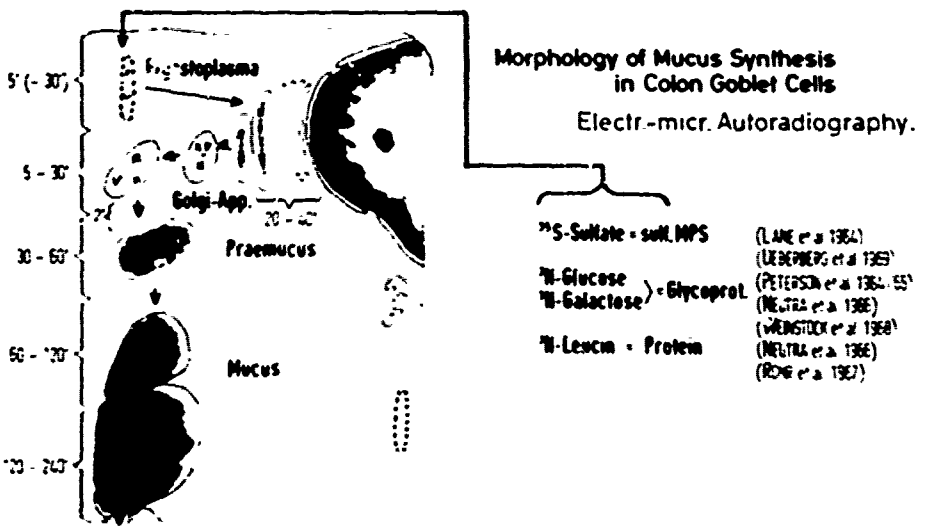


Figure 68: Summary of the results of the most important electronmicroscope studies on the morphology of the epithelial synthesis of mucus, especially the acid glycosaminoglycans; using the colon goblet cell as example.

also followed by the extrusion of labeled GAG - in this epithelial as well as in any mesenchymal GAG synthesis, of smooth muscle cells included.

^{35}S-sulphate is the best labeling precursor to use for locating and quantifying sulphated GAG synthesis because in contrast to other precursors, ^{35}S-sulphate is not involved in the synthesis of neutral polysaccharides or the polysaccharide moities connected to protein or lipid. For the same reason ^{35}S-sulphate is suitable for locating and quantifying sulphated GAG synthesis of connective tissues.

Within the first five minutes after the administration of the labeled precursor, only sporadically labeled ergastoplasm can be found; in contrast the Golgi complex of the goblet cells, or other mucogenic gastric and enteric epithelial cells takes up the label within the first 5-30 minutes. This is where the real synthesis and polymerization of neutral and acid mucus takes place. As has been especially well shown by the studies of Neutra and Leblond the loops of the Golgi apparatus are renewed with such speed that each loop consisting of 7-12 vesicles is completely replaced about every 20-40 minutes. Every 2 minutes one Golgi vesicle is transformed into a so-called premucosal vesicle (and mucigen granule). Labeling increases for the first 30 mins after the beginning of incorporation. The Golgi vesicles are transformed directly into the so-called premucosal saccules and granules out of which the mature mucus granules are then formed. At the end of 60-120 mins these may be labeled and may be delivered into the intestinal lumen through superficial fissures in either intact epithelial cells or in those with defective mucosal membranes (see summary Fig.68). This short time measurement of ^{35}S-sulphate incorporation may prove a better method for the assessment of these synthetic processes than the direct measurement of the specific activity of the fractionated glycosaminoglycans which was also carried out on this material. Early optimism which arose from the apparently successful transfer of methods for the evaluation of microscopic autoradiographs to a study of electron microscopic autoradiographs, has now been replaced by a more critical assessment of their value. It has proved essential to adhere to standard conditions such as the consecutive randomized measurement of the various parameters. These include resolving power, relative thicknesses of section and emulsion, the absolute sensitivity of the method, and a consideration of the ratio of the dimensions of the silver grains and other considerations appropriate to the apparatus employed. Therefore it is apparent that the study of electron microscopic autoradiographs and above all their quantification must be supplemented by more suitable quantitative methods. Here again the measurement of the rate of incorporation of ^{35}S-sulphate is best suited for routine application. So as a routine indicator method we are using the ^{35}S-sulphate incorporation for location and for quantification. The results of the so-called

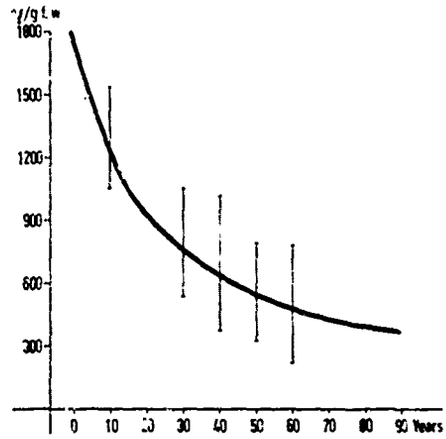


Figure 69: Age depending decrease in cell content of the human aorta (quantitative biochemical DNA analyses).

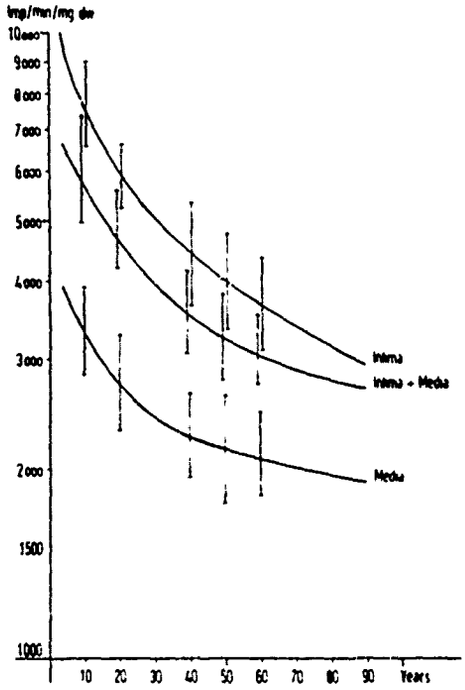


Figure 70: Decrease in the rate of ^{35}S-sulphate incorporation during maturation and aging of the human aorta; examined separately for the intima (upper tracing) and the media (lower tracing) as well as for both layers of the vascular wall together (middle tracing).

^{35}S-sulphate incorporation rate measurement are comparable to the results of assays of these enzymes which are responsible for the sulphate activating and transferring (PAPS). Using this method we find evidence of vascular aging of humans and rats. Indeed in a good preparation we see more than 90% smooth muscle cells in the aging rat aorta.

The decisive fact is, that the total cellular content of the arterial wall, particularly that of the aorta, decreases (Fig.69), no matter whether the results have been calculated in terms of total protein content or total nitrogen content, fresh weight or dry weight. This holds true for the intima as well as for the media.

In contrast with morphological investigations, biochemical changes in the vascular wall which are caused by aging and which depend on the structure of the individual layers, have not yet been examined in an adequate manner. Evidence for the fact that in the isolated intima and media of the human aorta an age-dependent decrease occurs from the beginning of maturation to very old age could only be obtained by means of the indicator technique, that is by measuring the rate of incorporation of ^{35}S-sulphate. This is always less in the media than in the intima (in terms of dry weight) (Fig.70).

Since the DNA content of the human aortic wall decreases in approximately the same manner from maturation to very old age as a consequence of aging, the conclusion can be drawn, that the synthesis of sulphated glycosaminoglycans, per cell does not decrease with age, but remains essentially the same, or may even be raised temporarily.

A comparable aging process has been demonstrated in vessels from experimental animals. Fig.71 shows an example from the rat. This decrease in the rate of incorporation of ^{35}S-sulphate into the aorta during maturation and aging corresponds to the decrease in the activity of sulphate-activating enzymes in the cardiovascular connective tissues during aging. The first graph of Fig.71 shows the decrease in the rate of ^{35}S-sulphate incorporation by the rat aorta during maturation and aging up to the third year of life (in terms of dry weight). The second graph shows that the decrease in the DNA content of the rat aorta occurs predominantly during the period of maturation and the first year of life; later on only a small decrease was found by us. The values expressed in the second graph of Fig.71 are related to fresh tissue weight. The third graph shows the rate of ^{35}S-sulphate incorporation in terms of the DNA content, again demonstrating a corresponding decrease during aging. This result is comparable with the findings obtained in two other connective tissues, namely skin and

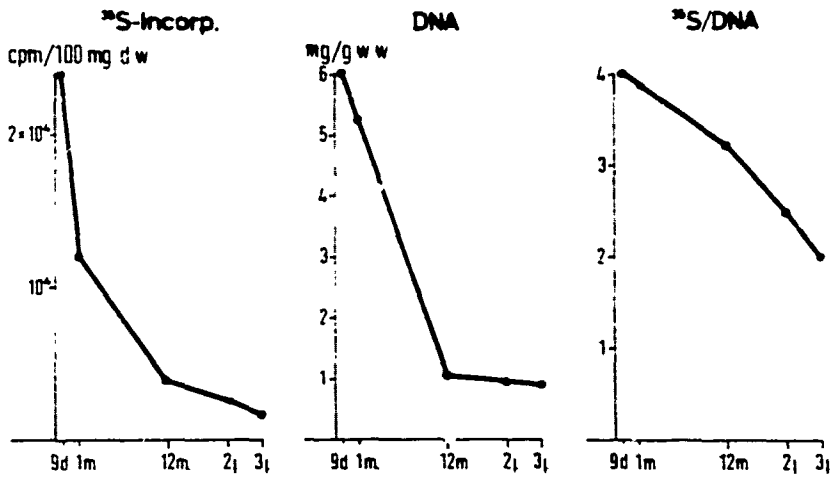


Figure 71: ^{35}S-sulphate incorporation and DNA content of the rat aorta during aging.

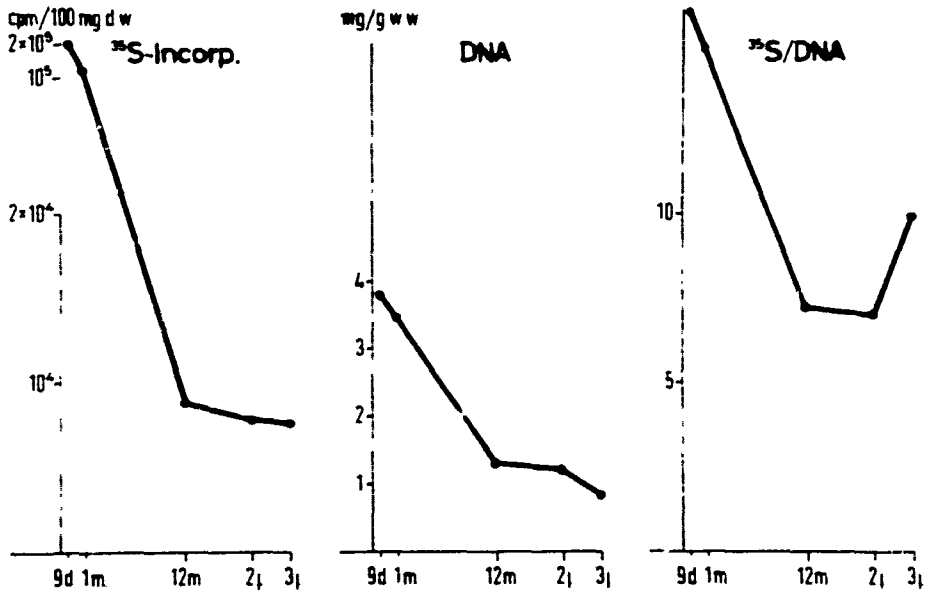


Figure 72: Course of ^{35}S-sulphate incorporation and DNA content of rat cartilage and cutaneous tissue during aging (compare with fig. 71).

cartilage. Thus Fig.72 records the decrease in the rate of ^{35}S-sulphate incorporation by cutaneous tissue of the rat during aging. By comparison of the three graphs in Figs.71 and 73 it can be seen that, whereas there is a marked fall with age in the rate of ^{35}S-sulphate incorporation expressed in terms of DNA content in the vascular tissue, there does not appear to be any comparable change in this parameter in the cutaneous tissue.

In cutaneous tissue (Fig.73) the synthesis capacity per cell is the same in younger as in older rats. In cartilage, as shown in Fig.72, GAG synthesis-capacity increases with age in rats. The validity of all statements concerning age-dependent alterations in various types of connective tissue, including the arterial wall, depends on whether synthesis, degradation, total content turnover or biological half-life times of the proteoglycan and collagen fractions have been examined.

Findings with respect to age changes in human material in these metabolic entities of arterial connective tissue are tabulated in Fig.74. Until now biochemical analyses of the alterations in arterial connective tissue during aging are insufficient, particularly component analyses with determinations of the total content of components of the intercellular matrix because (1) as a rule, no adequate separation of the effects of aging and atherosclerosis has been carried out, and (2) whole vessels i.e., pools of larger vascular moieties, have been examined for methodological reasons, thus levelling out possible analytical differences between individual components.

When acid and neutral polysaccharides, which are estimated together by determinations of total hexosamines are separated, it can be shown that the content of neutral polysaccharides occasionally rises more than the content of acid polysaccharides. With accurate analyses an age-dependent increase in total uronic acid content can be demonstrated. Thus, if the course of polysaccharide changes from prenatal development through postnatal development, maturation, and aging up to very old age is plotted, we find at first a large and then a smaller decrease in the uronic acid, hexosamine and galactosamine content of the human aorta as a quantitative measure of the decrease of the total amount of neutral and acid polysaccharides, and thus of groundsubstance of the vascular wall in old age. This is in contrast with atherosclerosis, which has been discussed before, in which a corresponding increase of the groundsubstance in the intima and media occurs. It is in agreement with the incorporation rates shown above. Alterations in the total content of the glycosaminoglycan fractions dependent on aging are caused by decreasing synthesis (in contrast with increased synthesis in atherosclerotic plaques) (Fig.74).

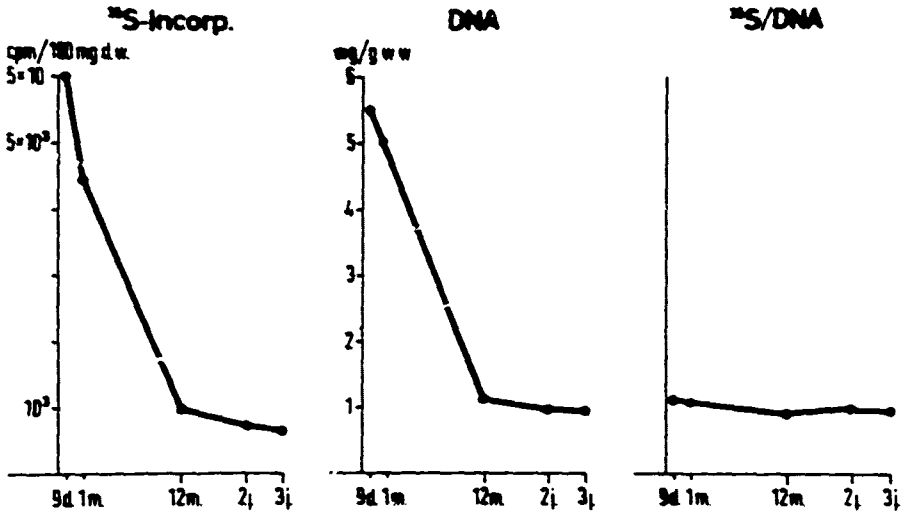


Figure 73: Skin aging.

VASCULAR AGING	SYNTHESIS	DEGRADATION	TOTAL CONTENT	TURNOVER	HALF LIFE TIME
GAG: Hyaluronic acid; C-4-S (A); Derm.sulf.(B); C-6-S (C); Hep.sulf.; Ker.sulf.	↓	↑↓	↓ ↑	↓	↑
COLLAGEN: soluble; insoluble	↓	↓	↓ ↑	↓	↑

Figure 74: Synopsis of the findings on the alterations of the individual metabolic entities of the arterial connective tissue depending on aging.

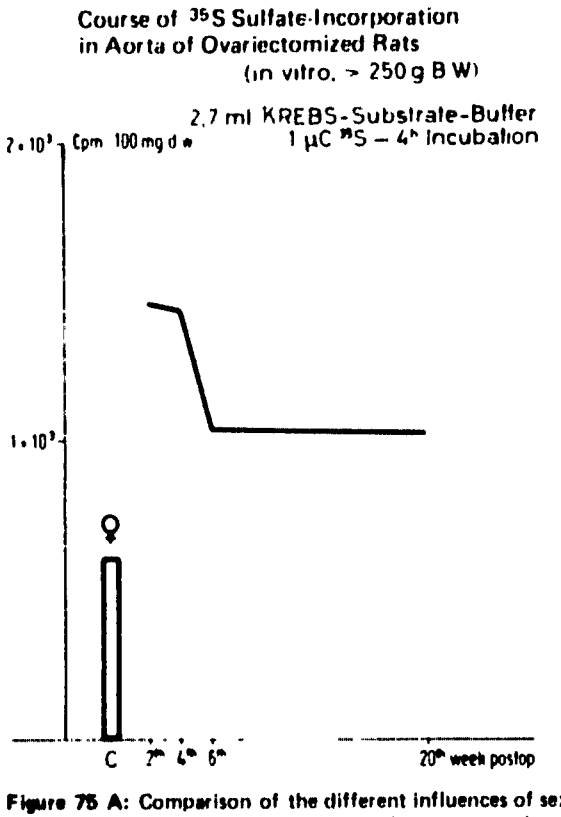


Figure 75 A: Comparison of the different influences of sex hormones on vascular connective tissue, here as example on aorta of the rat a) after ovarectomy.

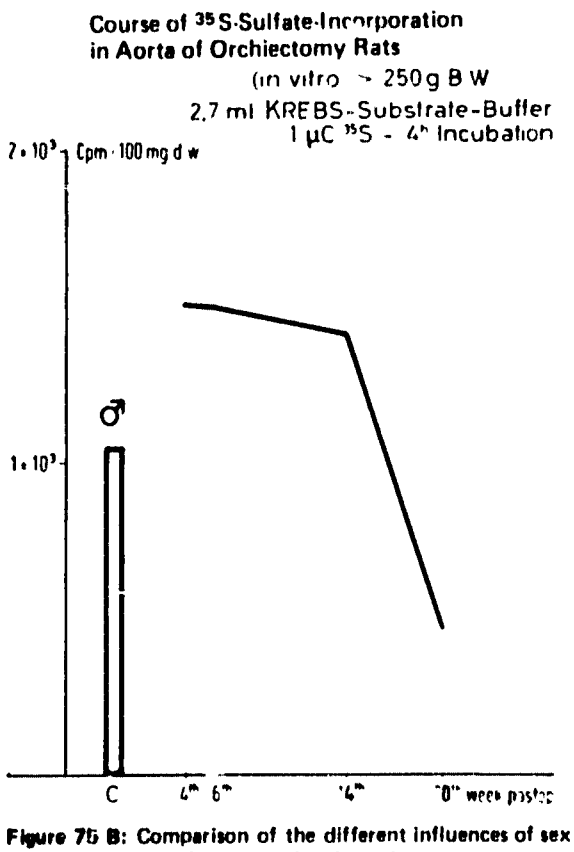


Figure 75 B: Comparison of the different influences of sex hormones on vascular connective tissue, here as example on aorta of the rat b) after orchiectomy until to the 20th week past operation.

Figure 76: Human fetal aorta. Units of elastic tissue consist of a central round core of unstained homogeneous substance (white in photograph) and surrounding coat of microfibrils of the extracellular space. Some microfibrils are cut tangentially and others are seen in cross section. Units fuse with each other to form larger elements of elastic tissue. Glutaraldehyde-osmic acid fixation; Epon-812 embedding; uranyl acetate-lead citrate staining; magnification = x 58,000

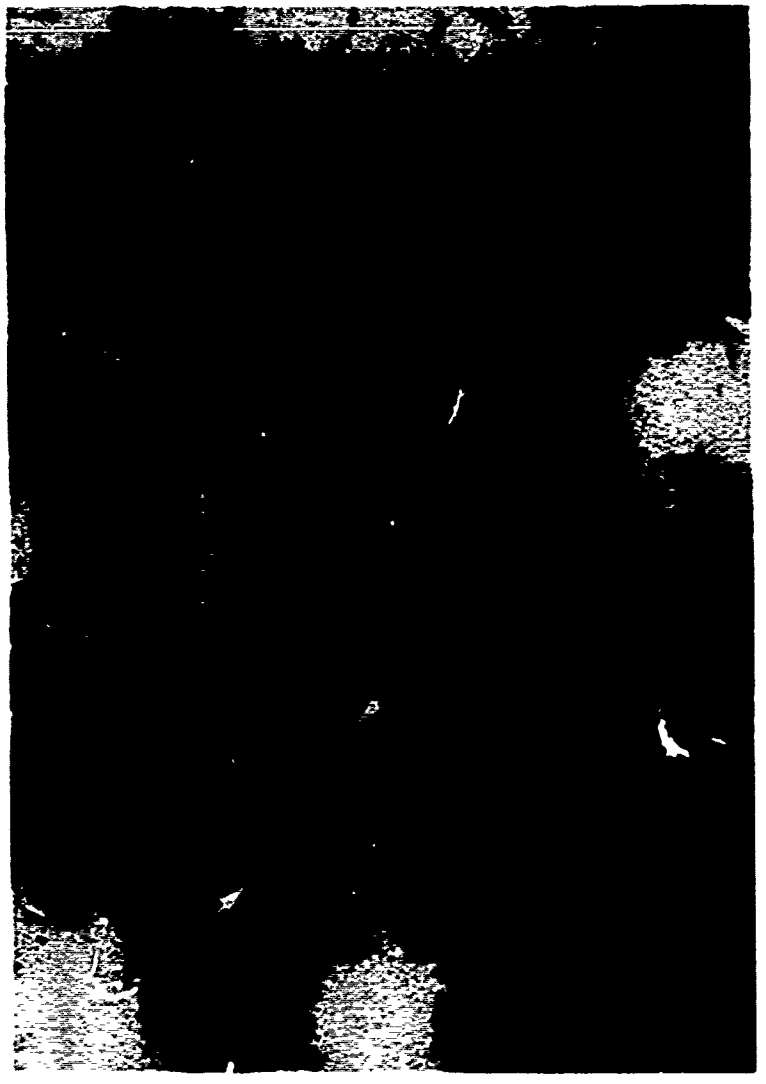


Figure 77: Porcine newborn aorta. Elastic tissue appears black in this photograph. Black stained cores of units fuse to form larger elements of elastic tissue leaving at first spaces between themselves which are filled with the microfibrils (arrows). At a later stage (owing to the process of molding?) these last vestiges with microfibrils disappear from the substance of elastic tissue. Glutaraldehyde-osmic acid fixation; Epon-812 embedding; uranyl acetate-lead citrate staining; magnification = x 72,000

We also have data concerning the influences of sex hormones on ^{35}S-sulphate incorporation into the vessel walls. Fig.75a and b indicates the proteoglycan synthesis in the aorta of ovariectomized and orchiectomized rats. Ovariectomy (Fig.75a) leads to a more than 50% increase in ^{35}S-sulphate incorporation during the first four weeks post operation, followed by a decrease to the sixth week and then a constant rate of synthesis until the 20th week post operation, which is nevertheless 150% higher than the control.

In contrast the synthesis rate in the aorta of orchiectomized rats (Fig.75b) increases and persists at that level until the 14th week post operation. Then there follows a steep decrease to 50% of the control value up to the 20th week. The possible relevance of these findings to changes observed in human vessels after the female climacteric should be explored. There are insufficient data available on the degradation of single mucopolysaccharide fractions. They appear to increase initially during aging, and then to decrease. The turnover of the mucopolysaccharide fractions in the vascular wall decreases with age, their biological half-life increasing.

Morphology of Elastic Tissue

DR. HAUST: I should like to address myself to the matter of terminology employed in the presentations regarding the morphology of elastic tissue. On several occasions reference was made to the translucent component of the elastic tissue. At the time when the concept of the elastic tissue unit was introduced some ten years ago (138, 139, 231) the unit was defined on a morphological basis as consisting of two distinct components: the central round core and the surrounding microfibrils of the extracellular space (135, 140). Initially, the central core did not take up stains in preparations for electron microscopy and it was therefore termed "translucent" (Fig.76). Subsequently, however, it was possible to stain the core at times (Fig.77) and thus, the term "translucent" can no longer apply. It would be advantageous if at this conference a decision were made no longer to refer to the core of the unit of elastic tissue as the translucent component. Perhaps it could be more properly replaced by the term "homogeneous". It is of interest that this homogeneous component remains the dominant feature of the various elastic tissue elements that result from fusion of the individual units, whereas the microfibrillar component disappears as a distinct morphologic element within larger elastic tissue elements (Fig.76).

DR. ROBERT: I am glad you brought up this problem because that will give me a second chance to clarify a problem of semantics which has been confusing the field of elastin for thirty years. Now many eminent people wrote a great deal of confusing things on elastic tissue, as you know. I certainly have to give credit to you, Dr. Haust, that you are one of those pathologists who did not

say anything confusing and I think your idea of the "elastic unit" was at that time a very wise proposition. Now the problem is that I don't think we can any more define the "elastic unit" in the same way because we believe that we have to distinguish between elastic tissue and elastin. Elastin, as we know now, is the polymer of a relatively well defined protein: proelastin, and as I have said, we have to start with this proelastin which was isolated from several animals. Now this is quite a polar protein and if you can get it pure I am sure it would react to everal of the electron microscopic staining agents, such as PTA fo· instance. The second component of elastic tissue is the structural glycoprotein-microfibril; this is again another protein completely different from elastin. You may find it completely independent of elastic tissue. That is what I was trying hard to get through yesterday. Now it happens that the smooth muscle cell secretes both of these proteins and the formation of elastic tissue has to start with microfibrils. On these negatively charged units, the proelastin which is positively charged can probably aggregate by coulombic forces and the cross-linking catalyzed by a specific lysine oxidase can go on in a precisely oriented manner. That is what we call "vectorial synthesis".

Otherwise if this cross-linking would go on in a homogeneous solution containing a mixture of proteins together with proelastin, you could never get such a thing as a structured tissue, as are the concentric elastic lamellae of the aorta. "Elastic unit" as defined by Dr. Haust referred to these two components (microfibril and proelastin polymer). As you know, many people described in "plaque elastin" or "atherosclerotic elastin" an increase in polar amino acids, that would have meant that the elastin is changing its amino acid composition in pathological conditions. What really happened is that they overlooked this microfibrillar component. Or else the glycoprotein component increases again in atherosclerotic aorta by a mechanism we still do not understand quite clearly.

The adjective "translucent" just means that polymeric elastin in its native state does not react with some stains used (uranyl acetate, etc.). It's like blue skies so I don't object to change any names to any other, until we define more precisely the molecular terminology of elastic tissue. Now again yesterday I showed you the picture where after 8M urea-0.1 M mercapto-ethantol extraction the elastic lamellae are no more "translucent" but do stain with uranyl acetate Pb or with phosphotungstic acid (Fig.78). No more "microfibrils" can be seen, they are in the urea-extract (Fig. 78C). We think that these urea-resistant structures might be the cross-linked elastic units (= proelastin) with the microfibrils gone.

DR. HAUST: Well, it may be appropriate for me, as one of those who originally introduced the designation "translucent" to state again that it is incorrect and should not be used. And in reference to some other comments made by Dr. Robert the following should be pointed out: (1) It was shown in the early sixties that the microfibrils are present "free" in the extracellular space apparently unrelated to the elastic tissue (135, 203) and it became even then a well recognized fact that several cell types, including the smooth muscle cells, are capable of their formation (135, 140). (2) The proposal that it is the microfibrillar component that induces or initiates the formation of elastic tissue is perhaps at present not at all an established fact. At best this may be true in some circumstances, but not necessarily in all. Indeed, at a recent Workshop on Arterial Wall Mesenchyme at New Orleans (April 1973), Dr. Daoud from Albany showed that in tissue explants proliferating smooth muscle cells contain in their cytoplasm round, small homogeneous structures having an appearance very similar to, or even identical with, the unit cores of elastic tissue. It is conceivable that, at least in tissue cultures, these intracellularly formed cores are extruded from the cells and acquire subsequently a coat of microfibrils. Perhaps Dr. Daoud himself would care to comment on that subject in more detail.

DR. ROBERT: Well, I can answer but it will really not take us very far. I could answer with another question. Did anybody demonstrate the formation of elastic tissue without microfibrils? Structural glycoproteins-microfibrils appear significantly earlier during phylogenesis than elastin: we could isolate recently such glycoproteins from several species of sponges (170) with a very similar amino acid composition to that of vertebrate structural glycoproteins. Elastin appears much later and all investigated elastic tissues do contain structural glycoproteins. Now I agree with the other half of your comment. We isolated the structural glycoproteins from every investigated connective tissue whether elastin was present or not and I was referring to that yesterday (284, 287, 288, 289, 290, 291). So I think we agree again on the essential issues.

DR. DAOUD: In tissue culture of explants we have observed clear units surrounded by microfilaments. In some of the cells we have demonstrated a translucent structure which has the same measurement and the same shape as the center of the unit. We attempted enzymetic digestion of these structures but were not successful. We are not yet sure whether these intracellular structures are really elastin or not, although they do have the same configuration and the same size.

DR. WIGHT: Yesterday, I think there was some confusion concerning the possible interaction of collagen and proteoglycans in

the arterial wall. Numerous chemical and morphological studies (11, 83, 91, 204, 206, 207, 213, 234, 340) have demonstrated that proteoglycans interact with collagen in vivo and in vitro. The majority of this work has utilized cartilage tissue to demonstrate this interaction. However a recent study (172) has suggested that proteoglycans interact with collagen in developing chick embryo aortas. We also have data to suggest that proteoglycans are bound to collagen in the arterial wall. Small segments of normal and hyperplastic iliac arteries from one year old pig tail monkeys (Macaca nemestrina) were fixed in the presence of ruthenium red, an electron stain that has been reputed to have some staining specificity for proteoglycans (206, 207) and processed for electron microscopy. Small 200-300 A° diameter ruthenium red positive granules were deposited on the outer edge of the collagen fibers at the major period band (Fig.79). Treatment of this tissue with testicular hyaluronidase prior to ruthenium red exposure eliminated the collagen associated ruthenium red granules leaving the collagen fibers intact (Fig.80). These findings indicate the proteoglycan nature of these ruthenium red granules.

Glycosamino-Glycans

MONKEY

I would also like to add one more observation concerning the arterial smooth muscle cell that came from these ruthenium red studies. Examination of unstained sections from arterial tissue fixed in the presence of ruthenium red revealed that a thin coating of ruthenium red positive material existed on the outer surface of the smooth muscle cell's plasma membrane (Fig.81). This coating was observed in addition to the characteristic basement lamina of the smooth muscle cell which also demonstrated an affinity for ruthenium red (Fig.82). Testicular hylaruonidase did not appear to alter this staining pattern. Although the chemical nature of the surface coat and basement lamina cannot be ascertained from these studies, such observations do suggest that the surface of the smooth muscle cell and associated basement lamina are strongly anionic. The anionic nature of the surface of this cell may be important in the selective uptake of certain macromolecules such as positively charged B lipoproteins (199).

DR. RUEGG: I should like to discuss the similarities and differences in the contractile mechanism of arterial smooth muscle as compared to that of skeletal muscle. We shall focus our attention on the protein myosin which is an ATPase. Professor Somlyo told you this morning that arterial muscle, like skeletal muscle, consists of thick and thin filaments which may slide past each other during contraction.

Contractile Proteins of Arterial Smooth Muscle and their Mechanism of Action

Figure 78 C: The urea-mercaptoethanol extract of a purified elastin preparation, dialysed and lyophilised. Notice the presence of "microfibrillar" structures.

Figure 78 B: Same preparation as in A but after repeated extractions with 8 M urea-0.1 M mercaptoethanol. Notice the swollen elastic lamellae and the appearance of a granular structure inside the lamellae reacting with the electronic strain (uranyl acetate-Pb, x 42,500).

Figure 78 A: Purified elastin from bovine ligamentum nuchae. Notice "microfibrillar" structures and amorphous elastic lamellae (uranyl acetate-Pb, x 42,500).

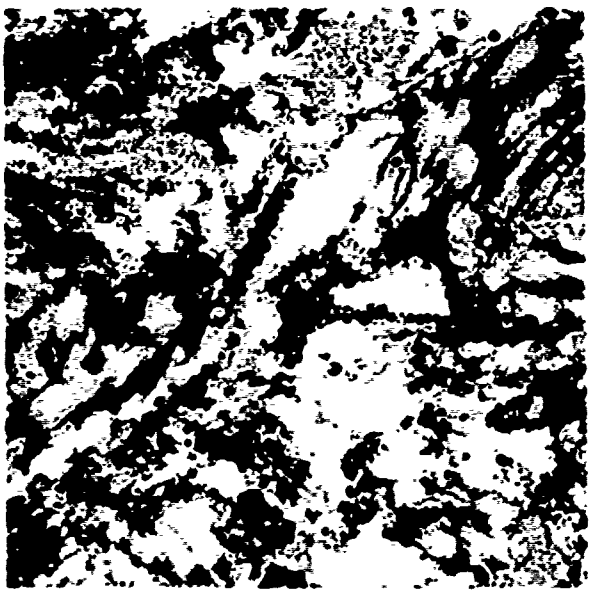


Figure 79: An area of extracellular matrix from a hyperplastic arterial intima, fixed in the presence of ruthenium red. Note the electron dense granules associated with the major period band of the collagen fibers. The section is stained with uranyl acetate and lead citrate. x 40,000.

Figure 80: An area of extracellular matrix from a hyperplastic arterial intima which has been treated with testicular hyalurinidase (0.1%) for 1 hour prior to fixation in the prevence of ruthenium red. The section is stained with uranyl acetate and lead citrate x 64,000.

Figure 81: An arterial intimal smooth muscle cell fixed in the presence of ruthenium red. Note the staining on the outer surface of the plasma membrane. The section is not stained with uranyl acetate or lead citrate x 74,000.

Figure 82: Portion of an arterial smooth muscle cell fixed in the presence of ruthenium red following testicular hyaluronidase treatment. Surface coat and basement lamina demonstrate affinity for ruthenium red. Section is not stained with uranyl acetate or lead citrate x 81,000.

The thin filaments contain mainly actin and in addition, the regulatory proteins tropomyosin and troponin. The thick filaments contain mainly, but not exclusively, myosin which is aggregated within the thick filaments in such a fashion that the shaft of the molecule lies within the filament, while the doublehead of the molecule and the neck region project out of the filament to form cross-bridges towards the thin actin filament. In the relaxed state these cross-bridges do not attach to thin filaments. In contraction on the other hand, the cross-bridges do attach themselves to the thin filaments, thereby forming cross connections between sliding thick and thin filaments. Under strictly isometric conditions the cross-bridges generate tension by performing a rotational movement, whereby the elastic region of the bridges becomes stretched by roughly 100 Å units, so that an elastic tension is produced.

If the conditions are not isometric, the stretched spring exerts a pulling action on the actin filament, which then slides past the thick filament and in this way movement is produced. Of course the movement produced by a single action of a cross-bridge is extremely small. But larger movements are produced if the myosin heads adhering to the actin filaments move not only once but many many times. In this way, they pull the actin filaments by a repetitive action of the bridges much like a ship's crew which can only haul in a long rope by repetitive heaves. A repetitive cross-bridge action based on the rope-hauling principle is not only occuring during shortening of muscle fibers but even under isometric conditions, when continuous tension is maintained. Here too, cross-bridges continuously attach and detach in cycles; but despite this cyclic action tension does not oscillate because cross-bridges act asynchronously.

Description of the Cross-bridge

The energy for the described repetitive mechanical performance of the cross-bridges is derived from the splitting of one or two molecules of ATP in each cross-bridge cycle. Presumably, ATP is split each time a cross-bridge interacts with actin and performs a cycle. Without the intervention of actin the ATPase activity would be very low under physiological ionic conditions, that is at low ionic strength, and in the presence of high concentrations of Mg. Indeed the ATPase activity of myosin alone is extremely low without actin, but it is greatly activated as soon as myosin is allowed to react with actin. As shown in Fig.83 this is also true for the arterial myosin ATPase. U. Mrwa succeeded in preparing pure myosin from arteries and together with D. Trentham of Bristol determined some of its enzymatic characteristics.

The lower curve shows the ATP splitting by myosin in the presence of Mg ions and in the absence of actin. Phosphate release

is shown as a function of time. Note that the time progress curve is linear after about five minutes. The rate is extremely low. Only about one mole of ATP is split by one mole of myosin in ten minutes. In other words, one molecule of myosin requires, under these experimental conditions, about ten minutes to split a molecule of ATP. In the presence of an excess of actin the rate of splitting is greatly increased. This signifies presumably that in arterial muscle too, cross-bridges split ATP only when they interact with actin in the course of the cross-bridge cycle. This kind of actin-dependent ATP splitting can only occur in the presence of trace Ca ions as in the case of skeletal muscle contractile proteins. At pH 7 the threshold of Ca concentration is about 10^{-7} molar, 50% activation is obtained at the Ca concentration of roughly 10^{-6} molar and maximal activation at the Ca concentration of 10^{-5} milar. A similar Ca dependence is found in actomyosin systems from skeletal muscle. There are, however, two important differences between the skeletal muscle system and the smooth muscle system. The first difference: in the arterial system but not in skeletal actomyosin the Ca sensitivity is strongly pH-dependent: if the medium is acidified to pH 6.5, which may well be metabolically possible, about ten times higher Ca concentration is required to produce the same degree of activation as at pH 7. For this reason we might assume that a metabolically induced acidification of the cell medium could induce an uncoupling of excitation and contraction in arterial smooth muscle, but the same metabolic acidification would not have such an effect in the case of the skeletal muscle contractile system.

A second important difference of the arterial and skeletal system concerns the absolute ATP splitting rate. In skeletal muscle actomyosin up to 10 moles of ATP are split per one mole of myosin in 1 second, which means that one molecule of myosin splits one ATP within about 100 msec. In smooth muscle the ATP splitting reaction takes at least 10 seconds, i.e. the ATP splitting rate is about 0.1 mole of ATP per mole of myosin per second. In other words the speed of ATP aplitting is presumably about 100 times slower in the smooth muscle actomyosin compared with the striated muscle actomyosin. What does this mean in terms of the cross-bridge cycle of smooth muscle?

It is likely that one molecule of ATP is split in each cyclic operation of a cross-bridge. The low enzymic activity of arterial actomyosin then probably means that cross-bridges of arterial smooth muscle operate only once every 10 seconds in contrast to frog sartorius, where they may operate about 10 times/second in order to maintain tetanic tension. During the tension maintenance the energy derived from ATP splitting is used for the internal work, presumably the work done in stretching the elastic parts of the cross-bridges during the rotational movement of a myosin head.

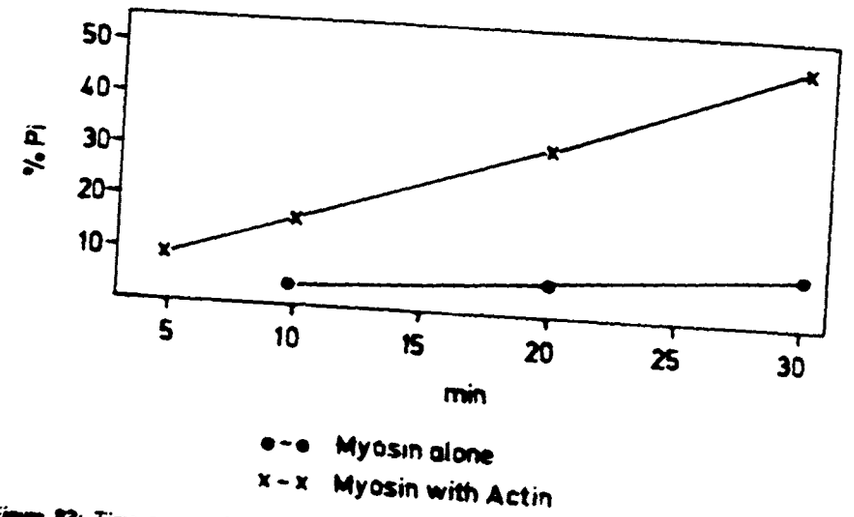


Figure 83: Time course of phosphate release and ATP split (in per centage of total ATP) arterial myosin alone, x with added skeletal muscle actin (incubation conditions.) 1 mM ATP, 10 mM Mg Cl_2, pH 7, ionic strength 0.1.

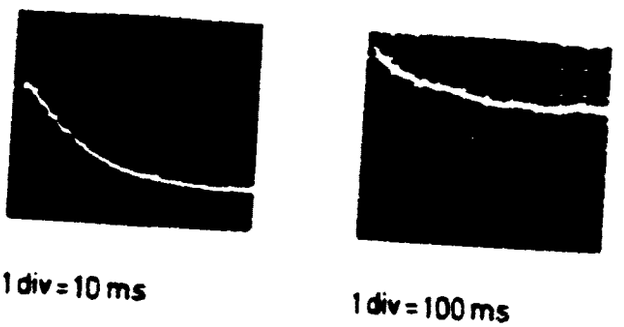


Figure 84: Time course of turbidity change of Actomyosin from skeletal muscle (left) and arterial muscle (right) after addition of ATP (0,2 mM). Conditions 5 mM Mg. Cl_2, pH 7 ionic strength 0,1 T = 20C.

In smooth muscle one cycle is performed within about 10 seconds and in this length of time one molecule of ATP is split. In striated muscle 100 molecules of ATP would be split and 100 cross-bridge cycles would be performed in the same length of time. This means that much more energy has to be expended to maintain tension in striated muscle than in the case of smooth muscle. Smooth muscle is indeed very economical in maintaining tone, because its cross-bridge cycling is so slow. Once the cross-bridges attach to the actin they obviously hold on to it for a very long time, they don't let it go as quickly as they do in the case of skeletal muscle. In other words, cross-bridges do not readily detach from actin once they are attached. The rate constant of detachment which is produced by the action of ATP should, if this schema is correct be much slower than in the case of skeletal muscle actomyosin. The detachment is - as we have seen - brought about by the intervention of ATP which separates the reaction partners, actin and myosin.

This reaction, namely the separation of actin and myosin by ATP, can indeed be investigated in vitro with isolated actin and myosin. The question arises then whether in the in vitro reaction too, the dissociation of the actin-myosin-complex by ATP is very much slower in smooth muscle than in striated muscle. That this is so has recently been demonstrated by Mrwa in collaboration with D. Trentham in Bristol.

The principle of this measurement is briefly the following: if actin and myosin are mixed together they form a highly viscous turbid actomyosin which lets very little light pass through. In other words the light absorbence is extremely high. Addition of ATP dissociates the actomyosin complex, therefore the light scattering decreases and hence more light passes through; in other words, the apparent absorbency decreases. The time course of the absorbence change reflects then the time course of the actin-myosin dissociation. Now let us see how this time course differs in actomyosin of skeletal and smooth muscle (Fig.84). The speed of the exponential reaction can be expressed by the time constant, i.e., by the time required to reach about two thirds of the final drop of absorbency. The reciprocals of these values reflect the first order rate constants. They are 20/sec in the case of skeletal muscle actomyosin and 4/sec in the case of smooth muscle actomyosin. Hence it may be concluded that ATP dissociates actomyosin from striated muscle much more quickly than in the case of the contractile proteins of smooth muscle. The rate constants are also dependent however on the concentration of ATP, increasing with increases in the concentration of ATP. In the myosins of striated muscle there is a dependency on ATP concentration also, but the ATP concentrations required to produce a dramatic dissociation effect are much smaller than in smooth muscle contractile

proteins and it is also seen that at all concentrations of ATP smooth muscle actin-myosin dissociation proceeds at a slower rate than the dissociation of striated muscle contractile proteins. This results from the tendency of the smooth muscle myosin cross-bridge to hold on to the actin for a long time, a property which is one of the reasons for the high holding economy of smooth muscle on the other hand, and for its slowness on the other hand.

What general conclusions can be drawn from these findings? First it is seen that in arterial contractile proteins there is a high ATP requirement of at least 5 millimolar for a proper function. If under certain metabolic conditions the ATP concentration should drop below this concentration in the living cell, the proper function of the contractile system is not guaranteed, the function of the contractile cross-bridge cycle may then be impaired, especially relaxation, which is the dissociation of actin and myosin.

Second, it has been shown that smooth muscle actomyosin dissociation by ATP is slower than striated muscle actomyosin dissociation. Obviously one of the essential rate constants of the smooth muscle cross-bridge cycle, i.e., the dissociation of the bridge from the actin by the intervention of ATP is very much slower than in the case of skeletal muscle. Of course, theoretically, this difference may be due to each of the two reaction partners, i.e. it may be due to either actin or myosin. But in fact it can be shown that the essential difference lies within the smooth muscle myosin and not within the actin, for the same slow kinetics of actomyosin dissociation can also be found in hybrid actomyosins made from arterial myosin and skeletal muscle actin. In addition we have seen that not only one of the essential steps of the cross-bridge cycle, i.e. the dissociation of the bridge from actin, is very slow in smooth muscle, but the whole cross-bridge cycle is indeed slower. This has been inferred from measurements of the enzymatic activity which have shown that the rate of ATP splitting by smooth muscle actomyosin is about 100 times slower than the rate of splitting by skeletal muscle actomyosin.

Again, in this case too, the essential enzymatic activity is due to a difference of the myosin partner and not to differences of the actin partner. One should then investigate in what respect the myosin of smooth muscle differs structurally from the striated muscle myosin. Due to the investigations of Mrwa and Trentham one already knows that presumably the content of tryptophane and tyrosin is lower in the smooth muscle preparation; also smooth muscle myosin contains only two so-called light chains, while striated muscle myosin contains three light chains per molecule.

In this brief report I have tried to show you some enzymatic differences between the myosins of smooth and striated muscle,

differences which may be functionally significant because they are presumably the reason for the slow cross-bridge cycling in smooth muscle contractile systems and hence for the slowness and the high holding economy of the smooth muscle. Using similar techniques it may also be possible later on to demonstrate not only differences between smooth and striated muscle myosin, but possibly also between myosins from normal and diseased smooth muscle. But this is for the future.

Histochemistry of Smooth Muscle

DR. ADAMS: I propose to compare the enzyme histochemistry of arterial smooth muscle, bronchial smooth muscle and two groups of phagocytes-reticuloendothelial macrophages and atheroma lipophages. The enzymes surveyed do not represent a complete range of available histochemical techniques, but rather were deemed relevant to a comparison of these various cell types.

As can be seen from Table XV, all four cell-types contain $NADH_2$-reductase and a variety of other dehydrogenases (1, 429). However, amongst those we have tested, it is only succinic dehydrogenase that discriminates amongst these cell types. Arterial smooth muscle predominantly respires anaerobically, as shown by its low content of succinic dehydrogenase. By contrast, bronchial smooth muscle contains an abundance of succinic dehydrogenase, which may reflect its proximity to atmospheric oxygen. Intestinal and ureteric smooth muscle occupy an intermediate position in their activities of succinic dehydrogenase (2). Most macrophages seem to respire anaerobically (175), but the pulmonary alveolar macrophage - like bronchial muscle - respire aerobically (175).

Non-specific esterase is an enzyme of uncertain function. It is distributed among all the cell-types under consideration, but its activity in arterial smooth muscle varies among different species (See footnote to Table XV). An interesting feature about this enzyme is that it is one of several enzymes which are present in high concentration in the arterial smooth muscle of atheroma-resistant species and, conversely, are relatively inactive in the muscle of atheroma-susceptible species (429). The high activity of non-specific esterase in the rat and low activity in the rabbit and man es exemplary. Wolman (427) considers that non-specific esterase at pH 5.5 is equivalent to lysosomal acid lipase, and it has been suggested that one fraction of this lipase can hydrolyse cholesterol esters (194). The esterase at low pH shows a similar species variability to the enzyme at neutral pH.

Non-specific cholinesterase distinguishes smooth muscle from the macrophage and atheroma lipophage, but the activity in vascular smooth muscle is too variable amongst the different species

examined to be reliable for this purpose (See footnote 2 to Table XV). Nobody knows what this enzyme's function is but perhaps muscle possesses the non-specific enzyme to protect its cholinergic receptors from being swamped and confused by an excess of non-specific choline esters (i.e., those that are not acetyl choline). Alternatively, non-specific cholinesterase may protect true cholinesterase from the action of non-specific inhibitors (197).

Phosphorylase also distinguishes smooth muscle from the two phagocytes being discussed, but again is variable in vascular smooth muscle among the species examined (see footnote 3 to Table XV). Acid phosphatase is a marker for lysosomes, and not unnaturally is prominent in phagocytes and undemonstrable in normal smooth muscle. Alkaline phosphatase is usually regarded as a marker for endothelium in smaller blood vessels and is absent from smooth muscle fibers (1). Pyrophosphatase, as shown by a technique being developed by Dr. Brian McArdle, is prominent in smooth muscle but absent from the phagocytes, but again the smooth muscle reaction varies between species (see footnote 2 to Table XV). Leucine aminopeptidase is absent from all four cell types.

The most useful enzyme for distinguishing the reticuloendothelial phagocytes (histiocyte and macrophage) from all smooth muscle and from the atheroma lipophage is the catalase reaction (2). The Novikoff-Goldfischer (241) technique with diaminobenzidine (pH 9) distinguished cyanide resistant catalase in macrophages from the cyanide-resistant benzidine peroxidase (pH 7) in granulocytes and erythrocytes. In practice, considerable cross-reactivity is seen in these cells when the "catalase" and "benzidine-peroxidase" techniques are applied without the relevant inhibitors, but this does not affect the value of catalase as a marker to distinguish macrophages from smooth muscle and atheroma lipophages. The relevance of this distinction to atherosclerosis is discussed on page

Aging and Hypoxia in Smooth Muscle Cell Cultures

DR. ZEMPLENYI: I will begin with a brief review of some of the evidence pointing to tissue hypoxia as an important factor in the intermediary metabolism of the artery.* Afterwards, I plan to discuss the reasons for choosing cultured arterial smooth muscle cells as a convenient model to clarify the mechanisms of the effect of hypoxia. Finally, I will show our results obtained with smooth muscle cultures.

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* This contribution was prepared as "Studies on the Effect of Hypoxia on Arterial Smooth Muscle Cell Cultures", J.F. May, T. Zemplenyi, W. J.Paule, V.K.Kalra, D.H.Blankenhorn, and A.F. Brodie, of the University of Southern California, Los Angeles, California.

TABLE XV

ENZYME HISTOCHEMISTRY OF SMOOTH MUSCLE

	Smooth muscle		Macrophages	Atheroma lipophages
	Arterial	Bronchial		
$NADH_2$-reductase	+	+	+	+
SDH	weak	+	weak	weak
Nonspecific esterase	+[1]	+	+	+
Nonspecific cholinesterase	+[2]	+	-	-
Phosphorylase	+[3]	+	-	-
Acid Phosphatase	-	-	+	+
Pyrophosphatase	+[2]	+	-	-
Alkaline Phosphatase	-	-	-	-
Leucine Aminopeptidase	-	-	-	-
Catalase	-	-	+	-
Benzidine Peroxidase	-	-	+	-

1. Rat strong, human and rabbit weak
2. Rat and rabbit strong, human -ve
3. Rabbit strong, human and rat -ve

It has been shown by Wolkoff in 1923 that in a fully developed adult coronary artery, the thickness of the subendothelial layer may exceed the thickness of the media (426). It consists not only of a musculo-elastic layer, but also of an elastic-hyperplastic layer and sometimes even of an innermost connective tissue layer containing a high proportion of collagen. Since the oxygen supply of such an artery is almost entirely dependent upon diffusion from the lumen, the thicker the intima, the more impaired will become the deeper layers of the arterial wall. The oxygen supply of the thin venous wall derived from the lumen is much more advantageous, and this can be inferred, for example, from a comparison of the lactate dehydrogenase isoenzyme pattern of arterial and venous tissue.

PIG Fig.85 shows the distribution of LDH isoenzymes in pig aorta and vena cava. The left bars represent averages of aortic, and the right ones averages of venous findings together with the standard errors of the means. One can see very clearly the prevalence of the faster-moving fractions in the veins with the absence of any activity in the last two slow-moving fractions. In the aortas, on the other hand, there is a definite shift of activity toward the more slowly-moving electrophoretic fractions (430).

For interpretation of this and similar data, we must, of course, bear in mind that, according to the theory of Dawson et al. (74), the slow-moving cathodic LDH fractions are the principle isoenzymes in anaerobically metabolizing tissues, while the fast-moving fractions are the most abundant isoenzymes in tissues where a steady supply of energy is maintained by oxidation. Despite some possible doubt as to the general validity of this hypothesis, the prevalent consensus is that LDH isoenzyme patterns reflect long-term metabolic conditions of oxygen availability.

HUMAN Fig.85 shows that in atherosclerotic human arteries the prevailing LDH isoenzyme fraction is the slow-moving anaerobic LDH electrophoretic band. This is in sharp contrast with the pattern observed in healthy human or pig arteries where the fast-moving aerobic fractions are dominant. These findings agree with data by Lojda and Fric (202) who observed, in addition, that in arteries of children the aerobic fractions showed the highest activities.

It appears that a slightly injured artery also has to depend more on glycolysis than does a healthy artery. For example, Lindy et al (201) observed that damage caused by pulling an inflated balloon catheter through the rabbit aorta resulted in elevation of anaerobic LDH isoenzymes. Hypertension had the same effect in the experiments of Will (421) using miniature pigs.

There exist, of course, other data corroborating the import-

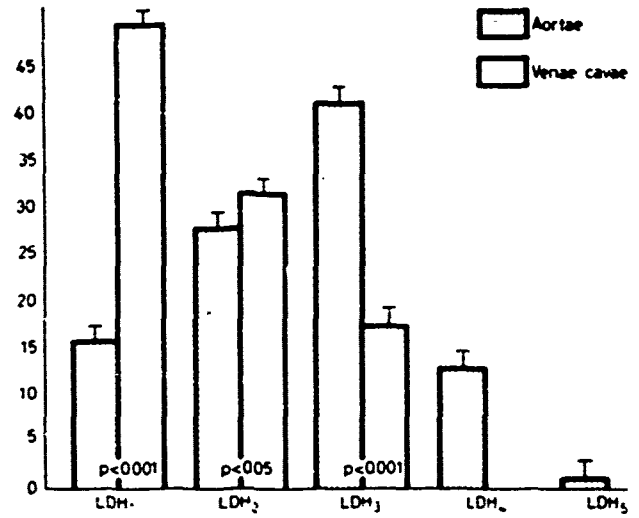


Figure 86 A: Distribution of LDH isoenzymes in 19 pig aortae and venae cavae. (From Zemplenyi and Blankenhorn, Angiologica 9:429, 1972, with permission of the editor and publisher of Angiologica).

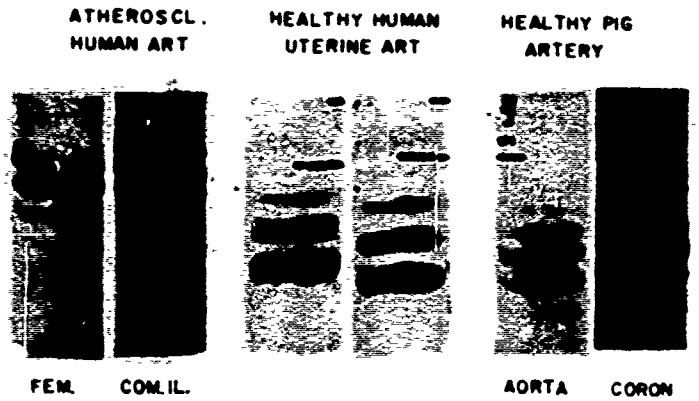


Figure 86 B: Cellulose acetate electrophoresis of LDH isoenzymes in normal human and pig artery and in atherosclerotic human artery. (From Zemplenyi and Blankenhorn, Angiologica 9:429, 1972, with permission of the editor and publisher of Angiologica).

ance of oxygen availability in atherogenesis. From the work of Astrup et al (17), Helin and Lorenzen (145), and others, it is known that tissue hypoxia is an essential factor in accelerating experimental as well as human atherosclerosis. It was suggested that the underlying mechanism consists of increased arterial permeabili y as a result of hypoxia. There is good evidence in this regard from the work of other investigators as well. However, insufficient oxygen supply was reported also to stimulate arterial lipogenesis. The work of Kresse et al (190) and of Filipovic and Buddecke (99) demonstrates, for example, that under hypoxic conditions in the CALF healthy calf aorta there can be detected an unequivocal increase of ^{14}C incorporation from labeled acetate into triglycerides and fatty acids. Furthermore, Howard (153) found a substantial increase of (2-^{14}C) glucose incorporation into total lipids under hypoxic conditions.

Let us now turn our attention to the arterial smooth muscle cell. It is clear to the participants of this symposium that although as early as 70 years ago some German pathologists suggested involvement of intimal smooth muscle cells in atherosclerotic lesions (for details see Geer and Haust, 113), such a role for smooth muscle cells has been considered unlikely and has been almost completely neglected. However, the application of electron microscopy and immunochemical techniques to this problem has confirmed that the arterial smooth muscle is a key component of the atherosclerotic lesion (113, 427).

Smooth Muscle Cells as Foam Cells

It is conceded that the great majority of the foam cells of the lesion are smooth muscle cells filled with lipid and that only a slight fraction are perhaps macrophages that might have ingested lipid prior to entering the artery from the blood. The formation of foam cells, which is a focal process is associated with proliferation of the intimal smooth muscle cells. Studies using tritiated thymidine or mitotic indices after colchicine injection have unequivocally demonstrated an increase in smooth muscle cell multiplication in response to local injury as well as to cholesterol feeding (216, 393). According to Buc'' (54), following vascular injury there is proliferation of smooth muscle cells in the media and entrance into the intima either through fenestrations or ruptures in the internal elastic membrane.

The proliferating smooth muscle cells produce connective tissue elements as well. This was demonstrated by incorporation of tritiated lysine into elastin and of labeled sulfate into mucopolysaccharides. The elegant experiments of Ross and Glomset (317) indicate that endothelial injury in vivo may promote smooth muscle cell proliferation by increasing the concentration of plasma proteins, especially low density lipoproteins, in the extracellular fluid of the arterial wall.

Figure 86: Medial smooth muscle cells after two weeks in culture. They range from fusiform to stellate in outline. Lipid droplets are present in the cytoplasm in addition to granules representative of mitochondria and lysosomes. (phase contrast, x 225).

Figure 88: Cultured medial cell, fixed and strained with silver to demonstrate the presence of bundles of myofilaments. (x 410).

Figure 87: Sparse population of medial smooth muscle cells after five weeks in culture. Most of the cells have acquired cytoplasmic striations suggestive of the presence of bundles of myofilaments. (phase contrast, x 260).

Figure 89: Ultrastructure of medial cells after five months in culture. N, nucleus; MF, myofilaments; M, mitochondria; ER, rough endoplasmic reticulum; BL, basal lamina; D, fusiform dense body or "attachment plaque." (x 15,700).

However, the stimulus for and the mechanisms of transformation of the smooth muscle cell into the lipid filled foam cell are not clear. The role of hypoxia in this regard is suggested from studies by Robertson (296). Using human and animal intimal cells, he observed that one type of flat polygonal cells ("atherophils") changed under unfavorable environmental conditions, including hypoxia, into "atherocytes". Oxygen concentrations below 5% induced significant increases in incorporation of exogenous cholesterol by these cells.

Hypoxia and Aging

In view of these and similar data, we felt it important to study in more detail the effect of hypoxia on the morphology and biochemistry of the arterial smooth muscle cell in culture. Smooth muscle cultures were obtained from the media of aortas of 3-4 week old piglets. After two weeks in culture, as shown in Fig.86 smooth muscle cells are fusiform to stellate. Nuclei are vesicular and are centrally located. Mitochondria and other organelles are also seen.

Fig.87 shows cells after five weeks in culture. They have a stellate appearance while their population is sparse, and they assume a fusiform shape as they increase in number. Most of the granules in the cytoplasm are mitochondria, but some are lysosomes. The filamentous cytoplasm is indicative of bundles of myofilaments. As the cultures age, the cells become confluent, line up in groups, and become polarized.

As shown in Fig.88 the presence of myofilaments was confirmed by the use of silver stain. This appearance remained stable over a period of more than five months. Fig.89 is an electron micrograph of cultured cells after five months. The cells have an appearance which is very similar to smooth muscle cells in the intact aorta. The nucleus (N) is oval and contains dense areas of chromatin. The cytoplasm is packed with myofilaments (MF) and small amounts of rough endoplasmic reticulum (ER). Characteristic exceedingly dense mitochondria of smooth muscle are seen at "M". A few lysosomes are evident, and a basal lamina (BL) is apparent. Extracellular fibrillar material, most likely elastin, is also to be seen.

In the experiments dealing with hypoxia, experimental flasks were placed in an incubator containing an atmosphere of 5% O_2, 90% N_2, and 5% CO_2, or of 2% O_2, 93% N_2 and 5% CO_2; control flasks were placed in an incubator containing an atmosphere of 95% air and 5% CO_2. All cultures were maintained at 37 degrees C.

It was observed that the hypoxia population exhibited a slower

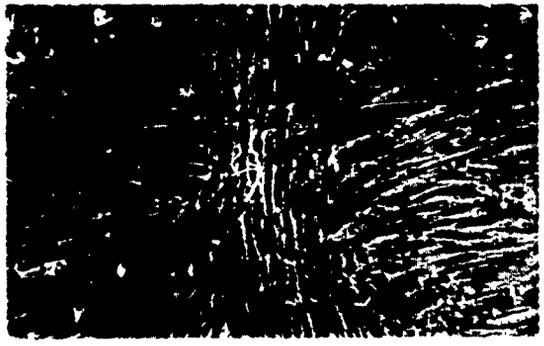


Figure 90: Control population of medial cells. During the study period they maintained a polarized pattern and occasionally grew in multiple overlapping layers (center). (phase contrast, x 100).

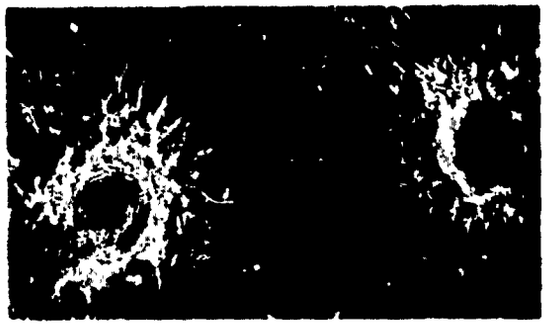


Figure 92: Hypoxic culture after three weeks exposure to hypoxic atmospheres. The refractile areas have enlarged into mound-like structures. (phase contrast, x 160).

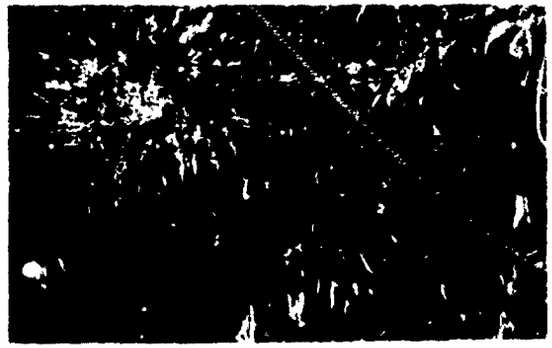


Figure 91: Hypoxic medial cells after exposure to 5% oxygen for six days. Focal refractile areas were formed containing lipid droplets (intra- and extracellular) in addition to some cells debris. (phase contrast, x 120).

Figure 93: Side view of a hypoxic medial cell culture containing multiple osmiophilic mounds. Many of these structures measured up to 150μ, in height by 120μ, in width. (x 8).

rate of growth, which was evident as soon as 24 hours after the beginning of the experiment. Fig.90 is an example of medial cells cultured under controlled conditions. As the cells attain confluency they assume an organized pattern with cells oriented parallel to each other. After becoming confluent, they then grow in multiple overlapping layers. During exposure to hypoxic atmospheres, as shown in Fig.91, the cells assumed a disorganized pattern and began to accumulate lipid in scattered focal areas throughout the flasks.

Figs.92 and 93 show hypoxic cultures at a later stage. The refractile areas have enlarged into mound-like structures. Oil red O confirmed the presence of lipid droplets in these structures and the cells surrounding them (Figs.94 and 95). Elastin (or pro-elastin) and mucopolysaccharide also accumulated as indicated by a positive reaction to aldehyde fuchsin and PAS stains.

Some of the biochemical findings in cultured cells are of interest. The results obtained thus far indicate a higher activity, expressed on a DNA basis, of glycolytic enzymes compared with Krebs-cycle enzymes in homogenates from the cells. The activities of glycerol-3-phosphate dehydrogenase and glycerol kinase are higher than the activities observed in homogenates from corresponding intact tissue. The mitochondria exhibit levels of cytochromes b, c, a, and a_3 comparable to those observed in aortic tissue. These mitochondria utilize succinate and NAD-linked substrates, the rate of oxidation being lower with the latter substrates than with succinate.

Hypoxia of such short duration as used in these experiments does not appear to induce unequivocal and consistent changes in the activities of the enzymes studied. A shift of the LDH isoenzyme pattern toward the more anaerobic fractions could be detected in all experimental studies thus far. Furthermore, hypoxia induces an increase in the activity of most enzymes so far investigated as long as the cultures are comparatively young (9 - 12 weeks). This effect is, however, more pronounced with glycolytic enzymes and as the cultures become older, the activity of Krebs cycle enzymes tends to decline under hypoxic conditions. In addition, as shown in Table XVI, in the hypoxic cells an increase of the glycerol-3-phosphate, lactate and NADH/NAD level and a decreased ATP/ADP ratio could be detected. All these changes can be considered favorable for the synthesis of triglycerides and phospholipids.

Although aging of cells as well as hypoxia appear to induce some changes necessary for increased lipid synthesis, such as the elevation of glycerol-3-phosphate levels, synthesis of lipids need not be the only or principle factor causing accumulation of lipids in the cells. The medium in which the cells are normally grown

Figure 95: Same as Fig. 94, focused midway up the mound to demonstrate the presence of lipid within this structure. (x 290).

Figure 94: A mound and surrounding cells, fixed and stained with oil red O to demonstrate the presence of lipid droplets. The mound, in the center, is out of focus due to its size (x 290)

TABLE XVI

Steady-State Levels of Some Metabolites in Pig Arterial Smooth Muscle Cells Cultured Under Hypoxic Conditions.

Metabolite*	CONTROL	HYPOXIA
Lactate	859·58 ± 112·70**	1926·00 ± 105·00
Lactate/Pyruvate	61·64 ± 6·10	144·79 ± 10·39
α-Glycerol phosphate	10·92 ± 0·56	27·09 ± 2·20
ATP/ADP	2·40 ± 0·14	1·02 ± 0·23
NADH/NAD*	0·16 ± 0·07	0·43 ± 0·25

* Nanomoles/10^6 Cells

**Mean ± S.E.M.

TABLE XVII

Incorporation of ^{32}P orthophosphate and ^{3}H oleic acid into normal and atherosclerotic rabbit aortas perfused simultaneously at physiological pressure with Krebs bicarbonate buffered Ringer containing 4 g/100 ml bovine serum albumin. Phosphate concentrations 0.944 μmoles/ml in all cases. The units are nmoles/g dry defatted tissue/hour, and each figure is the mean of six observations.

	0.1 μC/ml		0.5 μC/ml		1.0 μC/ml	
Lipid	^{3}H 18:1	^{32}P	^{3}H 18:1	^{32}P	^{3}H 18:1	^{32}P
PC	6.4	77.0	14.5	87.0	36.8	54.0
PE	1.1	0.6	2.4	0.6	4.8	0.4
FFA	9.1	-	32.5	-	146.7	-
TG	7.3	-	19.0	-	81.5	-
CE	0.4	-	0.4	-	1.4	-

PC = Lecithin PE = Phosphatidyl ethanolamine

FFA = Free fatty acid TG = Triglyceride CE = Cholesteryl esters

contains 10% calf serum. When the concentration of serum is de- CALF
creased, lipid accumulation - as seen microscopically - becomes less conspicuous. This may indicate that under hypoxic conditions increased cell permeability or a decreased metabolic barrier is the major factor causing accumulation of lipids. It is possible, however, that factors in the serum, similar to those inducing cell proliferation, stimulate the cell to synthesize more lipid. It is also possible that the cells under hypoxic conditions are unable to oxidize fatty acids, resulting in lipid accumulation. Further studies are evidently needed, and such studies are under way in our laboratories.

DR. DAY: I wish to add to the remarks made by Dr. Stein yesterday regarding the metabolism by arterial smooth muscle cells in tissue culture. He described the synthesis of phospholipid by the medial cell layer of the artery and by smooth muscle cells in culture using choline and fatty acid as precursors. It is interesting to note that when ^{32}P phosphate is used as a precursor for phospholipid synthesis in studies on arterial wall metabolism that, in the medial layer, most of the ^{32}P phosphate is incorporated into phosphatidyl inositol (237, 433). We have recently studied the uptake and incorporation of ^{32}P phosphate into various phospholipids by arterial smooth muscle cells in tissue culture (75). In these rapidly growing cells also phosphatidyl inositol turnover is extremely high. The significance of this high phosphatidyl inositol turnover is not clear but could perhaps be discussed.

Phospholipid Metabolism in Arterial Wall

I would also like to refer to other preliminary studies carried out with smooth muscle cells in tissue culture (75) where an attempt was made to assess the incorporation of oleic acid into the cholesterol ester fraction of the cell. As has been observed for normal aortic wall preparations only a small proportion of the fatty acid taken up by the cells was incorporated into the cholesterol ester fraction.

DR. BOWYER: I should like to comment on the question of phospho-lipid synthesis in the arterial wall. Peter Davies and I have made extensive studies of the incorporation of radioactively labeled precursors into the phospholipids of perfused segments of rabbit aorta. In these experiments the segments were perfused with ^{32}P orthophosphate and ^{3}H oleic acid together in the same perfusate. We measured the absolute incorporation of these precursors into phospholipids, separated by micro thin-layer chromatography, of aortas of normal rabbits at fatty acid concentrations varying from a low to a moderately high physiological level. The results are given in Table XVII, which shows the absolute incorporations into various phospholipids assuming that the specific activities of

RABBIT

precursors in the arterial wall are the same as in the perfusate.

As the fatty acid concentration is increased in the perfusate, there is an expected increase in incorporation into the phospholipids, but this is not accompanied by an increased incorporation of orthophosphate. This strongly suggests that acylation of lysophosphatides is occurring as shown by Stein. It is possible that the lyso-phosphatide, phosphatide cycle is involved in controlling the permeability of the plasma membranes of the smooth muscle cells (and possibly endothelial cells) and is altered both by the type of fatty acid and by hormonal influences.

DR. SMITH: I am particularly interested in Dr. Zemplenyi's demonstration that the lipid which appeared in the anoxic cultures appeared to be, to a large extent, an accumulation from plasma or from the medium rather than synthesis. It is now quite clear that the arterial wall contains large amounts of plasma components, thus these cultures seem to relate closely to the sort of situation that you might find in the wall. The intimal cells are bathed in lipoprotein and if they become anoxic this sort of accumulation might occur.

Arterial Lipids in Aging

I will be brave enough to mention the fatty streak because we agreed in Berlin that it was not really atherosclerosis. Fatty streaks occur in most young people, including healthy accident cases, and it is rather striking that many of the fat-filled cells are right on the extreme surface of the intima. It cannot be a question of penetration anoxia there, and one wonders if the cell has some sort of metabolic block to oxygen uptake; this is pure hypothesis.

HUMAN

This question of the large amount of plasma constituents in the arterial wall makes me feel that I must comment on Dr. Stein's presentation on the phospholipids yesterday. I feel that there seems to be some discrepancy between his observations on aging aorta in which the phospholipid increased relative to DNA whereas in the cultures the total phospholipids decreased relative to DNA.

Now as I understood it, in the intact wall, your hypothesis was that most of this increase was in fact due to increasing quantities of plasma membrane (380). But there is also a great increase in connective tissue components and in aging aorta lipid associated with elastic tissue is clearly demonstrable by crude light microscopy at a very early age. I feel that it is very important to separate the muscle cells from the connective tissue components and see whether this sphingomyelin increase is really mainly occurring in the cell, or is in the extracellular lipid which accumulates around fragments of elastic tissue.

I am sure that you are absolutely right that the relative imbalance in sphingomyelinase activity is a major cause of sphingomyelin accumulation (33). We agree with your calculation that although a large amount of plasma phospholipid is carried into the vessel wall in LD-lipoprotein only a small amount of it, relative to cholesterol, is retained. Thus large amounts of LD-lipoprotein phospholipid, which contains a high proportion of sphingomyelin, must be eliminated. With increasing lecithinase activity and static or decreasing sphingomyelinase activity it seems probable that a large part of the accumulating sphingomyelin is derived from plasma.

DR. HAUST: Dr. Stein, in view of your data on sphingomyelinase of the smooth muscle cells, I wonder whether you had a chance to study the activity of this enzyme in these cells in Niemann-Pick disease. As you know, this disease is characterized by the absence or decreased activity of a specific sphingomyelinase and consequent accumulation of sphingomyelins thus providing an experiment by Nature to study the activity of this enzyme in the arterial smooth muscle cells under the altered condition.

DR. BOWYER: Concerning the accumulation of sphingomyelin in the smooth muscle cells of the aorta in Niemann-Pick's disease, we have been able to investigate this in one subject, a girl aged 18 months. At autopsy an atherosclerotic lesion was formed in the aortic arch. Lipid analyses were made by micro-thin-layer chromatography and chemical assay of the undiseased intima and underlying media of the lesion and of the plasma. The results are shown in Table XVIII and compared with analyses of undiseased intima and underlying media and plasma of a child of the same age without Niemann-Pick's disease.

Niemann-Pick's Disease

HUMAN

In each case the undiseased intimal and medial samples were of very similar composition. In the samples from the case of Niemann-Pick's disease, however, the concentration of sphingomyelin was approximately thirty times greater than in the normal, constituting 70% of the phospholipids compared with 15% normally. Other phospholipids were slightly raised and there was an approximately ten-fold increase in free cholesterol, yet only a small increase in esterified cholesterol. This higher concentration of free cholesterol may have occurred because of a simple physiochemical association with the large amounts of sphingomyelin.

In the atherosclerotic lesion in the Niemann-Pick case, the sphingomyelin concentration was comparable with the adjacent undiseased tissue, but there was a high concentration of free and esterified cholesterol associated with foam cells.

TABLE XVIII

Lipid composition of aorta and plasma from a case of Niemann - Pick's disease: comparison with normal child of same age.

		LPC	SPH	PC	PS	PI	PE	FFA	TG	C	CE
Aortas mg/g wet											
Niemann - Pick											
UI		Trace	11.4	1.8	0.9	0.7	0.4	0.5	0.9	4.0	0.4
UM		Trace	7.0	1.4	0.6	0.6	0.7	0.3	0.4	3.0	0.2
DI		Trace	13.7	2.7	0.5	0.8	1.6	0.1	1.1	17.4	12.8
Normal											
UI		Trace	0.2	0.5	0.1	0	0.5	0.9	0.5	0.3	0.2
UM		Trace	0.4	0.5	0.3	0	0.1	0.5	0.3	0.4	0.1
Plasma mg/100 ml											
Niemann - Pick	8.5	44.4	116.9	0	7.1	19.3	53.9	262.9	54.4	109.3	
Normal	13.3	44.8	120.0	Trace	8.1	23.1	40.0	155.0	35.6	123.8	

UI = Undiseased intima UM = Undiseased media DI = Diseased intima

The plasma lipid concentrations in the Niemann-Pick case were normal and the sphingomyelin concentration was not raised. Thus it is almost certain that sphingomyelin accumulation occurred in the aortic smooth muscle cells, because of failure of catabolism as in other tissues. We were unable to estimate aortic sphingomyelinase activity because the tissues were not sufficiently fresh. It is interesting that the aorta of such a young child should have contained a foam cell fatty streak. The premature appearance of such a lesion may be related to the high concentration of sphingomyelin and free cholesterol in the arteries.

DR. Y. STEIN: As to the question put by Dr. Haust. Unfortunately we did not have the opportunity to examine the aorta of a child with Niemann-Pick's disease, so that I am not able to answer whether there is also an increase in the sphingomyelin content of the aorta in this condition. I would like to ask Dr. Ross whether with aging of the smooth muscle cell in the Petri dish there is an increase in the number of tetraploid cells?

DR. ROSS: Yes, the tetraploidy does increase with increasing numbers of cell divisions. We have not looked at tetraploidy in terms of keeping the cells in the dish for a longer period of time, only in terms of increased numbers of trypsinizations toward the end of the cells ability to divide. Whether that is aging or not, I cannot say.

DR. BJORKERUD: The term "aging" has been mentioned several times during this meeting and I would like to make a comment related to this concept. When it comes to tissue cultures perhaps the concept "aging" and "age" is rather clear, but difficulties arise when dealing with such a complex system as the arterial wall. When an artery consists of merely the endothelial layer, the media and the adventitia the problem is rather simple. However, when a subendothelial intimal layer is present it is not clear that all of the tissues in the arterial wall have the same age. If an intimal thickening is induced experimentally the age of the thickening corresponds to the time interval between the induction and the sampling, while the age of the underlying media corresponds to the age of the animal, if the media was not subject to injury and repair, or, if it was, the age is more similar to that of the intima. The age of the uninjured segments correspond to the age of the animal. This is readily demonstrated if one measures the incorporation of glucose into phospholipids in arterial samples in vitro because there is a linear correlation between the rate of incorporation per cell and the age of the tissue, but no correlation at all with the age of the animal. In contrast, if the incorporation of glucose into triglycerides is measured in the same tissue samples one finds a non-linear inverse relationship between

Age of Tissue versus Age of Animal

the rate of incorporation and the age of the animal, but no correlation whatsoever with the age of the tissue (33). In summary, there are different kinds of "age" in the arterial wall. If this is not taken into account when an experiment is designed "age"-correlated variables may turn out to be disturbing variables and may render the interpretation of the results difficult or impossible.

DR. ROTHBLAT: I would like to ask a questions concerning aortic smooth muscle cells in culture. These cultures have been described by Dr. Ross as having "hills and valleys" which represent areas where they are growing as monolayers and other areas where they are growing in multilayers. This might suggest that there is a heterogeneous population of cells, some of which continue to divide, others which may never divide. Is there evidence of heterogeneity in lipid accumulation and in thymidine labeling of cells in these cultures?

DR. FISHER-DZOGA: For the first question, as far as these hills and valleys and thymidine incorporation go, the whole culture is not homogeneous. I am talking now of primary cultures. Thymidine incorporation is not limited to either hill or valley nor is it limited to monolayer or not monolayer. Usually you can also find one or two sections in the culture which do not respond. We were thinking we had some fibroblast contamination there. But now after reading Benditt's work, I am not so sure any more (25). Maybe we have two different cell types. Morphologically you cannot tell them apart. Everything looks like smooth muscle cells. For the secondary cultures, I really cannot answer that question. Autoradiographs again indicate that proliferation is not very homogeneous. Many times it is localized in certain sections of the culture. Lipid uptake is not at all homogeneous. You will have it in one section of the culture much more than in another. Over-all it is increased if we extract lipids but not all cells respond in the same way.

DR. ROSS: This situation is really no different from any other cell culture situation, Dr. Rothblat. These are not synchronized cultures, in terms of either DNA synthesis or any other aspect of metabolism, because as you know, depending upon which particular macromolecules you are looking at as far as synthesis is concerned, there are both direct and/or inverse correlations between that and the DNA synthetic cycle. In any given cell, since the cultures are not synchronized, one would not expect to find all cells homogeneously doing the same thing at the same time, such as all cells necessarily taking up lipid at the same time. Now if that is what you are calling heterogeneous, O.K. If you mean are we looking at different cell types, smooth muscle cells versus non-smooth muscle cells, we are quite satisfied that we have uniform homogeneous cultures of smooth muscle cells, that there are no other cell types there because we know under our conditions endothelial cells

do not survive. In terms of any other heterogeneity, that is cell size and shape, this could be a manifestation of cells in different phases of the DNA synthetic cycle.

The Distinction Between Smooth Muscle Cells and Fibroblasts

DR. BURNSTOCK: An issue that worries me has come up in a number of talks, particularly those of Dr. Stein and Dr. Ross yesterday. Perhaps you can clarify it for me. It is the problem of identifying smooth muscle cells when they are undifferentiated or dedifferentiated. What are the criteria that you use to distinguish them from fibroblasts? One or two pictures were presented yesterday which were claimed to be smooth muscle, and no doubt they were, but I could not tell how they differed from fibroblasts. We have this trouble all the time in culture and also in problems like wound healing. For example, if you have a small wound, the cells around the wound surface dedifferentiate; they divide and as soon as they form a confluence they redifferentiate and the wound is completely healed. However, if it is a big wound, the muscle cells go on dividing and dividing, but they do not seem to be able to form a confluence, so they do not redifferentiate. Under these circumstances, they get more and more fibroblast-like and perhaps at this stage begin to produce collagen. Maybe there are fibroblasts also producing collagen at the same site, but one would like to be able to distinguish these from dedifferentiated smooth muscle. The kinds of criteria we have considered are: the presence of plasmalemmal vesicles, basement membrane, filaments, dark 'areas' and 'bodies', endoplasmic reticulum cell inclusions, and so on, but I would be very grateful if someone would clarify this situation for me.

DR. DAOUD: In our tissue culture system the cells look like fibroblasts during the first few days. After that, as you point out, in wound healing the dedifferentiate and in about three weeks almost all of them are mature smooth muscle cells. Autoradiography electron microscopy during early proliferation reveals very little or no filament formation. We can qualitatively demonstrate collagen or elastin synthesis only after about seven days. So actually it appears that when these fibroblast-like cells are dividing they are not secreting. And when they are secreting, they stop dividing. May be there is a phase between secretion of myofilament and secretions of material outside.

DR. ROSS: In one of the papers I wrote on the smooth muscle cell cultures there is a final paragraph devoted to the question of what is a smooth muscle cell. We really don't know. I think that what we have been using for the moment are phenotypic criteria to describe smooth muscle and you have described most of them already. We have used these to satisfy ourselves that these cells have these

traits. What we probably need are criteria which are more specific than the ones we have at the moment. Because we know that corneal epithelial cells make collagen, it is certainly not a property unique to fibroblasts. So we can't look at synthesis of extracellular proteins necessarily unless we can find one that only smooth muscle cells make. Thus far I don't know of one. We can't use a basement membrane because some smooth muscle cels have a continuous one, whereas with other smooth muscle cells the basement membrane is irregular or discontinuous. We certainly can't use rough endoplasmic reticulum development to describe cells as fibroblast either, because any cell that is making secretory protein is going to have a rough endoplasmic reticulum. So I have to also ask the question, "What is a fibroblast?" We really don't know the answer to that question either. What we are saying is that the criteria we have been using to identify cells are quite arbitrary in many ways and we are trying to find some common denominator.

Now I think there is an approach to this that should be investigated and probably will bear some fruit because it has worked for some investigators in helping to define cell types. Many cells have clearly defined cell surface antigens which are characteristic -- characteristic is the wrong word -- which are unique to those cells. For example, monocytes do. Neutrophils do, and platelets do, in addition to the histocompatibility antigens which these cells share, they do have unique antigens. We know nothing about whether smooth muscle cells contain such antigens and/or whether there are families of smooth muscle cells and fibroblasts.

I suspect that uterine myometrial smooth muscle cells are different from aortic smooth muscle cells, which may be different from coronary artery smooth muscle cells. We don't know. I believe that one way of getting at this problem is to begin to look at cell surface antigens to see whether they have unique properties. They may not and we may be back in the same black hole all over again if they don't in terms of answering the question definitively. That is a long winded way of saying that we don't have a definitive answer to your question.

What we have done is to take a series of criteria which we have arbitrarily applied and which for the moment, in the crude sense, is the best we can do. And I think we have to be honest and admit to ourselves that if you are going to pin us down and ask, "How do you really know that that is a smooth muscle cell?", then I have to say we don't.

DR. WERTHESSEN: Dr. Burnstock in his motion picture described an elegant way to make a certain identification of a cell as a smooth muscle cell. If a growing nerve fiber seeks it out, attaches and modifies the contractile rhythm, it is a smooth muscle cell.

Are there other ways of making such a reliable identification?

DR. WISSLER: I think we have a long way to go in terms of identifying smooth muscle cells definitively but Dr. Miriam in our laboratory and Dr. Fisher-Dzoga more recently in cultured cells have used immunohistochemistry to differentiate, we believe, between adventitial fibroblasts and smooth muscle cells. I don't believe we can differentiate between endothelial cells and smooth muscle cells at this point although we are making a valiant attempt to see if we can get an antigen out of arterial smooth muscle that will not stain endothelium.

I think Russell Ross's approach is probably a very good one to follow up on and try to find something on the surface of the cells. We have not done that. But for the moment the myosin or actomyosin in smooth muscle can be stained without staining whatever contractile proteins there are in fibroblast tissue. I am well aware that granulation tissue fibroblasts have a contractile protein that may cross react but we do not see that thus far in adventitial cells, in tissue culture or in the stained sections.

DR. ROBERT: The question was raised of cell membrane markers as better signs of differentiation than the "biological markers" such as contraction for example. I think that there is at least some evidence that the structural glycoproteins do carry organ specificity and that they might be considered as "differentiation antigens". This was shown in several different types of experiments. One of them being to sensitize rabbits with structural glycoproteins extracted from cornea of different species, then putting lamellar grafts in their cornea and watching rejection (292, 293). Such grafts can survive for a very long time on control animals. We have shown years ago that if you sensitize the rabbit with, for instance, a structural glycoprotein preparation from calf cornea then it will reject specifically this graft, but if the glycoprotein used is from calf skin then it will not (293).

Immunologic Specificity of Tissue

RABBIT

Years ago I met Dr. McCullough from Cambridge whose Ph.D. dissertation involved a study of elephants. We were really surprised to find that these animals suffer from severe arteriosclerosis and the histological modifications resemble the human pathology a great deal. Now I would like to remind you that elephants don't eat too much lipid. We extracted the structural glycoproteins by the same method used for the other tissues. That is what we put in the non-elephant aorta-extracts. As you see in Fig.9b, the antibody which we induced to human aorta structural glycoprotein did give two lines with this unpurified urea extract from the elephant aorta,

ELEPHANT

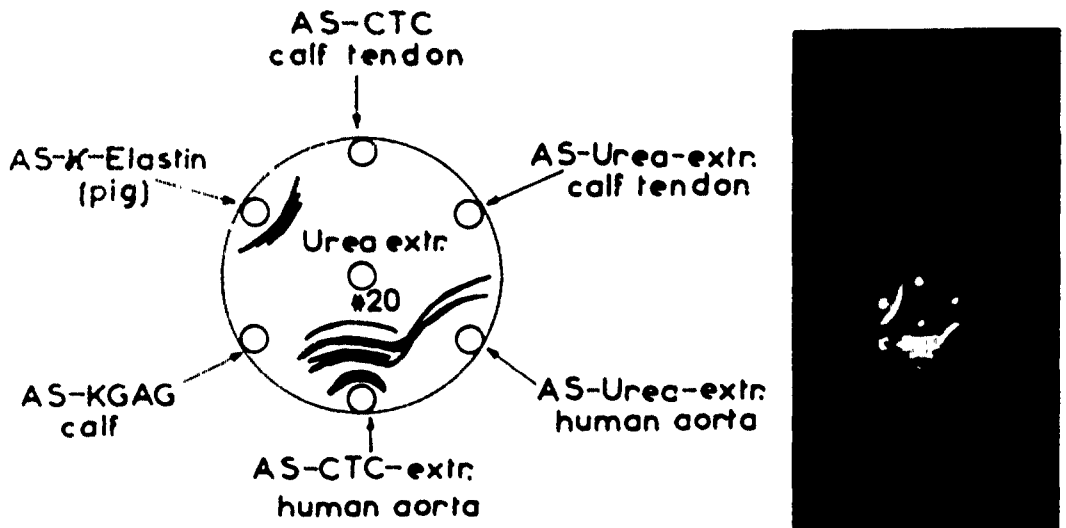


Figure 96: Diagram of the precipitation lines formed on an immunodiffusion plate containing elephant aortic urea extract antigen in the center well and rabbit antisera to various connective tissue extracts in the peripheral wells (AS-CTC-extract calf tendon: antiserum induced by injecting a 1M $CaCl_2$-extract of calf tendon to rabbits; AS-urea extract calf tendon; antiserum obtained by injecting a urea-extract, containing the structural glycoproteins of calf tendon; AS-urea extract human aorta: antiserum to the urea extract (structural glycoproteins) of human aorta; AS-CTC-extract human aorta: antiserum to the 1M $CaCl_2$-extract of human aorta; AS-KGAG-calf: antiserum to the urea extract (structural glycoproteins) of calf cornea; AS-K-elastin pig: antiserum to the K-elastin of pig-aorta; for details see Robert et al (1965) In "Structure and Function of Connective and Skeletal Tissue" ed. Tristram G.R., Butterworth, pp 406-412).

as well as an anti-serum which we obtained in rabbits by injecting the calcium chloride extract of human aorta. But there were no lines with the anti-serum to cornea or to tendon (214). So this and several other experiments which we carried out showed that although the overall aminoacid composition of the structural glycoproteins isolated from various tissues is quite similar (286) they do carry a good deal of tissue specificity. Now this might be taken as an argument showing that they behave as "differentiation antigens" and carry slight differences in their conformation and that might be a link between their immunological specificity and the morphological patterns of the tissues which synthesized them.

DR. ROTHBLAT: For a moment, I would like to come back to the question of heterogeneity in cultures of aortic smooth muscle cells. I don't think the differences among cells can be simply explained on the basis of a non-synchronized culture. This might explain the thymidine labeling results if short pulse labeling periods were used. It is more difficult to explain the patchy appearance of vacuoles, particularly if the hyperlipemic serum is present in the culture medium for a number of days. I don't think asynchrony can be the explanation for the multi-layered and mono-layered areas observed after many weeks in culture. Could the explanation be that there are various clones of cells present in these cultures?

DR. ROSS: Let me preface my answer by saying that this is an opinion. We have not really examined critically the question you are asking. Observations I have made suggest that the answer I am going to give is correct, but not necessarily so. I think what we are looking at is a growth pattern of the clone and that this is an ascending or "pile up" rather than the flat pattern one sees in a fibroblast culture. If you look at fibroblast cultures you can identify those clones because they become concentric whirls of cells. You actually see where the whorls intercept, separating clones, not cleanly but in a fashion one from another. I think the difference we are seeing is a phenotypic aspect of smooth muscle. They tend to grow vertically by piling up but what they tend to do first is occupy the space available to them. I think we are seeing clones. We have not worked with hyperlipidemic serum. Now Dr. Fisher-Dzoga has.

DR. DAOUD: An explant grown in the absence of serum shows essentially no growth at the periphery. In the presence of serum growth is such as to suggest the possibility of clone but we do not know.

DR. ROBERTSON: The question raised by Dr. Ross is both important and well recognized by those of us working with vascular cells in vitro. In order to avoid some of the possible metabolic and functional changes induced by proteolytic enzymes on cell surfaces

during isolation and harvesting, for the last several years we have used a method based upon the tendency of growing vascular cells to attach to glass surfaces (297, 298). Our "double coverslip technique" consists of fusing the ends of two rectangular coverslips together leaving between them a space of 1 mm. or less to hold a cross section of the blood vessel to be studied. This allows the endothelial surface to be in intimate contact with one glass surface and the adventitial or medial layer, depending on the thickness of the vascular wall, with the other (Fig.97). In approximately two to three weeks for primate arteries (the time is considerably shorter for other species and tissues) two distinct cell populations are established and the coverslips can then be separated and transferred as monolayer cultures to individual stationary or rotating culture flasks for further growth. This technique has two other major advantages over enzymatic isolation which are worth mentioning: (1) it allows comparative metabolic and morphological studies of cell populations from different layers of a multilayer organ such as the vascular wall; (2) an autologous environment may be used throughout the entire experiment, allowing the use of immunological techniques for identification of cellular changes.

By accident, we learned in our laboratory that only endothelial cells are able to survive and divide in 100% whole human serum. We have found this very helpful in identifying vascular cell types. It is possible, therefore, if you have a mixed population of endothelial and other vascular cell types, including smooth muscle elements, to identify the former by incubation in whole homologous sera. The procedure is both simple and effective and could be considered an alternative method to the use of surface antibodies suggested by previous speakers.

DR. BECKER: We have recently been culturing endothelial cells. We found that they contain blood group antigens. Smooth muscle cells and fibroblasts don't. So, if you are working with monkeys and/or human tissue in culture you can determine the donor's blood type and at least test for endothelial cells.

DR. KNIERIEM: I think we should go back shortly to the problem of identification of smooth muscle cells which was mentioned by Dr. Ross. When I worked with Dr. Wissler seven years ago, we were able to demonstrate smooth muscle cells by direct immuno-histochemistry using labelled antimyosin and actomyosin (182, 183, 184, 424, 425). In Fig. 98a you can see quite well smooth muscle cells in an artery of the bovine kidney stained by fluorescein-labelled specific anti-bovine myosin. You can see the internal elastic membrane stained white, the smooth muscle cells of the media green and outside the blue fluorescence of the collagen. The

Immunologic Identification of Smooth Muscle Cells

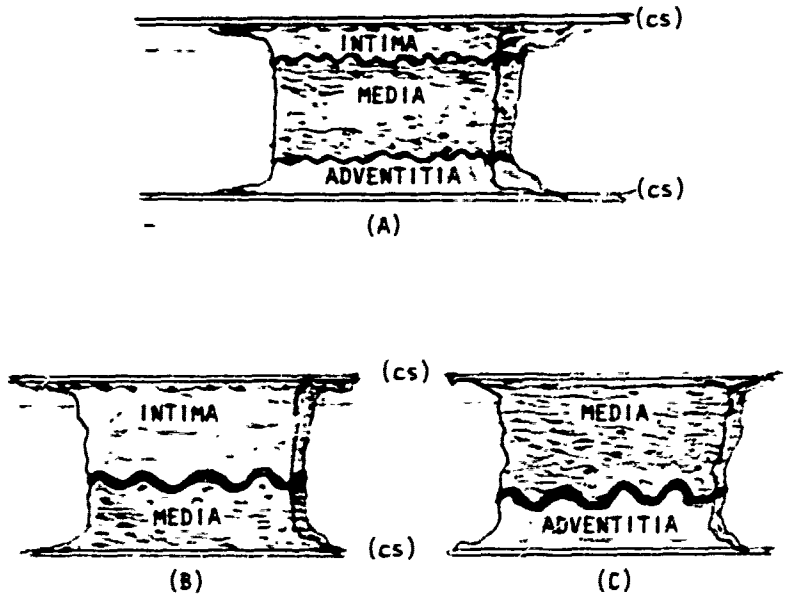


Figure 97: Schematic demonstration of the "double coverslip" technique used to isolate vascular cells from different arterial layers. (A) shows isolation of intimal and adventitial cells from a small muscular artery such as the epicardial branch of coronary artery between coverslips (cs). (B) and (C) demonstrate a similar method for thicker vessels such as the human aorta in which intimal and medial layers (B) and medial and adventitial layers (C) are cultured separately. After 10 to 16 days growth in Leighton tubes, the fused coverslips are separated and matching stationary cultures prepared from cells which have grown independently on each coverslip.

COW bovine aorta has very densely packed smooth muscle cells, only a few collagen fibers and less elastic material (Fig.98b). We had HUMAN great difficulty in getting myosin from human aortas because even so-called healthy persons had large amounts of lipids in their aorta. Thus we could not obtain clean myosin from them. Therefore, we used human skeletal muscle as antigen. The human aorta of a three month old fetus showed the distributions of smooth muscle cells with little collagen interspersed and no elastic material at all. Also within the human aorta you can demonstrate very well fluoresceinated smooth muscle cells. I think it is of great importance to differentiate the different proteins of smooth muscle cells which act as antigens. There are often cross-reactions between the different antimyosin sera. The differentiation of these antigenic properties may be also important for the understanding of different immunopathies particularly for the clearing up of vascular changes in cases of muscular diseases like dermatomyositis.

Finally, an additional remark to the question of staining endothelial cells by antimyosin or anti-actomyosin sera. At first we thought this might be an artifact but later I was reminded by an electron microscopist who studied tonofibrils in endothelial cells that there are probably actomyosin filaments. Therefore, I believe that those incidental findings were not artifacts but real. In reference to the importance of the various antigenic properties of muscular proteins, I may call Dr. Groschel-Stewart who has new findings on different cross-reactions of vascular muscle proteins.

HUMAN DR. GROSCHEL-STEWART: We have extracted actomyosins from human smooth (uterine) and striated (pectoral) muscle and we obtained in rabbits anti-bodies against these proteins that were directed against the respective myosin unit and did not cross-react (127). When we applied the FITC-labelled gamma-globulin-enriched fraction of these antisera to unfixed frozen sections of human uterus, the smooth muscle fibers stained brilliantly with the antibody against smooth muscle actomyosin, but not with the antibody against striated muscle actomyosin (Fig.99). The antibody against uterine actomyosin also reacts with the smooth muscle fibers of the vascular system and with the contractile elements in non-muscular cells, such as fibroblasts (128) (Fig.100), thrombocytes, megacaryocytes and macrophages (126).

DR. ROSS: I don't mean to imply that the beautiful work that both Dr. Knieriem and Dr. Becker have done with raising these antibodies and localizing these in smooth cells and endothelial cells is not a valuable approach. It is very valuable. It is the best we have. My only comment was that in terms of really separating out cells as Dr. Groschel-Stewart has just shown that fibroblasts cross-react, it may not be good enough if we are getting down to asking critical questions.

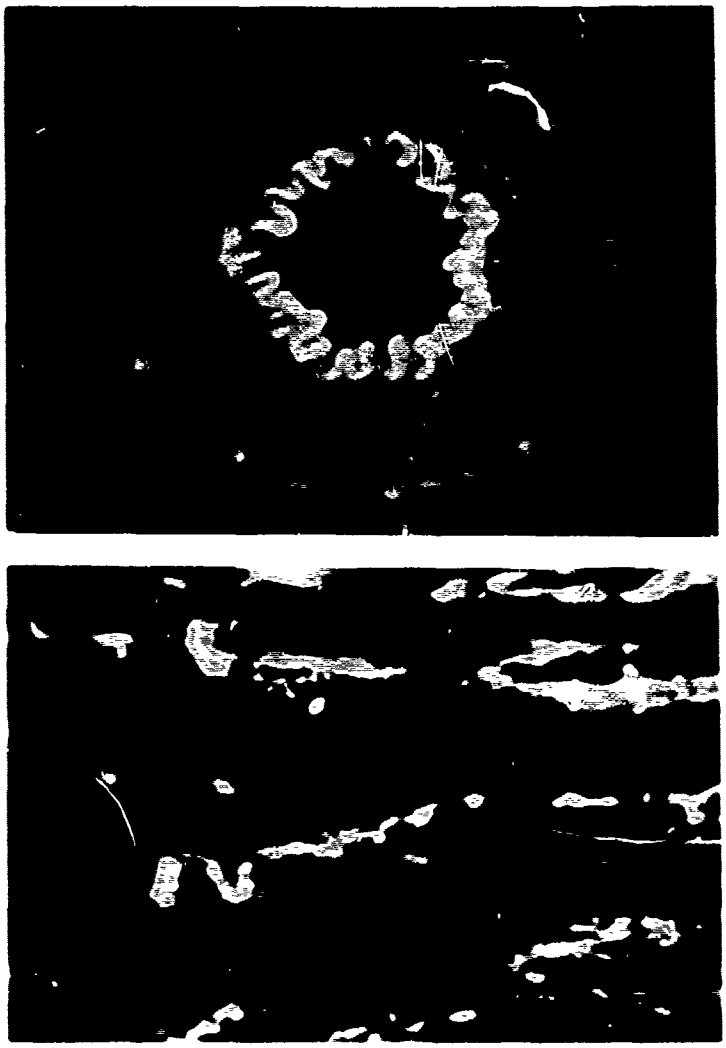


Figure 98: a) Normal bovine kidney treated with fluorescent antibovine myosin serum. A medium sized artery shows green fluorescence of its concentric oriented muscle cells and white fluorescent internal elastic membrane as well as blue stained collagen in the adventitia x 400

b) Normal bovine aorta treated with fluorescent antibovine myosin serum. The white fluorescent elastic lamellae separate bundles of green fluorescent smooth muscle cells in the outer half of the media. x 700

DR. A. P. SOMLYO: Perhaps I am the only one who is confused. It seems to me that Dr. Groschel-Stewart's data showed that skeletal myosin, antibody to skeletal myosin, does not cross-react with smooth muscle myosin. I also obtained the impression from Dr. Knieriem that he was showing the reaction of antibody to skeletal muscle myosin with vascular smooth muscle presumably myosin. I would just like to know if there is really a conflict here and, if so, any suggestions how it can be resolved.

DR. GROSCHEL-STEWART: It might be possible that Dr. Knieriem had antibodies against actin in his antisera. These antibodies will react with both smooth and striated muscles (61) since actin is very similar or even identical in the two types of muscles.

DR. BECKER: I would agree with Dr. Groschel-Stewart that there might be either anti-actin or anti-tropomyosin because anti-tropomyosin will stain both smooth and striated. When we were making anti-cardiac antibodies (anti-cardiac antimyosin antibodies) the earlier preparations after a short period of immunization only had antibodies that would stain striated muscle. If we continued to immunize for longer sometimes some rabbits would make antibodies that would stain smooth muscle as well. I think the reason for that is that when you grind up a heart, or skeletal muscle, and extract it although you are extracting mostly striated muscle you are also extracting smooth muscle. We are giving them a mixture of antigens. When we use uterine muscle we are only dealing with smooth muscle. That, I think, is how it can be resolved.

DR. ROTHBLAT: Dr. Wissler has previously indicated that the accumulation of lipids in aortic smooth muscle cell cultures is a reversible process. Since much of the accumulated lipid seems to be cholesteryl ester, the clearing of the cellular lipid suggests that cholesteryl esters can leave the cells. Is there any information on whether the cholesteryl ester is released from the cell as free or as esterified sterol?

Intra- and Extra-Cellular Migration of Cholesterol

DR. WISSLER: I will talk a little bit tomorrow, I hope, on our work with hyperlipidemic serum and low density lipoproteins in tissue culture. I do not believe that we have any definite evidence about the form in which the cholesterol and cholesterol esters leave the cells but I will show some of the evidence that they do leave and the supposition is that they are hydrolized first.

DR. KRITCHEVSKY: I would like to make a few comments regarding cholesteryl ester synthesis and hydrolysis in the aorta. Dr. Stein has quoted Charles Howard's data which indicate that about half of the esterified cholesterol found in the artery can be

Figure 99: 4 μ cryostat section of human uterus (cervical portion), stained with FITC-labelled antibodies. Left: antibody against striated muscle actomyosin. Right: antibody against smooth muscle actomyosin.

Figure 100: 4 μ cryostat section of human umbilical cord, stained with FITC-labelled antibodies against human uterine actomyosin. Note the staining of the smooth muscles fibres in the umbilical vein and of the fibroblasts (arrows) in Wharton's gelatin.

derived from cholesterol and locally synthesized fatty acids. In experiments in which they used elaidic acid as a marker, Hashimoto and Dayton concluded that half of the aortic cholesteryl ester might be derived from the circulation. Zilversmit has said that his data show that all the arterial cholesteryl ester is derived from the circulation.

Aortic Cholesteryl Esterase

There are a number of investigators using various systems for the study of cholesteryl ester synthesis and hydrolysis by arterial tissue. There are almost as many systems as there are investigators. Working with Dr. Himanshu Kothari we have been using an acetone powder of the artery and this preparation contains both hydrolytic and synthetic activities. The hydrolytic activity is most pronounced at pH 6.6 and proceeds best when the substrate is presented as a micelle. Synthesis is carried out at pH 6.2 with an emulsified substrate (189). It is interesting to speculate that the micelle is small in size, like an alpha lipoprotein and the emulsion resembles a beta lipoprotein.

Table XIX presents comparative data on synthetic and hydrolytic activity in the aortas of eight animal species. It is important to note that both synthesis (S) and hydrolysis (H) of cholesteryl esters are taking place and it is the ratio of one to the other which is important. It is obvious that the ratio of synthesis to hydrolysis is considerably higher in those species which are more susceptible to atherosclerosis - man, baboon, pig, rabbit, and chicken. The average S/H ratio for the five "susceptible" species is 84% higher than it is for the others.

One of the criticisms levelled in this field is at comparison of metabolism in different species. We have, therefore, compared two breeds of pigeons -- the White Carneau, which exhibits spontaneous atherosclerosis in the distal portion of the aorta and the Show Racer which is free of spontaneous atherosclerosis. In studies of acetone powders of whole aorta we found the S/H ratio to be 1.23 for the White Carneau pigeon and only 0.73 for the Show Racer.

To compare the S/H activity in the proximal and distal portions of the White Carneau and Show Racer aortas we obtained fifty aortas of each breed (courtesy of Dr. T. B. Clarkson) and prepared pools of the distal and proximal portions. These pools were coded and given to Dr. Kothari for analysis so the experiment was carried out in a double-blind fashion. As can be seen from Table XX, the S/H ratio of Show Racer distal and proximal aorta is the same. The S/H ratio in White Carneau proximal aorta is higher than it is in either half of the Show Racer but the S/H ratio in White Carneau distal aorta is significantly higher than in any of the other portions.

TABLE XIX

CHOLESTERYL ESTER SYNTHESIZING (S) AND HYDROLYZING (H) ACTIVITIES OF ACETONE DRY POWDER PREPARATIONS OF AORTAS OF VARIOUS SPECIES*

Species	Experiment 1			Experiment 2		
	S	H	S/H	S	H	S/H
Human	6.5	8.3	0.78	6.6	6.9	0.96
Baboon	6.2	7.5	0.83	5.4	6.2	0.87
Swine	5.5	4.8	1.15	6.7	7.0	0.96
Rabbit	6.5	7.0	0.93	5.8	6.0	0.97
Chicken	8.0	8.1	0.99	6.3	6.5	0.97
Rat	10.0	16.0	0.63	10.6	19.6	0.54
Mouse	9.2	14.0	0.66	7.3	12.7	0.57
Dog	4.0	14.5	0.28	6.5	18.0	0.36

*Synthesis and hydrolysis of cholesteryl ester is described as nmoles of substrate converted per mg of protein per hour.

Substrate for synthesis: 15.5 μmoles (^{14}C) cholesterol, 445 μmoles oleic acid, 31.0 μmoles sodium taurocholate, 100 μmoles NH_4Cl in 0.154 M phosphate buffer, pH 6.2. Final volume 1.5 ml.

Substrate for hydrolysis: 1 mg (^{14}C) cholesteryl oleate, 1.8 mg sodium taurocholate, 3 mg lecithin in 1 ml of 0.154 M phosphate buffer, ph 6.6. Sonicate 15-20 kc sec for 15-30 min.

Incubations carried out for 2 hrs at 37°C. Analysis by thin layer chromatography.

TABLE XX

RATIO OF CHOLESTERYL ESTER SYNTHETASE (S) AND HYDROLASE (H) ACTIVITY (S/H ± S.E.M.) IN PROXIMAL AND DISTAL PORTIONS OF AORTAS OF WHITE CARNEAU AND SHOW RACER PIGEONS

Aorta Portion	No. Pools	Strain of Pigeon	
		White Carneau	Show Racer
Proximal	5	0.65 ± 0.08[a]	0.48 ± 0.03[b]
Distal	5	0.95 ± 0.10	0.52 ± 0.05[b]

[a] vs WC Distal, $p < 0.05$ [b] vs WC Distal, $p < 0.01$

HUMAN In man, the ratio of aortic free to esterified cholesterol changes dramatically with age, falling markedly.

RAT Table XXI presents data obtained using aortas of young (2 months) and old (12 and 24 months) rats. The synthetic rate is up 4% at 12 months and 77% at 24 months. In contrast, the hydrolytic rate rises 245 and 1060% at 12 and 24 months respectively. The result is that the S/H ratio falls. Whether this is a generalized phenomenon or peculiar to the rat is under investigation.

Cholesterol Synthesis and Esterification in vivo and in vitro

DR. SMITH: I feel rather unhappy about the synthesis/hydrolysis ratios because there seems to be a good deal of evidence that synthesis may only occur when the cholesterol molecule is in the right place, spatially. This rather curious data has arisen in several recent studies (238, 239, 278). If you supply an intact vessel or a piece of tissue with labelled fatty acids it rapidly esterifies these to cholesterol, but if, instead, you present the tissue with cholesterol there is a very long delay before it is esterified with fatty acid and the suggested interpretation of this is that the cholesterol has to be in the correct binding site, for lack of a better word, before it is esterified. Therefore, actual rates observed in isolated systems or extracellular preparations may not really bear much resemblance to the rates within the intact cell.

DR. BOWYER: I do agree with Dr. Elspeth Smith on this point, that we have to distinguish between enzyme activity as measured in vitro on the one hand and on the enzyme action in vivo on the other. We have tried to address the question of the physiological rate of cholesteryl ester turnover in the arterial smooth muscle, by perfusion of a segment of artery in which the normal anatomical relationship of endothelium and smooth muscle cells is undisturbed. Using oleic acid as precursor, we were able to show that in arteries of rabbits with various stages of induced fatty streaks, the fatty acid incorporation increased with increasing severity of lesions. If, however, as is shown in Table XXI, the incorporation into cholesteryl esters is plotted against the cholesteryl ester concentration in the artery, there is a strong positive correlation. Thus, the percentage of the metabolically available pool of cholesteryl esters (i.e. the turnover) remains constant or falls slightly in the larger fatty streaks.

DR. LOFLAND: I think it can hardly be doubted that the arterial wall can synthesize cholesterol ester. Using a perfusion system that exposed only the intima, we showed that even when you use acetate as substrate in a medium which contains no plasma, you get very, very active synthesis. This is a function of the state of the artery.

TABLE XXI

INFLUENCE OF AGE ON SYNTHESIS AND HYDROLYSIS OF CHOLESTERYL OLEATE BY RAT AORTA PREPARATIONS

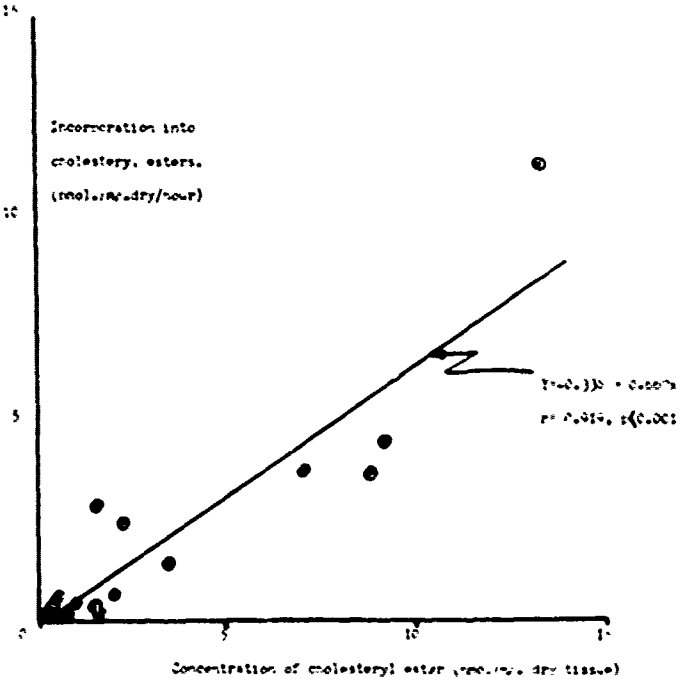
Age (mo)	No. Pools	Synthesis* (S)	Hydrolysis* (H)	S H
7	4	$3.9 \pm 0.17_a$ ‡	$2.0 \pm 0.62_a$	1.95
12	3	5.5 ± 1.62	$6.9 \pm 2.30_b$	0.80
24	4	$7.0 \pm 0.41_a$	$21.2 \pm 2.46_{ab}$	0.31

* nm·mg protein hr

‡ standard error

Values with same subscript are significantly different.

TABLE XXII



(from Davies J.F., Ph.D. thesis, in preparation)

DR. Y. STEIN: In answer to Dr. Smith's question: The cholesterol that enters the cell is usually in the form of free cholesterol. After it gains entrance into the cell, it then exchanges quite rapidly between the different cellular membranes and so the labelled cholesterol is diluted in a large pool of unlabelled cholesterol. If instead, we present a cell with a labelled fatty acid, it will enter into a very small cellular pool and therefore will be readily available for the esterification of cholesterol. I think it is important to keep in mind that the difference in pool sizes of free cholesterol and fatty acid will determine the rate of appearance of label in cholesterol ester, when the labelled precursor is either the free cholesterol or fatty acid.

DR. KRITCHEVSKY: I would like to amplify Dr. Lofland's remarks. We have also found that the rate of cholesterol esterification can rise dramatically after only a few days of cholesterol feeding. We have observed 40-100% increases in aortic cholesteryl ester synthesis after feeding rabbits cholesterol for only 5 to 7 days. The Bowman
PIGEON Gray group observed the same phenomenon in pigeon aorta after 10 days of cholesterol feeding, at a time when lipemia was moderate and no lesions were visible. Although these results have been obtained in aorta preparations they probably reflect the in vivo situation. It would appear that the feeding of an atherogenic diet, in some way triggers this remarkable increase in cholesterol esterification. Whereat has shown that aortic fatty acid synthesis is enhanced significantly after a few days of cholesterol feeding. Whether increased esterification is a physiological attempt to "detoxify" or store cholesterol or whether it is an attempt to remove fatty acids isn't clear, but I prefer the former explanation. What is evident, however, is that a few days of dietary insult are sufficient to provoke a great increase in aortic metabolic activity.

CALF DR. WERTHESSEN: During the 1950s we used the calf's aorta to study cholesterol accumulation and sterol synthesis from acetate in vitro. Diluted, defibrinated cow blood was pumped through the organ. Pressure was controlled. Experiments were run for 24 hours.

When high pressure (200/180) was used it was easy to show an inverse relation between the initial cholesterol concentration and the change in concentration while the organ was perfused. There was a maximum concentration achievable. So, if on excision from the donor the concentration was low a considerable change in concentration occurred or minimal if the initial level was high.

Bondjers (see page 190) was able to show with statistics that a similar relationship held at low pressure (110/80). But it was also possible in these studies to compare C^{14} acetate

incorporation into sterols. It was significantly higher on the high pressure runs.

What all these data said is: (1) that an excised and thus traumatized aorta in this sytem will accumulate cholesterol and synthesize sterols; (2) when made to work hard the aorta will demonstrate these capacities better than when it can loaf; (3) the metabolism is a function of the load put upon the organ.

If I am not mistaken this load factor has not been given much consideration in biochemical studies of late.

DR. SMITH: With regard to the work that the system is doing, I am fascinated to learn from Dr. Avril Somlyo (first day) that the aorta is capable of contracting and I am wondering if there is any information on the extent to which it does contract in vivo.

Arterial Contraction

DR. WERTHESSEN: When you speak of aortic smooth muscle cells contracting, or being capable of doing so, I wonder if we have ever had clearly defined for us just what they do "in situ". I used to watch our calf and swine aortae when they were maintained on the perfusion pump system and hoped to be able to study them some day on a more cellular basis. For these reasons: One could see the pressure wave of the pulse beat extend the aorta in both directions and then the contraction which followed during diastole. It was obvious that the tissue was always under load and that it contracted when the pressure pulse went down. It was not at all like a piece of rubber tubing set up in the fashion.

What I thought such studies would show was the manner in which these cells maintain the diastolic pressure. I attribute to the smooth muscle cells the fact that the blood pressure in our extremities is so close to that in the aortic arch. As you know, no hydraulics engineer has devised a comparable system. I think, but cannot document, that they "help" the pulse along in a kind of peristaltic action.

DR. SMITH: I feel that this is very important, because if the vessel is contracting vigorously it must make nonsense of static studies of hydrodynamic factors. Imagine also what happens where a branch comes off - if each vessel is contracting there will be a most traumatic situation where the two contraction waves meet. This could be at least as important in the development of plaques round branches as changes in flow patterns.

DR. ZEMPLENYI: Dr. Werthessen, I think that before we leave the problem of arterial metabolism, it has to be mentioned that we

know very little about the bioenergetics of the arterial wall and especially the role of oxidative phosphorylation in this regard. In our laboratories a good deal of attention is paid to this problem. Recently Drs. Kalra and Brodies presented evidence that mitochondria isolated from aortas of atherosclerosis-resistant Show Racer and susceptible White Carneau pigeons differ in this respect. Oxidative phosphorylation and respiratory control with succinate and other NAD-linked substrates is the same in both types of mitochondria. However with alpha-glycerol phosphate as substrate, respiratory control and ATP formation is observed only in mitochondria from the atherosclerosis-resistant pigeon arteries. The deficiency of the glycerol phosphate shuttle is also important for lipid synthesis because it may lead to accumulation of alpha-glycerol phosphate in the cytoplasm and alpha-glycerol phosphate is needed for the synthesis of triglycerides and phospho-lipids (173).

Arterial Energy Metabolism

PIGEON

DR. WERTHESSEN: Would you be willing to imply that the genetic work that the Bowman Gray observers did could be based on what you might call now a genetic lack of an enzyme?

DR. ZEMPLENYI: This is precisely what we wish to answer in our experiments. The results which I just mentioned together with other data were obtained in older birds and the differences observed could very well be a consequence of aging or of atherosclerosis in the more susceptible strain. Therefore we repeated just a few days ago some of our studies with aortas of pigeons only a few weeks old. I do not have so far the results because many of the data have to be calculated on the protein, DNA and fat-free, dry-weight basis. We hope that the results will answer the question whether metabolic differences between the atherosclerosis-susceptible and resistant pigeon arteries are of an inherited (genetic?) nature.

DR. BJORKERUD: This is just a comment on Dr. Kritchevsky's suggestion that the "atherosclerotic" smooth muscle cell might form fatty acids suitable for cholesterol esterification. There is experimental evidence for this suggestion. It seems to be a general consensus that glucose is not converted to fatty acids in the normal arterial wall. I am especially referring to Vost's work. It is possible to induce intimal thickenings with a high content of lipid and thickenings with low lipid content selectively by choosing in the first case, male rabbits and, in the second, female rabbits. In the female intimal tissue there seems to be no conversion of glucose to fatty acids and no transfer of C^{14}-glucose label to cholesterol ester or sphingomyelin. In the male, however, glucose is converted to lipid. In some samples this may be very marked and the C^{14}-glucose label may predominantly be confined to the sphingo-

RABBIT

myelin and the cholesterol ester fractions, which is markedly abnormal (33, 34).

I would like to make a point here. The intimal tissue in question, especially in the male, is not completely covered with endothelium. It is therefore flooded with fatty acid-containing albumin. A shortage of fatty acids per se cannot be responsible for the lipogenesis. Perhaps the cells need other types of fatty acids than those supplied in the serum.

DR. KRITCHEVSKY: Dr. Lofland has commented on experiments in which administration of labeled acetate yielded labeled ester cholesterol. Dr. Charles Howard (Beaverton, Oregon) has shown that conversion of acetate to cholesterol and its esters proceeds readily in normal monkeys, but not in cholesterol-fed monkeys. On the other MONKEY hand, when cholesterol-fed monkeys are given labeled glucose they are able to synthesize cholesteryl esters labeled in the fatty acid moiety.

Chapter 3
RELATIONSHIP OF ENDOTHELIUM TO SMOOTH MUSCLE

OVERVIEW

Dr. Soren Bjorkerud

This review will be focused on those properties of arterial endothelium which might influence the underlying smooth muscle cells. The review will have a rather short time perspective with regard to the age of the information. The late Dr. French gave an excellent review on portions of the subject at the Lindau Conference (109).

The disposition follows roughly that of our own life, first to be discussed will be some obstetric and pediatric cell problems, then properties of the adult cell, and finally, cell regression and death.

That arterial endothelium has the capacity of rapid regeneration after experimental trauma is known from the work of Poole et al (255), Cotton et al (70), and Gottlob and Zinner (124). Not too long ago it was unclear and indeed, questioned by some, if arterial endothelium of adult animals was subject to turnover. Autoradiographic demonstration of incorporated tritiated thymidine in arterial sections (374, 375, 393) made it clear that endothelial cells replicate even in "normal" adult endothelium, although slowly.

Progress in this field has been hampered by technical difficulties due to the fact that endothelium is a monolayer. Thus, very little information on the whole cell population can be obtained from single sections. These difficulties were circumvented by isolation of the endothelium with a modified version of the old en-face, "Hautchen", technique and subsequent autoradiography of the isolated layer as first described by Obaze and Payling Wright (242). Improved modifications of this technique were described by Sade and Folkman (322) and recently by Schwartz and Benditt (335).

The endothelium is heterogeneous with regard to frequency of cell divisions both in different areas within an aortic segment, and in different segments along the aorta.

The drawing in Fig.101 depicts the average percentage of labelled cells in different segments along the guinea pig aorta as found

GUINEA PIG

by Payling Wright (250). As an explanation for the larger rate of mitosis in the aortic arch, in the upper abdominal aorta and in the bifurcation area, she proposed hemodynamic damage, leading to endothelial injury and repair (250, 251). The rate of replication would correspond to a life span of 60–120 days in branching regions and 100–180 days in unbranched segments. Her finding that the rate of mitosis was increased in and above an artificially created constriction of the guinea pig aorta (250) indicates that the hemodynamic damage explanation may be correct (Fig.102).

RAT

Essentially similar results from unmanipulated rats were recently presented by Stephen M. Schwartz and Benditt (335). A refined technique permitted a detailed mapping of the mitotic rate of the endothelium within each segment of the rat aorta. In Fig. 103 is shown such a map of a segment of the thoracic aortic surface of a three month old rat. The left pictures illustrate the orientation of the map. The middle figure is the map where each dot represents one tenth of a per cent of labelled cells within a unit square of the map. In the right figure the data of the map is integrated. It can be seen that the mitotic rate is larger in the dorsal sector which was the rule for the thoracic aortic segments. No such apparent reproducible pattern was found for the abdominal aorta (the mouth of the intercostal arteries are situated along the dorsal sector of the descending thoracic aorta).

The mitotic rate of arterial endothelium does not only vary with location but also with age of the animal. Sade et al (323) measured the in vitro labelling of endothelial cells of the aorta of rats of varying age. As can be seen from their results, as shown in Fig. 104, the labelling of cells decreases greatly with age. Similar results were obtained by Stephen M. Schwartz and Benditt (335) after in vivo labelling. Newborn rat aortas exhibited an over all rate of labelling about ten times that of three month old rats. The pathophysiological implications of an age-related decline of the capacity for endothelial regeneration is obvious.

Endothelial Cell Turnover and Smooth Muscle Proliferation

The results just mentioned from Payling Wright's works (250, 251, 252), suggesting a relationship between increased hemodynamic strain and increased endothelial cell turnover, have recently been confirmed and extended in the sense that regions with increased permeability for albumin-Evans blue complex were shown to have a higher mitotic rate by Caplan and Colin Schwartz in 1973 (57). It should be mentioned that a marked stimulation of the mitotic rate was observed after cholesterol feeding both in rabbits (216) and in swine (393) where this effect was demonstrated after only three days of cholesterol feeding (103). They concluded that some

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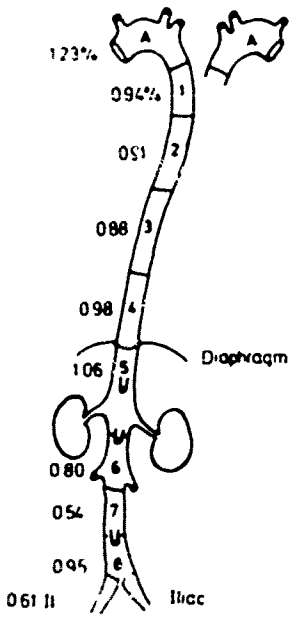


Figure 101: Schematic illustration of the average percentage of labeled endothelial cells in different segments along the guinea pig aorta. From Payling Wright, 1970.

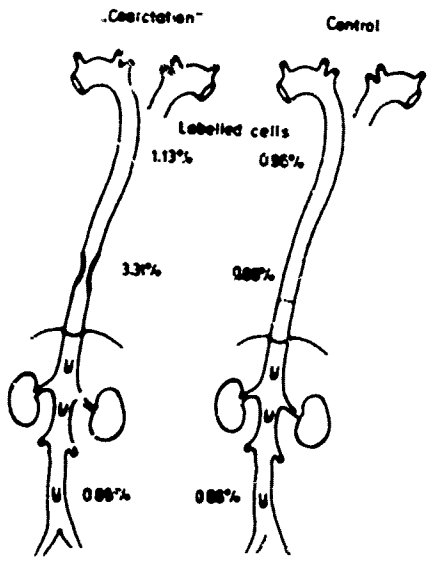


Figure 102: Average percentage of labeled endothelial cells in control guinea pig aorta and in an aorta with an artificially created constriction. The mitotic activity in the endothelium is markedly increased in and above the constriciton. Payling Wright, 1970.

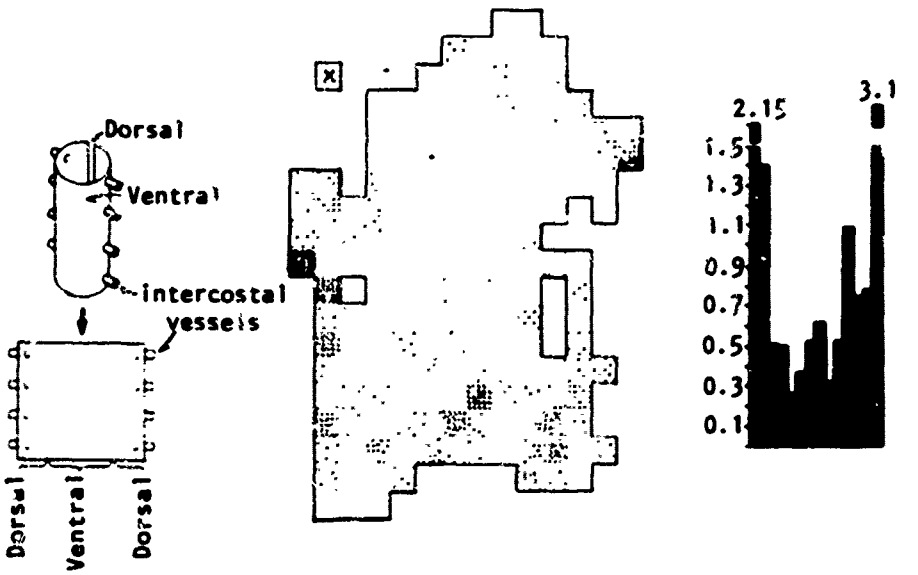


Figure 103: Mapping of mitotic activity in rat aortic endothelium. A refined technique was used to isolate the endothelium as a "Hautchen" preparation and to trace mitotic activity by autoradiography. The left figures illustrate the orientation of the endothelial sample. The mitotic rate at different sites in the endothelial sample is depicted in the middle figure where each dot represents one tenth of a per cent labeled cells within a unit square of the map. The data on each "longitude" is integrated in the right figure. The mitotic rate was, as a rule, larger in the dorsal sector in thoracic segments. Modified from Schwartz and Benditt, 1973.

PERCENTAGE OF LABELED CELLS IN AORTIC ENDOTHELIUM OF RATS OF VARYING AGE AFTER IN VITRO INCULATION WITH LABELED THYMIDINE (VALUES FROM SADE ET AL., 1972)

AGE/WEIGHT DAYS GRAMS	PERCENTAGE OF LABELED CELLS
21/50	0.5-1.3
42/120-150	0.05-0.80
180/600-700	0.01-0.10

Figure 104: The mitotic rate of arterial endothelium in rats decreases greatly with age as shown by Sade et al., 1972.

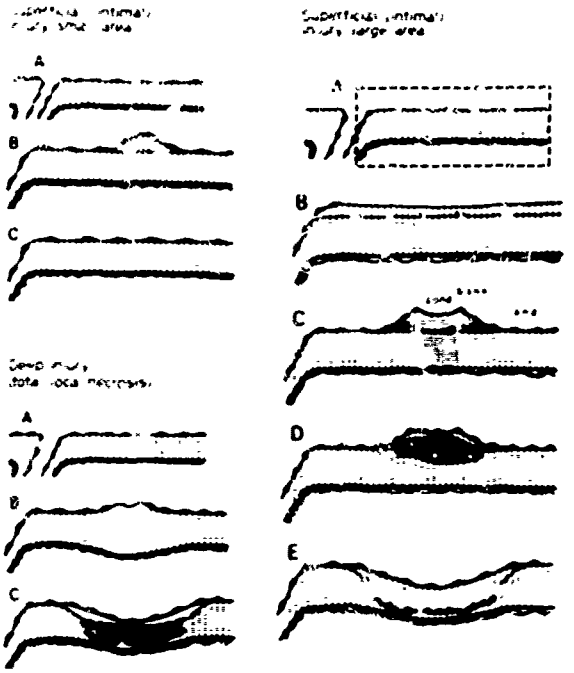


Figure 105: Rapid regeneration of experimentally desquamated aortic endothelium follows when the initial distance from sources of intact endothelium is short (superficial injury, small area and deep injury). When sources of intact endothelium are more remote the rate of endothelial regeneration slows down markedly (Bjorkerud and Bondjers, 1973a, b).

Figure 106: An experimentally denuded aortic segment is rapidly covered with platelets (Haudenschild and Stuber, 1971).

constituent of the blood of the cholesterol-fed swine is acting directly on the endothelial cells, and suggested that the same factor may be responsible for the proliferation of the smooth muscle tissue. The increased multiplication of endothelial cells was probably balanced by an increased elimination of the cells, as the density of the cell population seemed not to increase. An important question in this context is: "What are the factors which promote or inhibit endothelial regeneration?" Very little information is available on this topic. It has been obtained primarily from experimental situations where the endothelium has been subjected to extreme challenge.

Endothelial Regeneration Following Injury

From the earliest studies of endothelial regeneration, following experimental injury, it was apparent that large differences exist with regard to the speed of endothelial regeneration (for discussion see Bjorkerud and Bondjers, 35). Species differences were proposed as an explanation, but the different methods used for the desquamation of the endothelium could also be important. The latter explanation has gained strong support.

It is possible, quite reproducibly, to selectively produce areas with rapid or with very slow endothelial regeneration in the aorta of the same animal. It is done by the infliction of two different types of defined mechanical injury using a special catheter (36, 37, 38, 39). Sequential comparative studies of the two types of lesions indicate that the speed of endothelial regeneration is related to the initial distance of the injury from sources of intact endothelium (Fig.105). Sources are the mouths of aortic branches, or islands, with intact endothelium. Areas near the source that can be covered within two to three weeks, heal. The overgrowth slows down markedly when the endothelium reaches more remote areas (35, 39, 40). The initial fast regeneration and the subsequent delay of endothelial covering has been confirmed by Christensen and Garbarsch (65) observing extensive lesions induced with the balloon catheter. There is as yet no definite clue to the cause(s) of the retardation.

Very rapidly after endothelial desquamation the surface is covered by platelets as shown by Haudenschild and Studer (133) in the rabbit (Fig.106). Polymorphonuclear leukocytes and monocytes eliminate the thrombotic material in a few days and seem to stop further platelet deposition (Fig.107) (133). It is possible that the subendothelial surface "cured" in this way permits rapid re-endothelialization. It is also conceivable that this property may be lost after a critical period of prolonged exposure to blood. For the normo-lipidemic rabbit this critical period seems to be 3-4 weeks. After this time interval there is a marked retardation of the reendothelialization.

Figure 107: The thrombolic material is removed with a few days after the infliction of injury, apparently by the action of polymorphonuclear leukocytes (PMN) and monocytes, which also seem to prevent further platelet deposition (Haudenschild and Studer, 1971).

Figure 108: En face semimicrophotograph of a rabbit aortic segment where an experimental lesion was induced mechanically two weeks earlier. The initial injury was shallow with a large surface area. New endothelium spreads from [illegible] inches. The proximal and distal rims of the regenerating endothelium (proximally and distally) have advanced to the markedly thickened regions denoted B. Interposed areas denoted P still lack endothelium. Multiple microthrombi are present predominantly along the edge of the endothelium as clearly shown in inset (Bjorkerud and Bondjers, 1973 a).

Figure 109: Low-power electron micrograph of section through experimentally induced aortic lesion with retarded reendothelialization. The sectioned region corresponds to non-reendothelialized regions denoted P in fig. 108. The surface lining consists of smooth muscle cells separated by large clefts containing fibrin-like osmiofilic material (Knieriem et al., 1973).

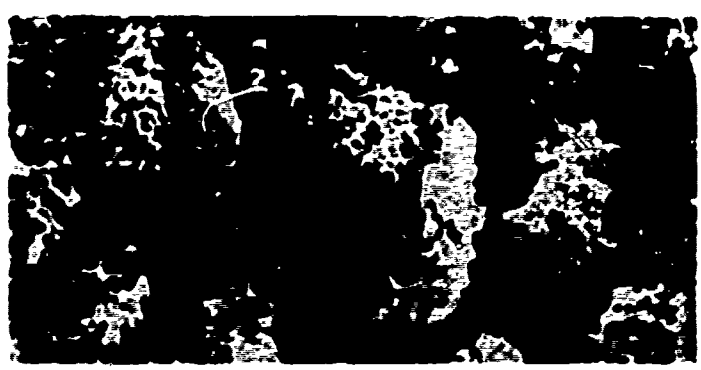


Figure 110: Low-power scanning electron microphotograph of dog pulmonary artery. Single endothelial cells are indicated by asterisks. Note the presence of a multitude of processes on the cellular surface (Una Smith et al., 1971).

RABBIT Fig.108 presents a semi-microscopic surface of a rabbit aortic segment two weeks after the induction of wide-spread superficial aortic injuries by the type of mechanical trauma which reproducibly leads to retarded reendothelialization (39). It is possible that the microthrombotic material at the front of the advancing (?) endothelium induces the retarded overgrowth. It is difficult to tell if platelets are involved in this process; this would be consistent with Ross's report on the presence of growth-stimulating factors in platelets (see page 76). An alternative explanation would be exaggeration of the intimal thickening by formation and organization of thrombi.

Tissue cultures have proven valuable in studying the influence of different factors on cell populations. Perhaps some of the questions just mentioned can be resolved with this technique. Endothelium like cells have been isolated by enzymatic dislocation both from human umbilical vein (112,211) and from the larger arteries of the rabbit (55).

Robertson isolated cells with the so-called double coverslip technique (299) without the use of enzymes. The cells survived about 20 passages and were diploid. Recently arterial endothelium was isolated by Csonka et al. (72) in Jellinek's group in Budapest also without the use of enzymes. The cells survived 50-60 passages and showed both light and electron microscopic properties of arterial endothelial cells. The cells were confluent at some areas and had a flattened appearance. Electron microscopy of junctional regions seemed to demonstrate both tight junctions and flaps - typical elements of endothelial cells in vivo (see below).

Under certain circumstances endothelial regeneration is accompanied by covering of denuded subendothelium with a strongly aberrant cellular surface layer which is referred to as pseudoendothelium (265). Tsao (396) concluded from electron microscopic observations that myointimal cells could possibly migrate towards the lumen and redifferentiate to endothelial cells. Dr. Robertson will expand upon this subject later. From our observations it appears that flattened smooth muscle cells can serve as a provisional surface lining for long periods in those instances where resurfacing with endothelium is retarded. There is some evidence suggesting that monocytes may also form a fairly continuous provisional surface lining (45, 65, 265).

The central part of the mechanically induced lesions displaying retarded healing in the rabbit is covered by flattened smooth muscle cells (39) (Fig.109). Very large clefts between the cells contain fibrin-like osmiophylic material (185). We have measured the transfer of cholesteryl ester from the blood (which can be regarded as a rough measure of lipoprotein transfer) into the underlying tissue.

It averaged 32 times greater in regions devoid of endothelium than in regions covered with intact endothelium.

Finally, as regards the origin of cells at the luminal surface, it should be mentioned that a proportion of the subendothelial cells may be of endothelial origin, as Borgers and coworkers (47) concluded from a combined cytochemical and electron microscopic study on developing coronary collaterals. Mixed origins of both endothelial and subendothelial cells would, indeed, not facilitate the understanding of the biology of these tissues.

Turning now to surface properties of arterial endothelial cells. These have been difficult to observe in transmission electron microscopy, but are apparent in the scanning electron microscope. One such is the presence of large numbers of irregular processes directed towards the lumen. The presence of processes was noted by Rhodin (269) and studied more extensively by Una Smith and coworkers (353). Fig.110 shows a low-power scanning electron micrograph of the luminal surface of the dog pulmonary artery. The separate endothelial cells are denoted by an asterisk and have large numbers of irregular protrusions.

Morphology of the Endothelial Surface

Transmission electron micrographs of sections of similar areas illustrate the varying thickness of the processes, and the presence of pinocytotic vesicles (marked by asterisks) in the processes (Fig.111).

We have found similar processes although more fold-like, in the rabbit aorta using scanning electron microscopy. Areas with relatively smooth surface alternate with areas with rough surface which points to the possibility that the degree of surface roughness may be a dynamic and rapidly changing property. No definite conclusion can be drawn as yet on the possible functional role of these surface projections. However, it is clear that the projections markedly increase the luminal surface area of the cells, which could facilitate plasma membrane-mediated transport between the blood and the vessel wall. RABBIT

Spreading of endothelium by mere flattening of the cells without cell replication has been observed in cell culture (112), but it is unclear if an endothelial lining can extend by such a mechanism in vivo. However, it is possible that the cell processes represent a plasma membrane reserve that might be utilized for amitotic spreading of the endothelium or for other plasma membrane requiring processes, as e.g. endocytosis. Una Smith and coworkers (353) suggest that the projections might produce a boundary layer of retarded flow of cell-free plasma along the surface of the cell body. Such a slow-flowing boundary layer could provide favorable

Figure 111: Transmission electron micrograph of section of endothelium in dog pulmonary artery. The endothelial processes vary in thickness. Pinocytotic vesicles are present in the processes (asterisks). From Una Smith and coworkers (1971).

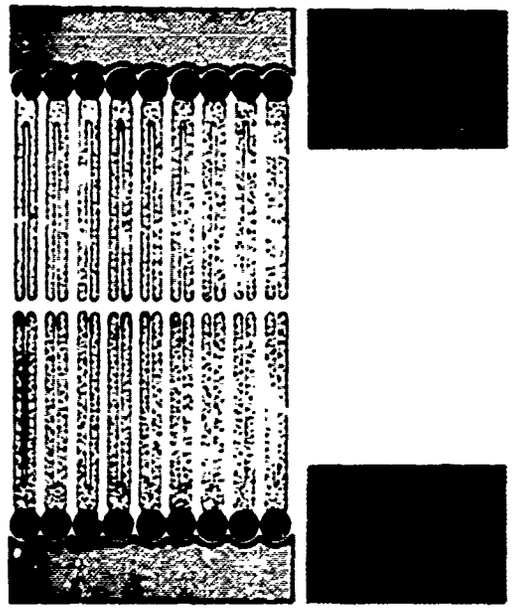


Figure 112: Scheme illustrating Robertson's modification of the Danielli-Dawson model of the unit membrane (From Bjorkerud and Sjostrand, 1971). When freeze-fracturing a tissue the fracture tends to follow preformed surfaces in the tissue, as e.g. cell membranes. The fracture line occurs between the hydrophobic ends of the bimolecular phospholipid leaflet of the membrane. The right figure illustrates the transmission electron microscopic image of the unit membrane.

conditions for transfer of substances between the plasma and the endothelium. On the other hand, a boundary layer could also act as a transport barrier through which material has to diffuse to reach the endothelium. Results from studies by Caro and Nerem (58), designed to test this theory, were inconsistent with transport controlled by a diffusion boundary layer. This does not mean, however, that a boundary layer does not exist. They only indicate that a boundary layer was not a rate-limiting factor for transfer between serum and arterial wall of the material in question which was lipoporotein-bound C^{14} labelled cholesterol.

Turning now to more qualitative aspects on the endothelial cell plasma membrane. At the Lindau Conference the presence of a ruthenium red-stainable surface coating, and its possible analogy to the so-called glycocalyx, was discussed by the late Dr. French (109). As the degree of specificity for carbohydrates of the ruthenium red method was uncertain, the question of the presence of a glycocalyx could not be settled. However, since then a specific cytochemical method for carbohydrate compounds is available. It is based on the affinity of concanavalin A for such compounds (27). Dr. Weber's group (403) have presented evidence obtained by this technique which strongly indicates the presence of glycocalyx on the surface of the arterial endothelial cell.

It is quite possible that some of the functional characteristics of the endothelial cell, as for instance plasma membrane permeability, and the physical properties of the cell surface in the serum - cell interphase could depend on the glycocalyx as suggested by French (109). Dr. Weber will treat this subject specifically. I will not expand on it, but only mention a study by Buonassisi (55) where arterial endothelial cells reared in tissue culture were found to synthesize various species of sulphated mucopolysaccharides. Some evidence was obtained for an incorporation of a major fraction of the mucopolysaccharides into the cell wall. Other smaller fractions of heparin-like mucopolysaccharides were released into the medium which could reflect an antithrombogenic mechanism in these cells. It was shown recently that the basal membrane subjacent to the endothelium is probably an endothelial product (336). Therefore, it is possible that the in vitro production of sulphated mucopolysaccharides just mentioned may reflect also the formation of material for the basal membrane.

Transmission electron microscopy of sections has yielded rather limited information on the plasma membrane per se. Lately, with the use of freeze-fracturing, shadowing, and subsequent electron microscopy, it has been possible to obtain more detailed information. When freeze-fracturing a tissue, the fractures seem to occur predominantly between the hydrophobic ends of the bimolecular phospholipid leaflet of the unit membranes (49). On the left side of

Fig.112, which depicts Robertson's modification of the Danielli-Dawson model of the unit membrane, the fracture would occur in the free interspace between the hydrocarbon chain palisades. The right figure depicts the transmission electron microscopic image of the unit membrane.

Fig.113, from a paper by Dempsey and coworkers (79), illustrates the two types of surfaces which are exposed when the fracture occurs in the plasma membrane. The exterior surface of the inner leaflet of the membrane is designated A-face and the interior surface of the outer leaflet B-face. Different fracture faces are demonstrated in Fig.114. Globular units are found between the plasma membrane leaflets of the capillary endothelial cell and the erythrocyte, some adhering to the A-face, others to the B-face (79). It has been suggested that the number and distribution of the intra-membraneous particles are related to the physiological activity of the membrane (49), and that they may represent enzyme clusters (354).

It seems as if fusion of membranes is preceded by a rearrangement of the intramembraneous particles to rings or plaques. Fig. 115 from a paper by Satir and coworkers (326) illustrates schematically the probable events before and during the membrane fusion occurring when a secretory granule is discharged from a Tetrahymena. A similar rearrangement of the intramembraneous particles has been observed in pulmonary endothelial cells and it was proposed that the rearrangement of the particles was related to membrane fusion occurring during pinocytosis (354).

I will now leave the free endothelial surface for a moment and discuss aspects relating to the intercellular parts of the plasma membrane. Rearrangement of intramembraneous particles seems not only to be related to communication between the interior of the cell and the external environment as in secretion and pinocytosis, but also to communication between adjacent cells. Plaque-like regions with the plasma membranes of adjacent cells in close contact can be found along the cellular interface. Figs.116 and 117 are illustrations from a paper by Gilula and coworkers (116). In the left part of Fig.116, which shows a section from the region between two fibroblasts, the plasma membranes are in, as it seems, close contact for a considerable distance. A surface of this kind is a so-called "gap junction". Freeze fracturing reveals a plaque-like cluster of intramembraneous particles in the gap junctional region as shown on the right side of Fig.116. Recently the presence of gap junctions in rat arterial endothelium was suggested by Stermerman and Ross (389) and demonstrated convincingly by Huttner and coworkers (154) who have studied them in great detail. As in other tissues, these junctions do not restrict the passage of, e.g., colloidal lanthanum intercellularly

Endothelial Cell Junctions

RAT

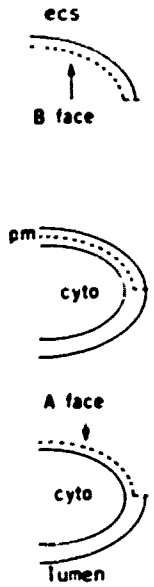


Figure 113: Scheme depicting the different membrane surfaces obtained after freeze-fracturing (From Dempsey et al., 1973).

Figure 114: Electron microphotography of endothelial membrane surfaces (A;B) obtained after freeze-fracturing of a fenestrated capillary. Globular units (intramembraneous granules) are present between the plasma membrane leaflets. rbc - A; rbc - B denote fracture faces of red blood cell in the capillary (Dempsey et al., 1973).

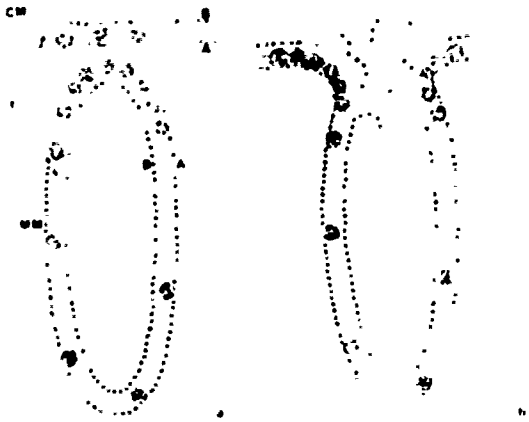


Figure 115: Scheme depicting the rearrangement of intramembraneous granules preceeding fusion of membranes when Tetrahymena discharges a secretory granule as proposed by Satir and coworkers (1972). Similar arrangement of intramembraneous particles was observed in pulmonary endothelial cells (Smith et al., 1973).

Figure 116: Electron micrograph of section through gap function (left) and freeze-fracture face of gap junction (right) between two fibroblasts. The intramembraneous particles are arranged as a tight cluster in the junctional surface. From Gilula et al., 1972).

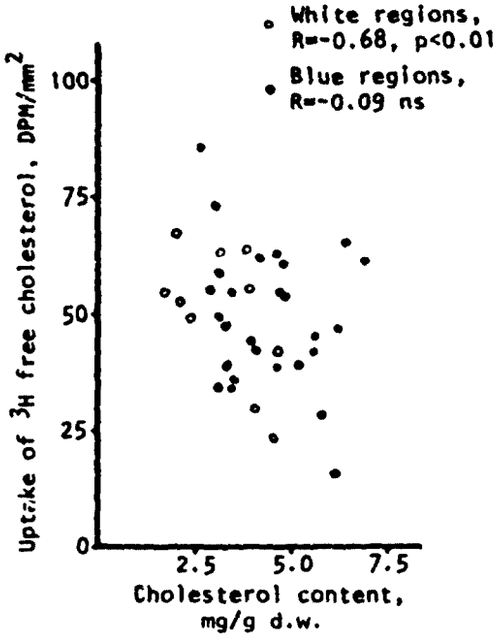


Figure 117: Transfer of tritiated free cholesterol from blood to aortic wall in vivo in normolipidemic unmanipulated rabbits versus the local aortic content of cholesterol. For regions with a low degree of endothelial injury (white regions) the transfer of cholesterol from the blood compartment was inversely related to the cholesterol content of the aortic region in question. The inverse relationship was not found for regions with spontaneously injured endothelium (blue regions). From Bondjers and Bjorkerud (1973a).

Figure 118: Electron micrograph of section showing different endothelial cell-to-cell contacts. Tight junctions are present at sites indicated with arrows and gap junctions at GJ. From Huttner et al., (1973a).

This can be explained by their macula-like configuration and presence of an about 20 angstrom gap between the cell membranes in the gap junctional region. Therefore, they are not thought to be important for the control of intercellular permeability, but are considered to represent the structural basis for communication between cells both with regard to electrical coupling and exchange of ions and metabolites (116). It might well be that they are necessary for possible synchronized functions of the cells in the endothelium, as suggested by Huttner and coworkers (154).

Rhodin (270) and Richardson and Beaulnes (273) suggested that endothelial cells may transmit signals from receptors on their surface to underlying smooth muscle cells. Gap junctions are present between arterial smooth muscle cells (161) and taken together with the presence of gap junctions in endothelium, it is possible that the entire endothelial and smooth muscle apparatus in the artery forms a coupled system as suggested by Huttner and coworkers (154). Actually, some experimental data lend support to such an hypothesis
CALF Werthessen and collaborators (417, 418) perfused calf aortae with a serum-containing medium at high pressure (200 mm. of mercury) and found an inverse relationship between net uptake of cholesterol during the perfusion and the initial concentration of cholesterol in the tissue. Data from experiments with lower perfusion pressure (Werthessen et al (419) as recalculated by Bondjers (45)), did also indicate an inverse relationship between uptake of cholesterol and tissue cholesterol content. They concluded: "the aortic content of cholesterol ... is subject to control." (417).

We found that the in vivo uptake of labelled free cholesterol into aortic segments with intact endothelium in unmanipulated
RABBIT rabbits also was inversely correlated to the cholesterol content of the segment (Fig.117) (46). These results strengthen the concept of the presence of a mechanism for the control of arterial cholesterol content.

As the inverse relationship was not found for regions with spontaneously injured endothelium (denoted blue regions in the diagram) it is likely that the controlling function is related to the presence of a continuous undamaged endothelium (46). These results suggest that the control may involve adjustment of cholesterol transfer from plasma in accordance with local requirements of the tissue (46). It is possible that the underlying mechanism for this "metabolic" coupling between endothelium and smooth muscle tissue with regard to cholesterol uptake and content could be based on communication via a gap junction system.

Another type of cell-to-cell contact known for a long time is the so-called tight junction or zonula occludens. It is an area of cell contact in which there is apparent fusion of plasma mem-

brane outer leaflets with an obliteration of the extracellular space (94, 95). Its structural properties in sections are illustrated in Fig.118 taken from a recent paper by Huttner and collaborators (154).

Freeze-fracturing also reveals that the tight junction comprises rearrangement of intramembraneous particles. Fig.119 was published by Goodenough and Revel (122) and shows the fine structure of the tight junctions present lateral to a bile canaliculus in the liver. The tight junction consists of chain-like clusters of intramembraneous particles situated in the same plane as the outer membrane leaflet. Interlacing grooves in the inner membrane leaflet correspond to the chains of granules. In the lower picture a bile canaliculus is fractured and the grooved aspects of the two junctions which seal the canuliculus are visible.

There is solid experimental basis for the opinion that the tight junctions represent intercellular permeability barriers. However, there seems to be a general consensus that the tight junctions in arterial endothelium (104, 155, 156, 336, 382) do not form a continuous intercellular barrier. Small intercellular clefts permit low molecular compounds and small macromolecules (around 40000 M.W.) to pass from the blood stream to the subendothelial zone. Larger molecules, as for instance serum lipoproteins, seem to be transported exclusively via vaceolae and vesicles (383). Dr. Olga Stein will treat the subject of the transport of macromolecules through the endothelium in more detail.

"That tight junctions may be not so tight under some conditions" was stated by Florey in a survey on the endothelial cell in 1966 (105). The permeability-increasing effect on capillary endothelium of certain metabolic inhibitors, as for example dinitrophenol, and the widening of junctions in inflammation and under the action of certain vasoactive substances were quoted as examples.

Endothelial Cell Contraction

That the permeability of venules may increase due to endothelial cell contraction with widening of the intercellular clefts seems to be well established (169). Although contractile fibers and actinomyosin (21) have been demonstrated in arterial endothelial cells, the property of such cells to contract actively is far from proven. Characteristic changes that may indicate active endothelial cell contraction are (1) nuclear deformation; (2) bulging of the cell towards the lumen; and (3) the widening of intercellular clefts, all of which are illustrated in Fig.120 from a survey by Majno (208). Such changes, however, can be induced by other factors.

As shown by Haudenschild and collaborators (134) the structure of the endothelium is highly dependent on how the initial fixative

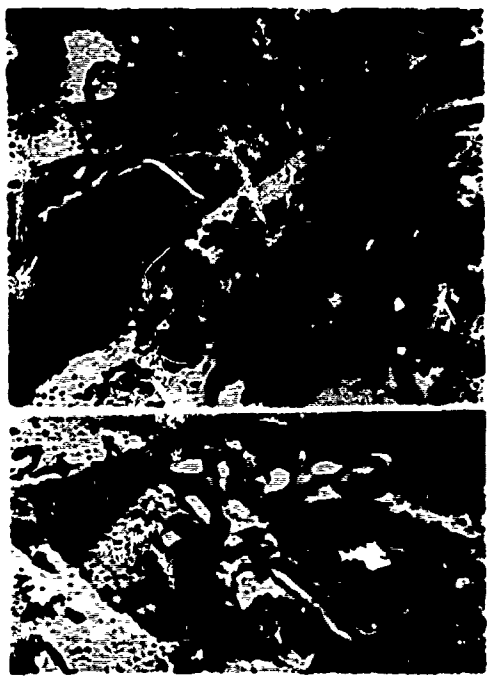


Figure 119: Freeze-fracture faces of tight junctions exhibit rearrangement of the intramembraneous particles, as for gap junctions but as chain-like clusters (cf. fig. 116). Bile canaliculus region in liver. From Goodenough and Revel (1970).

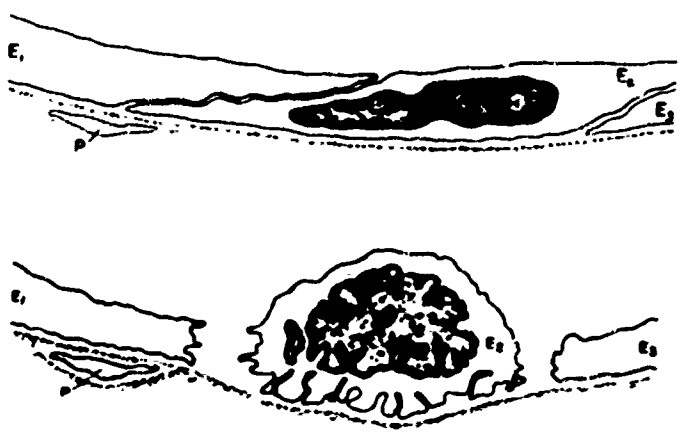


Figure 120: Schematic illustration of cellular changes accompanying active endothelial cell contraction. From Majno (1970).

is applied. This is illustrated in Figs.121 and 122 showing electron micrographs of endothelial cells from rabbit aortae fixed in situ. The perfusion pressure was high enough to straighten the elastic lamina (62 mm. Hg) when the aorta used for Fig.121 was fixed. If the aorta was fixed with perfusion at a lower pressure, the cell nucleus had indentations similar to those occurring in actively contracted venular endothelial cells (Fig.122).

The second criterion, bulging of the cell into the lumen is not specific. It occurs in other situations as e.g., after injury (41, 149, 169, 185), which is illustrated in Fig. 123. Extreme bulging of a cell in the abdominal aorta after mechanical injury is seen in Fig.124 (149). To avoid misunderstandings I should stress that these considerations do not exclude the possibility of active endothelial contraction in arteries. However, I think they should lead to the following conclusions: (1) The existence of active endothelial cell contraction in arteries has not been convincingly demonstrated; (2) special caution is necessary when interpreting changes which are non-specific as representing active endothelial cell contraction.

Various Stages of Endothelial Cell Injury

Even in normal animals, blood constituents as albumin (23, 57, 167, 215, 245) and plasma phospholipids (192) enter the arterial wall with an uneven distribution. There is an increased uptake in certain reproducible areas as around the mouths of branches and in bends or constrictions, i.e., such regions where increased hemodynamic strain on the wall can be expected. Such areas often show intimal edema, microthrombi (245) and clearly damaged or missing endothelial cells (41, 167). We have extended these observations with a technique by which the aortic endothelium is subjected to a functional test in situ which enables us to evaluate the selectivity of transfer of substances through the endothelial plasma membranes. The test is based on the well documented capacity of uninjured cells to prevent certain indicator dyes from passing the plasma membrane barrier and enter the cytoplasm. Such indicators are trypan blue, uncomplexed Evans blue or nigrosin. It should be emphasized that Evans blue in this context is used in uncomplexed form and not bound to albumin. Evans blue if bound to albumin serves merely as a visible marker for albumin and has been used by a number of groups to follow the uptake of plasma albumin into the arterial wall and other tissues.

Comparisons between endothelial cell morphology with interference contrast microscopy and the uptake of stain indicator showed that nigrosin exclusively stained cells changed to a degree which would indicate cell death while trypan blue and Evans blue stained cells with all degrees of injury. There is information available

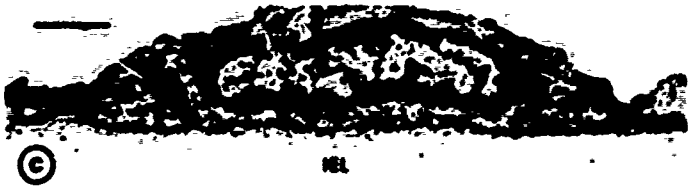


Figure 121: Electron micrograph of rabbit aortic endothelium after fixation by perfusion at a perfusion pressure high enough to straighten the elastic lamine (Haudenschild and collaborators, 1972).

Figure 122: Rabbit aortic endothelium fixed by perfusion at a low perfusion pressure. The nucleus has indentations similar to those occurring in actively contracted venular endothelial cells. From Haudenschild et al., 1972.

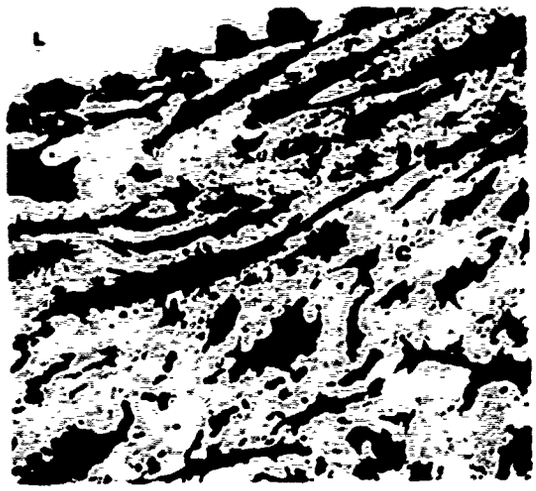


Figure 123: Electron micrograph of edge of poorly regenerating endothelium in chronic type of mechanically induced aortic lesion in the rabbit. The injured endothelial cells at the edge bulge markedly into the lumen (Knieriem et al., 1973).

Figure 124: Extreme bulging of endothelial cell in previously injured aortic segment (Hoff, 1970).

from the fundamental work of Bessis (29) on structural changes related to different degrees of cell injury in general, and by Hoff and Gottlob on experimental arterial endothelial cell injury in particular (for review see 149). There seems to be a strong relationship between endothelial injury and areas with increased hemodynamic strain. Severely injured endothelial cells show a decreased selectivity of plasma membrane permeability with uptake of nigrosin. The heavily stained cells bulge markedly into the lumen. Most conventional light microscopic cell characteristics are absent, indicating marked cell injury. The electron micrograph of an experimentally injured endothelial cell from a paper by Hoff and Gottlob (150) can be used as reference (Fig. 125). Note the extrusion which is larger than the original cell.

At this point it should be emphasized that the presence of injured endothelial cells in all probability does not reflect a static condition. On the contrary, taken together with the patchy distribution of intense endothelial cell regeneration referred to earlier, it probably reflects areas with very rapid endothelial cell turnover. In addition to serving as a plasma membrane permeability indicator the penetration of the stain used for dye exclusion tests below the endothelium can serve as an indication for an increased permeability of the endothelial barrier, at least for a low molecular weight compound. Related to the presence of injured endothelial cells, with nuclei showing up as dark-blue spots, is a diffuse blue background staining which is due to penetration of the marker to the subendothelium and inner media indicating increased endothelial permeability.

It has been shown that the uptake of cholesterol into areas with uninjured endothelium probably is an active transport process, involving hydrolysis of cholesterol ester as a primary step, a process that might be adjusted to the requirements of cholesterol of the tissue as mentioned earlier (46). It is possible that this process is equivalent to the transport of large macromolecules or molecular aggregates by cytoplasmic caveolae and vesicles noted by others.

Transport across Injured Endothelium

The active transport process of cholesterol is also present in areas with a large proportion of injured endothelial cells. But here the additional movement of both free and esterified cholesterol is probably due to easier filtration of lipoproteins (46). These considerations suggest that to the transport of lipoproteins by intracellular vesicles and possibly by intercellular clefts should be added a third route in the normal artery; namely, filtration through discontinuities due to injured endothelial cells.

But it appears that a considerable barrier function is still

Figure 125: Electron micrograph of experimentally injured arterial endothelial cell with large extrusion (X) and vacuolization of the cell body (Wolff and Gottlob, 1967).

present in spontaneously injured endothelium. Although the uptake of cholesteryl ester, and presumably lipoprotein, was significantly higher through spontaneously injured endothelium in the rabbit, it was on average not more than 2.5 times that into uninjured areas (46). This phenomenon seems quite normal and could reflect a physiological increase in flux of nutriments and oxygen. The presence of a partially retained barrier function is seen in the difference between transfer rates in completely denuded areas which averaged 32 times that of normal endothelium-covered areas. Restated for emphasis: If we assign a value of 1 to a normally endothelialized artery filtration rate, that of the spontaneously injured endothelium becomes about 2.5 and where there is no endothelium the value is about 32. We have no evidence for specific routes for transport through injured endothelial cells, but tend to believe that it occurs by mere filtration through discontinuities in and besides the cells. Scanning electron micrographs of endothelial cells from areas with spontaneously injured endothelium in rabbit aortae suggest that this assumption may be correct. Large openings are present in the distended cell. The presence of such discontinuities may not only explain the increased permeability but also the greater tendency for formation of microthrombi on injured arterial endothelial cells (41).

RABBIT

DR. LINDNER: If I understood Dr. Bjorkerud correctly he said that re-endothelialization may extend over an eighteen month period. What evidence do you have for that statement? All the research in the re-endothelialization and turnover of endothelial cells with thymidin by several groups, Haus and my group included, show a very, very high increase of the turnover rate after any kind of alterations. You showed a picture with Knieriem and Bondjers that indicated widening of the spaces between the smooth muscle cells and you postulate that this material between in the spaces is fibrin. Have you seen the periodicity of fibrin?

Endothelial Repair

DR. BJORKERUD: I haven't but I think that Dr. Knieriem has. Did you see it, Dr. Knieriem?

DR. KNIERIEM: We call it fibrin-like and proteinoceous material (185). There are no typical fibrin bands with periodicity. The material is more floccular and quite loosely arranged.

DR. BJORKERUD: And to your first question. It can be answered very easily because if the endothelium does not reach a desquamated region, it doesn't matter if the endothelium present in other regions has a very high mitotic index. In addition to the possible explanation mentioned earlier in my lecture, I would like to mention the possibility that the potential for mitotic multiplication of the cells may be exhausted. Ponten and collaborators have presented

evidence obtained from work with tissue cultures that each tissue has a limited "supply" of mitotic cycles which it can undergo. With a very high rate of mitotic division this limitation could play a role for the marked delay of the re-endothelialization.

DR. KRITCHEVSKY: You showed aortic wall continuities chrough which cholesterol might filter. Of course the cholesterol is presented as lipoprotein and I would like to ask if the cholesterol infiltrates while the protein remains behind. Does the size of the discontinuity permit you to calculate what might be happening?

DR. BJORKERUD: The size is large enough to let even chylomicrons in. As you know the parameter we used for estimating the entrance of lipoproteins was the transfer of cholesterol esters. This approach is to a great deal based on results from you and Dr. Rothblat rel. .ing to the degree of physical exchange of free cholesterol and esterified cholesterol. However, estimating lipoprotein transfer by measuring transfer of cholesterol esters is probably too unprecise to permit a more detailed quantitative analysis.

DR. ROSS: How was the tissue prepared? Was this critically point dried tissue or just frozen dried tissue. I am talking about the scanning electron microscope. This is a very important issue.

DR. BJORKERUD: I agree about the importance of the question. We found it very difficult to preserve the integrity of the endo-helium with any type of technique if special care was not taken to avoid the formation of artifacts. Artifacts may be formed during any step of the preparation procedure. Mere exposure to the air of the laboratory may induce artifacts as contamination with dust particles, uncontrolled drying with shrinkage, and formation of cracks or other defects by exposing the dehydrated sample to the moisture of the air. I don't think it is possible to avoid artifacts and I do hardly believe that any procedure would be ideal. Therefore, we have tried to obtain some insight as to what is artifactual or not, by studying several samples which have been prepared each with a different preparative procedure. Structures present in samples prepared with different techniques would more likely represent true structures. Structures only appearing after one type of preparation are more likely to be artifacts.

DR. ROSS: But you did not use critical point drying.

DR. BJORKERUD: Yes, I think we did.

DR. ROSS: Dr. Arnold Tamarin at the University of Washington and I have also been doing scanning electron microscopy. Without critical point drying you introduce enormous artifacts into tissue.

It must be critically point dried. Freeze drying by itself is not sufficient. You have to use a bomb to do this.

DR. BJORKERUD: I recall we actually used critical point dehydration a few times as described by Anderson (13) with a homemade critical point dehydration equipment. The actual preparative work with this technique was carried out by Dr. Hans-Arne Hansson at our Institute. The results were very similar to those obtained by freeze drying under the conditions used by us.

DR. MINICK: I would like to comment briefly about the importance of the techniques used to process arterial tissue for scanning electron microscopy and demonstrate changes that can be induced in the endothelial lining of arteries by immunological arterial injury.

In preparations of cultured endothelial cells processed by critical point drying, many blebs and micro-villi are seen on the surfaces and some cells extend long thin processes toward adjacent cells. These details cannot be seen in air dried material. Endothelial cells lining normal arteries are covered with similar blebs and microvilli which are more prominent in the endothelium of the ascending portion of the aorta than in coronary arteries. Microvilli and blebs appear to be most prominent overlying nuclei of cells as well as near the junctions between cells (Fig.126). There is no evidence of openings between cells; however, there are occasional craters within endothelial cells which might be confused with openings between cells but we believe represent ruptured blebs. As in the case of endothelial cells in vitro, microvilli and blebs are not prominent in air dried material. Moreover, we have seen instances of what appear to be prominent openings between endothelial cells near arterial branch sites in air dried material but to date we have not observed these openings in critical point dried material. Thus, prominent features of the endothelial surface are quite variable depending on techniques used to process the tissues, a fact that is important to remember when interpreting observations made by scanning electron microscopy.

Next, I would like to demonstrate some of the changes produced in the endothelium of rabbit coronary arteries by immunological injury, specifically, by immune complex disease induced by repeated injections of horse serum (223). Preliminary results of our experiments demonstrate distinct alterations in the structure of the endothelium in coronary arteries and aortas of rabbits repeatedly injected with horse serum. In some areas platelet aggregates are loosely attached to the endothelium. In adjacent areas platelets are firmly attached to arterial lining in the area of junctions between endothelial cells; adherent platelets are sometimes associated with openings between endothelial cells (Fig.127). It cannot be determined by scanning electron microscopy whether platelets

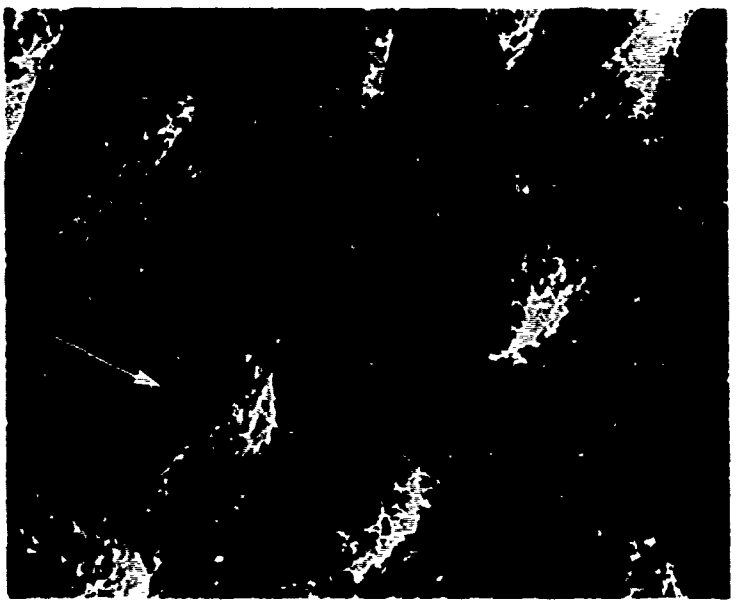


Figure 126: Scanning electron photomicrograph of endothelial surface of ascending thoracic aorta of normal rabbit. Oval elevations (arrow) represent portion of cell overlying the nucleus. Endothelial surface of ascending aorta is characterized by numerous vesicles and microvilli. No openings are present in the area of intercellular junctions. (x 1980)

Figure 127: Scanning electron photomicrograph of endothelial surface of coronary artery of rabbit # 12-662 that received two intravenous injections of horse serum at 16-day intervals. Platelets adhere to the endothelial surface in the area of the junction between endothelial cells. Note openings between endothelial cells adjacent to adherent platelet (arrow). (x 1730)

adhere to the surface of endothelial cells or to underlying material in the subendothelial area exposed by contraction of endothelial cells. Adjacent to other openings in the endothelium are fragments of membraneous debris which presumably represent remnants of disintegrated platelets. Leukocytes also adhere to the endothelium and fill large gaps or defects in the endothelial lining (Figs.128, 129). Some defects are surrounded by thinned and attenuated endothelium; often leukocytes can be visualized beneath this attenuated endothelium. In some instances portions of altered endothelium have sloughed leaving denuded areas covered by leukocytes and platelets (Fig.130). Eventually denuded areas are covered by other cells which may represent endothelial cells or smooth muscle cells.

Thus, immune complex disease may lead to alterations in the endothelial lining that may account for increased permeability of endothelium to large macromolecules, e.g., immune complexes or lipoproteins. Moreover, results of our experiments furnish direct evidence that platelets and leukocytes play an important role in the pathogenesis of the arterial lesions found in experimentally induced immune complex disease. This is in harmony with a large quantity of indirect evidence obtained by other investigators (67, 180, 181). Finally, increased endothelial permeability resulting from endothelial changes such as those illustrated here may be of prime importance in the pathogenesis of the experimental atherosclerosis induced by the synergy of immunological arterial injury and lipid rich diet (224, 225).

DR. OLGA STEIN: I would like to present some of our findings, then tell you about some of our speculations as to how the structure of normal aortic endothelium seems to be adapted to the process of macromolecular transport. The two transendothelial routes shown in Fig.131 can be defined as intercellular or transcellular and have been defined in very many endothelia of capillaries. Since we shall be drawing some comparisons between aorta and capillaries, I would like to point out that there are some analogies between the two types of endothelium and I am referring to the presence of continuous endothelium in the aorta which is similar to that of some capillaries. In the aortic endothelium of rat and mouse, both of which we have investigated, there is a close apposition of cells and the intercellular clefts, which are quite narrow are pursuing tortuous routes (Fig. 132). The aortic endothelium is also very rich in plasmalemmal vesicles which can be seen on both the luminal and abluminal sides and in the middle of the endothelium. In regions in which there is very close apposition between the leaflets of the two plasma membranes of adjoining cells, a punctate junction is formed (Fig.133). It is really not quite certain whether these are permanent tight

Transendothelial Transport

Figure 128: In some areas, leukocytes (arrow) were attached to the endothelial surface (Fig. 128) while in adjacent areas, leukocytes, often as many as 10 or 20 were present within openings (arrow) in the endothelium (Fig. 129). Note numerous villous processes characteristically seen on surface of leukocytes.

Figure 129: In some areas, leukocytes (arrow) were attached to the endothelial surface (Fig. 128) while in adjacent areas, leukocytes, often as many as 10 or 20 were present within openings (arrow) in the endothelium (Fig. 129). Note numerous villous processes characteristically seen on surface of leukocytes.

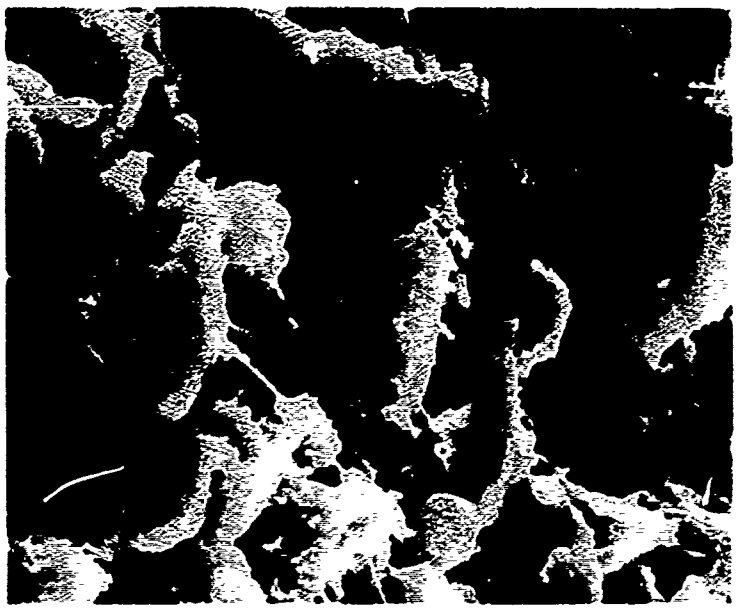


Figure 130

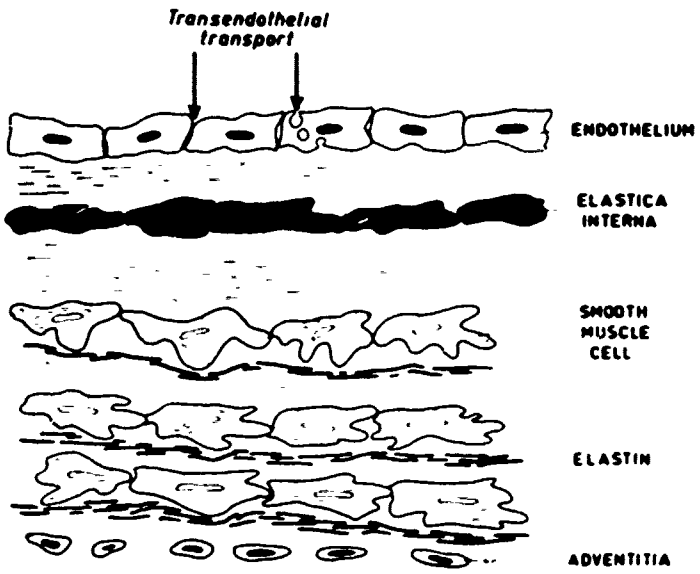


Figure 131: Schematic representation of aortic wall; the arrows indicate the two routes of transendothelial transport - through the cells and between the cells.

Figure 132: Electron micrograph of a section of rat aortic endothelium. Two adjacent cells, rich in plasmalemmal vesicles are separated by a long and narrow intercellular cleft x 45,000

Figure 133: Section of aortic endothelium showing multiple points of contact between the endothelial cells. Fig 3a x 135.00, Fig. 3b x 270.000.

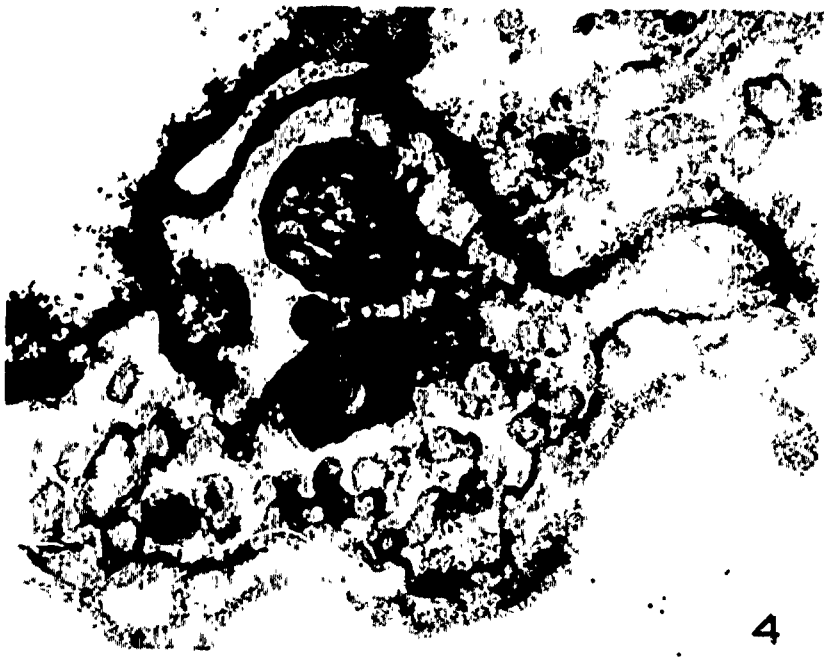


Figure 134: Section of aortic endothelium stained with lanthanum. The tracer is seen in the endocapillary layer and extends into a part of the intercellular cleft. x 72.000

junctions but they seem to provide barriers to the penetration of several markers including colloidal lanthanum (Fig.134). Certain other regions, which can be probably designated as gap junctions, are penetrable by lanthanum (384). In order to learn about the problem of macromolecular transport we also used isolated lipoprotein fractions which were labeled with I^{125}, predominantly in the protein portion of the molecule. With the help of radioautography we were able to demonstrate that in a perfused system there is transport of lipoprotein particles through normal endothelium (387). HDL particles were shown to penetrate more readily (Fig.135) than LDL particles (Fig.136) and the least readily taken up were the VLDL particles. Radioautography did not permit us to delineate the route of transport and to that end we have used markers of known molecular weight such as horseradish peroxidase and lactoperoxidase.

Horseradish peroxidase is transported very readily from the luminal surface to the abluminal surface and both the plasmalemmal vesicles and the intercellular clefts are passable by this marker (Fig.137). When we repeated these studies with lactoperoxidase (82,000 molecular weight), the reaction product was seen in the lumen, in plasmalemmal vesicles, but not in the intercellular cleft. These findings provide some information as to the size of molecules which would transverse the endothelial cell via the plasmalemmal vesicles or pass through the intercellular clefts. Thus even though from radioautography one cannot really conclude conclusively whether the developed grain is present inside the small vesicle, it seems plausible that since HDL particles are at least twice as large as the lactoperoxidase the mechanism for their transport lies in the plasmalemmal vesicles.

Having shown that there is some transport of particles in the form of HDL and LDL across normal endothelium, the question was asked next whether vesicular transport of these lipoproteins might be sufficient to account for the accumulation of cholesterol with age. Since we shall discuss vesicular transport of particles it seems pertinent to point out the difference in the amount of cholesterol contained in the particles. It is such that the number of HDL particles which will contain one microgram of cholesterol is much smaller than the number of LDL particles.

The next question was whether accumulation of cholesterol can be accounted for by the transport of either of the lipoproteins or by both, via the endothelium. We must also ascertain if we have enough vesicles required to transport such an amount of cholesterol (381). We have taken the liberty to use some of Dr. Smith's data (345, 346) about the thickness of the intima at certain ages and also about the amount of cholesterol which accumulates in an intimal segment during a certain amount of time (Fig 138). Assuming that the endothelium of the aorta is similar enough to that of

Figures 135 & 136: Radioautographs of aorta perfused with ^{125}I-HDL (Fig. 135) and ^{125}I-LDL (Fig. 136) x 1440

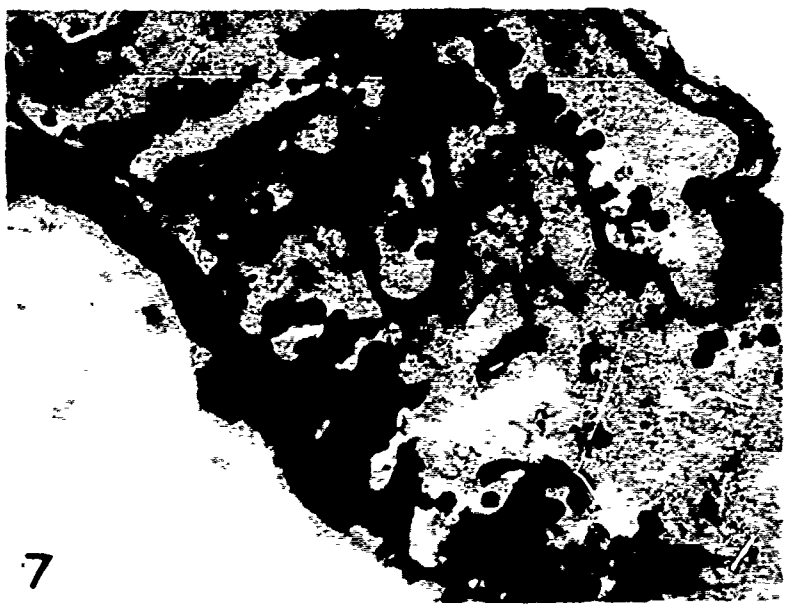


Figure 137: Aortic endothelium 1-2 min after injection of horseradish peroxidase. The tracer is seen in plasmalemmal vesicles, in the intercellular cleft and in subendothelial space. x 46.000

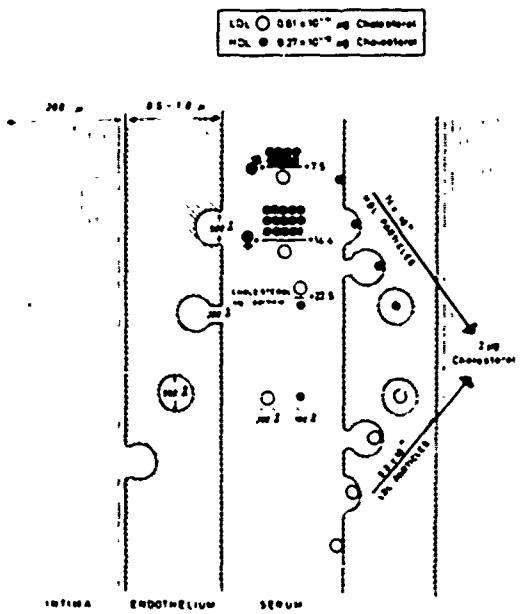


Figure 138: Schematic representation of vesicular transport of cholesterol in the form of HDL and LDL particles through the aortic endothelium into the intima.

capillaries of skeletal muscles or tongue, we might postulate that the number of vesicles that were estimated in those capillaries by Brooms and Palade (51, 52) and Casley-Smith (59, 60) is of the same order of magnitude as that present in the aorta (Fig.139). We have also estimated a range for traverse and attachment time of the vesicle which means the time it takes for the vesicle to form, to move and to traverse. Thus it became possible to calculate the number of vesicles which will be available from a certain intimal segment during a certain time period.

Assuming that those vesicles have to transport about two micrograms of cholesterol (the amount which is accumulating in that time interval) and if we assume that one vesicle will transport one particle then we may predict that in order to transport this amount of cholesterol we would need only one out of ten thousand vesicles to transport all the LDL particles and one out of five hundred vesicles to transport HDL particles in order to account for the amount of cholesterol which has accumulated. Of course, many assumptions and approximations were made in these calculations, but the point I would like to make is that the vesicular transport which we have demonstrated to be present in the aortic endothelium could account for the transport of cholesterol which accumulates with age.

DR. A. P. SOMLYO: When you are treating tissue with colloidal lathanum how often do you find that the abluminal vesicles also contain colloidal lanthanum? In comparison, for example, with horseradish peroxidase. For those who are not electron microscopists I think I should qualify this question by saying that horseradish peroxidase is applied prior to fixation and colloidal lanthanum after fixation. The question is how many of the abluminal vesicles really communicate through some kind of channel with the luminal side?

DR. OLGA STEIN: In our experiments we usually found only colloidal lanthanum in the luminal vesicles and I really don't recall finding them in the abluminal vesicles.

DR. SMITH: This table compares the ratio of albumin, low-density lipoprotein and fibrinogen in plasma and in normal intima. HUMAN This is intima from middle aged humans and is comparable to the intima from which the figures for cholesterol accumulation, which Dr. Stein used in her calculations, were obtained. There is a remarkable change in the ratios of the plasma constituents between the plasma and the intimal compartments. Whereas in plasma there is fourteen times as much albumin as lipoprotein, in the intima there is only about twice as much, measured as concentration of intact plasma protein per unit weight of intima. I am very puzzled as to what sort of mechanism could produce this effect. My own hypothesis is that "whole plasma" crosses the endothelium in pino-

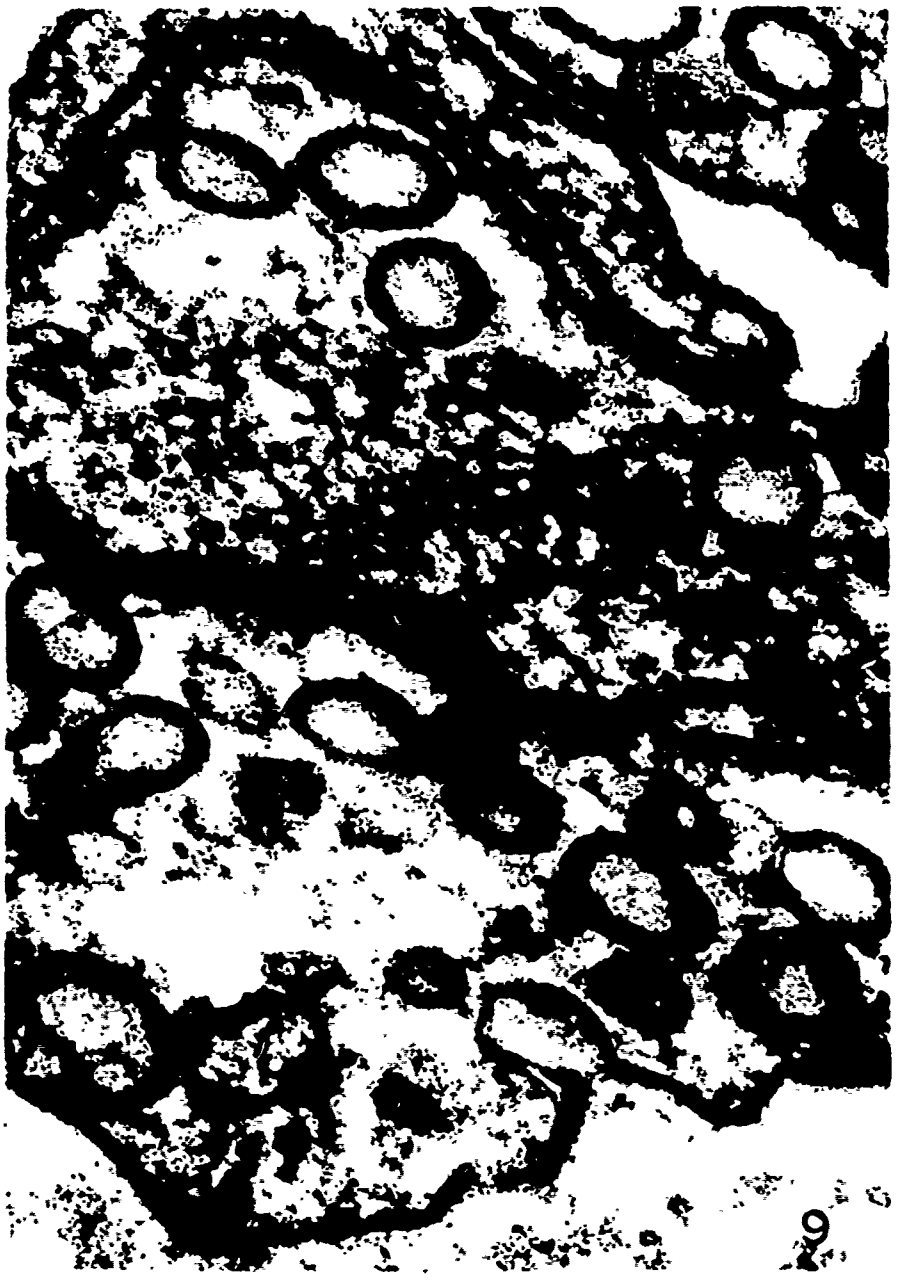


Figure 139: Section of aortic endothelium showing plasmalemmal vesicles and a portion of an intercellular cleft × 180 000

cytotic vesicles; small molecules such as albumin then pass rapidly through into the media whereas the large lipoprotein gets stuck, or can only move out at a slower rate. This idea is, I think supported by some of the Steins' data (387). But whether this concept of a vesicle containing whole plasma is tenable, I don't know. And this is a thing on which I would very much like to hear the Steins' comments.

DR. Y. STEIN: I think the first possibility you mentioned, that the small molecules are rapidly traversing through the media and are leaving through the adventitial side, is quite possible. But the question you really asked was whether a quantum of plasma is taken up into the vesicle or is there some selective uptake of lipoproteins. I can't answer this with great certainty, but I could envisage a process by which lipoproteins could be bound selectively to specific sites on the endothelium and following membrane invagination lipoproteins would be interiorized to a greater extent than the other serum components. This is something we have to investigate in the future, but I think at the moment that both possibilities are open.

DR. BJORKERUD: It is somewhat puzzling that the information obtained with transmission electron microscopy seems not to be consistent with some of our results obtained with scanning electron microscopy and interference contrast microscopy. Does anyone know if branching regions have actually been studied to an extent similar to unbranched regions? It is possible that most electron microscopists would think that structures which do not conform with the traditional concept of endothelial structures represent artifacts and tend to discard samples containing abberant structures. Could it be that the concept of an absolutely continuous arterial endothelium could be based on biased sampling? Are samples from branches regions studied as often as samples from unbranched regions?

DR. OLGA STEIN: I was not able to see channels of this size, and I am not aware that they have been described in the literature. Going back to the question of lipoprotein transport, there is quite good evidence that the larger lipoproteins, such as chylomicrons and VLDL are transported less readily than the smaller lipoproteins. Also, if you want to extrapolate to clinical conditions, high chylomicron levels are not accompanied by enhanced development of atherosclerosis while in the presence of elevation of the lower sized lipoproteins, this condition is much more frequent. During the past decades, physiologists and morphologists have been battling about the interpretation of the small pore equivalent, and the large pore equivalent in various capillary endothelia. Do we still have to deal with yet another large, large pore? Well, perhaps this is something for the future.

DR. BJORKERUD: Just a short comment. Olga Stein and I are talking about slightly different things. I am not referring to the highly organized subcellular endothelial structures that we have described recently (42) but possible cellular defects due to injury. Such defects or discontinuities could either be due to damage of the cell body or to the intercellular junctions.

Relating to Dr. Smith's lecture I would like to ask, are the results presented absolute or relative amounts? It is difficult to imagine that it would be a smaller influx of albumin into a gelatinous lesion than into normal endothelium.

DR. SMITH: In reply to your question, that slide showed only the relationship between the components. I have not spoken about the gelatinous lesions, the concentration of all components is greatly increased, but lipoprotein and fibrinogen are increased to about twice the extent of albumin. Total permeability seems to increase and the retention of lipoprotein seems to increase relatively more.

DR. STEIN: I just wanted to straighten out the misunderstanding. I was referring to something else. Now discontinuities in endothelium, when I see that I think there is something wrong with my preparation.

DR. WERTHESSEN: I think from where I stand outside of the discussion here that a couple of points are being missed. Dr. Stein just mentioned one. When she sees an apparently dead cell in her electron micrograph she thinks there is something wrong with the preparation. Dr. Bjorkerud has made the point that dead endothelial cells are normal. I would appreciate a resolution of this point if it can be done. Can you do it? You have three minutes to do it.

Presence of "dead" Cells in Normal Endothelial Lining

DR. SCHWARTZ: I would like to comment that Dr. Bjorkerud's findings with respect to spontaneous cell injury and death have been confirmed by our own group, but specifically in areas which accumulate Evans Blue dye in vivo. These areas of dye accumulation show significant structural differences which can be interpreted as cell injury in a broad sense. One often finds endothelial cells in which the organelles have been lost while others are swollen and vacuolated. Within the normal artery we have, therefore, a non-homogeneity and areas can be identified which exhibit focal endothelial injury and an increased endothelial cell turnover.

EDITORIAL COMMENT

The preceding discussion illustrates the strategy of a "Lindau-type" conference in which discussion centers on divergent views and interpretation of the same data. Thereby problems may be delineated and the limits of knowledge and understanding clearly laid out.

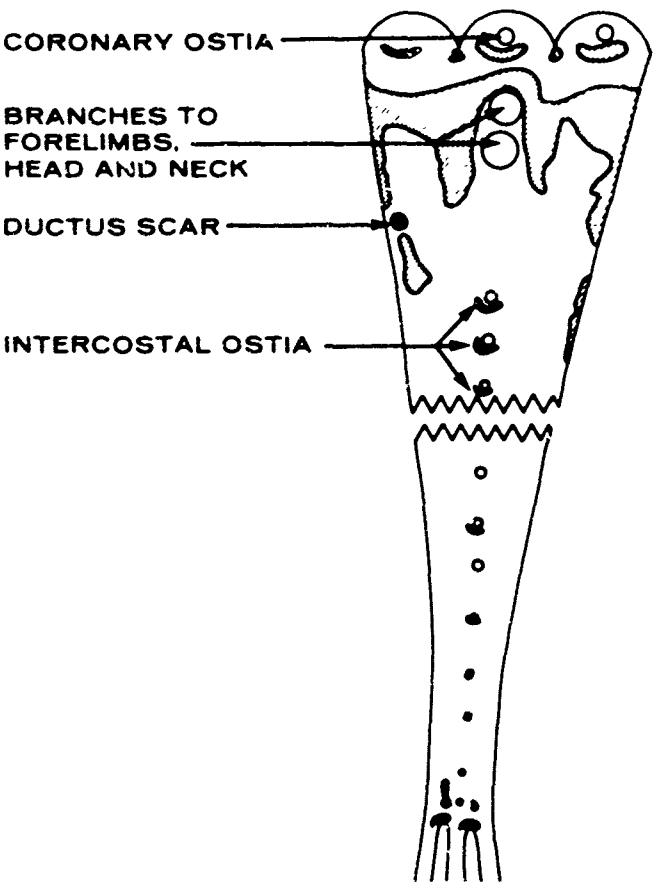


Figure 140: Schematic illustration of uptake of Evans blue dye by the wall of the aorta

DR. SCHWARTZ: I would like to describe some aspects of our work on endothelium; in particular the problems of macromolecular influx and the focal nature of differences in permeability that occur spontaneously in vivo in the young pig aorta. PIG The experimental model that I should like to describe in some detail is the Evans blue model. When I inject Evans blue in vivo it does not stain the aorta diffusely, but has a relatively consistent focal uptake pattern as shown schematically in Fig. 140 with the dye uptake occurring principally in the aortic sinus of Valsalva region, in relation to the brachiocephalic branches in the arch, and near the intercostal ostia.

Macromolecular Influx through the Endothelial Lining of the Aorta

We have examined this model in a variety of ways. Initially, Somer and Schwartz (355) studied the aortic uptake in vivo of tritiated cholesterol in such areas and observed a significantly greater uptake and turnover of unesterified ^{3}H-cholesterol in areas of Evans blue dye accumulation than in contiguous areas showing no dye accumulation. This we have interpreted as reflecting a greater propensity for the exchange of tritiated unesterified cholesterol in areas demarcated by Evans blue dye accumulation.

Transmural gradient studies by Somer and Schwartz (356) showed that the slope of the gradients and the greater activity in blue areas were due to unesterified rather than esterified cholesterol radio-activity.

We now believe that the focal uptake pattern of blue dye in vivo reflects the response of the vascular endothelium to hemodynamic injury. In one study we attempted to change the hemodynamic status of the aorta by the surgical induction of aortic coarctation (357). This model is currently being used to study endothelial influx to a variety of macromolecules. With aortic coarctation one can see a very significant increase in the extent of dye uptake in the aorta proximal to the line of coarctation, and little bluing distal to the line of coarctation. In a sense, these findings confirm the fact that altered hemodynamic status can modify the pattern of Evans blue dye uptake.

We have also looked at a variety of other factors or other parameters associated with this focal spontaneous dye uptake pattern. For example, we have examined endothelial cell turnover using Hartchen preparations with ^{3}H-thymidine autoradiography and found that areas of dye uptake or blue areas show a significantly greater endothelial cell turnover than contiguous areas from the aortic arch showing no dye uptake (57). Interestingly enough, if one injures the aorta in a variety of ways, and one such way is the production of endotoxin injury, one can observe a significant increase in

endothelial cell turnover. On the basis of the turnover studies, we can, I think, conclude that the areas of dye accumulation are areas of spontaneous in vivo hemodynamic injury.

We have also looked at endothelial permeability to macromolecules, and in particular to albumin and fibrinogen (24). Calculated ^{131}I-albumin influx in blue and white areas of the pig aorta in vivo were compared for white and blue areas from the thoracic arch, and for white areas from the upper and lower abdominal aortic segments. Influx, which has been calculated on the basis of micrograms/cm^2/hr was significantly greater in blue than in white areas. What is also important is the observation that the thoracic white areas had a significantly greater influx of albumin than either the upper or lower abdominal aortic segments. In other words, the normal young pig PIG aorta shows both focal and regional differences in permeability to radio-iodinated albumin.

A similar study was undertaken using ^{131}I human fibrinogen (22). As with albumin, influx in blue areas was significantly greater than in areas of no dye accumulation. Significantly less fibrinogen than albumin entered the aorta. Albumin with a molecular weight of approximately 69,000 and fibrinogen with a molecular weight of some 340,000 should, if molecular weight were the prime determinant of influx, show a five-fold difference in influx. The calculated influx for albumin and fibrinogen differed by some fifteen to twenty fold. This could indicate, perhaps, that we are not dealing with an influx which is directly proportional to molecular weight alone. In the case of fibrinogen, the influx appears to be consistent with a molecule of one million (mw), compatible with its physical behavior in gel filtration columns. An important point, however, is that the endothelium of the normal aorta does seem to exert some discriminatory capacity in controlling macromolecular influx.

The blue and white areas do differ morphologically and one of the striking features of the difference is the presence of a prominent sub-intimal edema in the areas of Evans blue dye accumulation.

We have examined a number of features of the blue and white areas with both en face preparations and scanning and transmission electron microscopy. Frequently, silver stained boundaries appeared thicker in blue areas than in white areas. Of particular interest, is the presence of a greater number of apparent discontinuities, or gaps, in the silver boundaries of cells in blue than white areas. These "gaps" were three times more frequent than in the white areas.

In other words, blue areas differ from white areas both with respect to the frequency with which they have gaps or discontinuities

in their cell boundaries themselves. Stigmata and stomata exhibited a similar frequency in blue and white areas. It would seem unlikely therefore that these are the determinants of the increased endothelium permeability in blue areas.

A number of other features were found in the silver stained preparations including rather large dark staining areas which appeared well circumscribed by what appeared to be cell outlines. We believe these probably reflect dead or dying cells, which can be seen with transmission electron microscopy.

With transmission electron microscopy, it was apparent that many of the fine silver granules in en face preparations could represent pinocytotic vesicles. With both transmission and scanning electron microscopy, blue and white areas have a number of differences which I shall not attempt to describe in any detail. Some of the junctions in blue areas are complex and the boundary gaps that we see may represent cytoplasmic processes overlying the junctions, perhaps preventing access of the silver. I would be very reluctant on the basis of our findings to ascribe any definite structural or ultrastructural differences to the difference in permeability which have been observed in the normal aorta in vivo.

DR. SMITH: I wonder if I could look at your influx data in a slightly different way, and that is in terms of the amount that is present in the plasma. On the figures you gave you had about twenty times as much influx of albumin as you did of fibrinogen. Your fibrinogen concentration was somewhere around 300 milligrams per cent and presumably the albumin was also at physiological levels.

DR. SCHWARTZ: Yes, perfectly normal. Yes, these were perfectly normal animal cells.

DR. SMITH: So that you had perhaps three grams per cent albumin. Now if you look at the amount that went in compared to the amount that is in contact with the endothelium then you get a rather different picture of the influx. And if you calculate it back into terms of volumes of the plasma from which they were derived you find a two-fold, or less than two-fold, difference in relative influx compared with a twenty-fold difference in absolute influx. I think that one really has to look at this sort of data in these terms; that would mean a two-fold difference due to the molecular weight rather than a twenty-fold difference.

DR. SCHWARTZ: Thank you. It is very difficult to know which way to express the data. The actual influx was calculated on the basis of median plasma specific activities determined over the two hour duration of the studies. The plasma specific activity for both albumin and fibrinogen was determined on the basis of a die-

away curve. I think the point you are making, however, is a very good one and needs consideration.

DR. DAY: The data presented by Dr. Schwartz relates to differences in permeability to albumin and fibrinogen in areas identified by Evans blue staining but it cannot necessarily be extrapolated to include differences in the entry or the accumulation of lipoprotein in these areas. It seems to me important to determine whether the entry of the larger lipoprotein molecules is different in the blue and white areas.

Permeability of Arterial Wall to Lipid

I had an opportunity recently in a current collaboration with Drs. Bell and Schwartz to investigate this accumulation of lipoprotein cholesterol in blue and white areas of normal and cholesterol fed pig aortas. The accumulation of cholesterol in blue and white areas of the intima (stripped from the underlying media) and in the subjacent media in normal fed and cholesterol fed pig aortas is shown in Fig.141 (76). After six weeks of cholesterol feeding there was a significantly greater accumulation of cholesterol (present originally of course as serum lipoprotein) in the blue areas compared with the corresponding white areas. These changes were not shared by the arterial media where there were no significant differences between the areas studied. When you push this comparison to 16 weeks of cholesterol feeding the concentration of cholesterol in both the blue and white areas of the intima are high. Again the blue areas contained appreciably more cholesterol than the corresponding low permeability white areas.

PIG

We were also concerned with the question of possible differences in the lipid metabolism of the intima from the high and low permeability white areas. In order to study this aspect the uptake and incorporation of both ^{32}P phosphate and ^{14}C labelled oleic acid into the lipids of the blue and white intimal and subjacent medial areas of the pig aorta were investigated. There was no evidence for differences between blue and white areas in uptake and incorporation of either oleic acid into lipid or ^{32}P phosphate into phospholipid in the normal fed pig aorta. After cholesterol feeding, however, there was a marked increase in the incorporation of oleic acid into phospholipid, triglyceride and in particular into cholesterol ester in the blue areas of the intima compared with the white areas. These studies are reported more fully elsewhere (77).

DR. LANG: Dr. Day, what is the evidence for your suggestion that the blue and white areas in the normal fed animals correspond to the blue and white areas in the cholesterol fed animals? Your data implies that the cholesterol was incorporated in the areas which were blue in the normal fed animals.

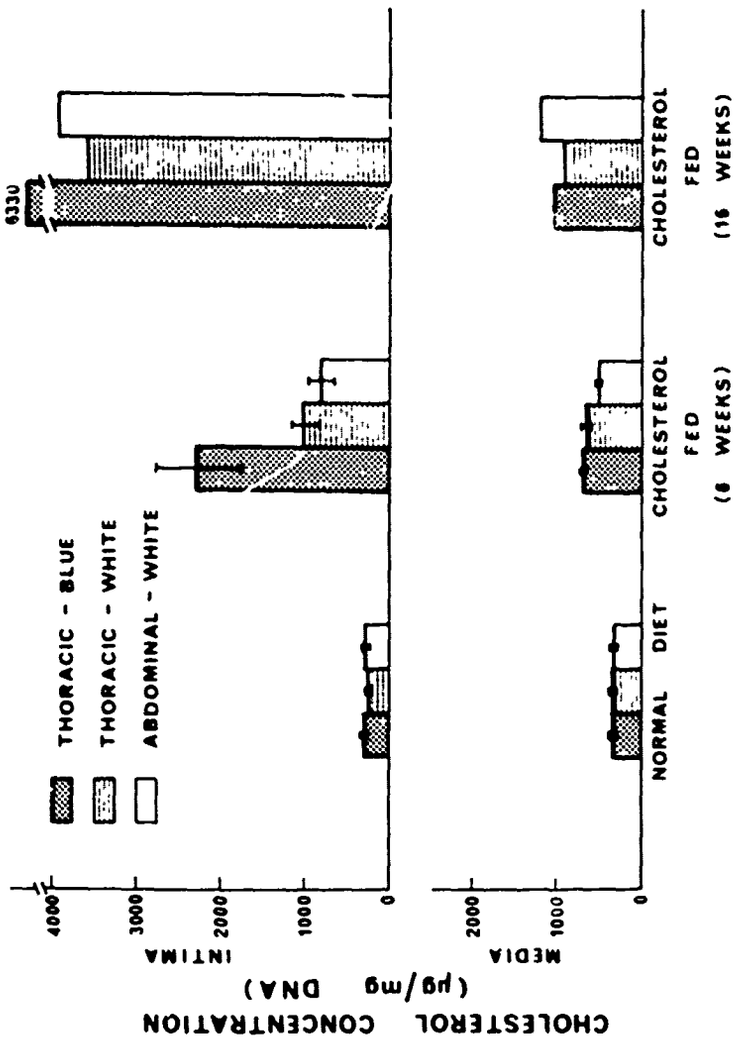


Figure 141: Cholesterol concentration (μg/mg DNA) in blue and white areas of normal-fed pig aortic intima and media (mean of 6 with the standard error of the mean) and in corresponding areas from pigs fed high fat, high cholesterol diets for six weeks (mean of 4 with the standard error of the mean) and for sixteen weeks (mean of 2). Reprinted from Day et al 1974a by permission of Editors of Experimental and Molecular Pathology.

DR. DAY: The methods used for staining and the areas taken for study were identical in the normal fed and in the cholesterol PIG fed pigs. The only difference was that in some of the cholesterol fed pig aortas the intensity of staining was not as great but we did, in fact, have the same portions of the intima in both series.

DR. BJORKERUD: In Dr. Day's study there was no difference between blue and white aortic areas with regard to content of cholesterol etc. This cannot be taken as evidence for an absolutely similar permeability in these different areas. One could recall the scientific rule that negation of an hypothesis does not prove anything but merely indicates that the hypothesis was false under the conditions in question. A negative outcome can be due to many factors, e.g., too insensitive measuring techniques or unsuitable sampling. We found actually differences between white and blue regions including different content of cholesterol (46). In this context it is pertinent to mention that supravital staining with uncomplexed Evans blue, a procedure which we used, gives an instantaneous detection of endothelial cell injury. The intravital procedure with Evans blue complexed to the serum albumin reflects the permeability of the endothelium for the Evans blue-albumin complex. The permeability is "recorded" over a much larger time interval (days) the cellular injury with the former technique (6 minutes). This difference should be kept in mind and taken account for when comparing results obtained with these techniques.

DR. OLGA STEIN: I think Dr. Schwartz's data is most interesting, especially since in the normal animal prior to cholesterol feeding, there was no difference in either cholesterol content or in the metabolic activities among the blue and white areas. I think this might indicate that unless you add insult to injury, those parts of the aorta behave quite normally. I just wondered what is the ultrastructural counterpart of the silver lines, if you would like to comment, Dr. Schwartz?

DR. SCHWARTZ: Dr. John Somer in our laboratory, has examined PIG blue and white areas in the normo-cholesterolemic pig. He has found that there are significant differences in the handling of acetate, glucose ethanolamine and choline. We have not yet been able to get together with Dr. Day to look at the implications of the different metabolic studies at this point but hope to do that shortly.

With respect to the thicker silver lines and the so-called discontinuities or "gaps" in the cell boundaries, I think that the thicker silver lines may reflect the presence of silver in the junctions, and also within saccular dilatations frequently seen at the junctional areas. The "gaps" we consider on the basis of our transmission studies to reflect the complex cytoplasmic pro-

cesses that overlie the cell junctions and perhaps interfere, as I indicated earlier, with the access of silver to the cell junctions themselves.

DR. ROSS: I should like to emphasize what Olga Stein has shown namely the complexity of the junctions between arterial endothelial cells as contrasted with capillary endothelial cells. Steve Schwartz in one of his papers pointed this out. In man and monkey arterial endothelial cells overlap much like the fingers of your two hands as you interlace them. Vesicles are often found along the junctional margins in arterial endothelium as Schwartz also pointed out in his study. This anatomical arrangement may explain in part differences in permeability between arterial and capillary endothelium.

Endothelial Cell Junctions

MONKEY

DR. WISSLER: I wonder if you would care to comment on how the areas which you pictured in the Archieves article (42, 195) compare with the ones Colin Schwartz was showing in the blue areas which he spoke of as stigmata and stomata.

The Question of Transendothelial Conduits

DR. BJORKERUD: We studied the characteristics of the structures you are referring to with a number of complementary methods including scanning electron microscopy, interference contrast microscopy, histochemical techniques, etc. It is difficult to know if the structures Dr. Schwartz was describing correspond to those described by us until more data than those obtained after silver staining have been presented. We found circular flap-like structures about 3-5 micra in diameter. The flaps were situated on or near the intercellular borders and were attached to the body of the endothelial cell with a thin stalk. Under the flaps was a circular region, approximately 1 micron wide, with increased permeability enabling it to serve as a marker for a low molecular weight substance such as nigrosin as well as 0.1 - 0.2 micra particles of colloidal coal. As judged from the distribution of the markers the circular region seemed to be continuous with a canal-like structure directed towards the media and with a length of about 5 micra. Interpretation, however, is difficult. There is no doubt that the circular flaps exist. The flaps are present in both white (low-injury) and blue (high-injury) regions. The form is more irregular and the size is often larger in blue regions. It is somewhat more uncertain if the canal-like structures actually represent preformed continuous canals through the endothelium. They may either represent canals or inter-cellular regions where the tight junctions are located at the extreme basal parts of the endothelial cells. At such sites the intercellular space could form a "pocket" where markers could be retained. Further research is required to settle this import-

ant question. Traditionally, the term "stigmata" is used to designate smaller circular silver-stained endothelial structures and "stomata" similar but larger structures (from about 10 micra to about half the size of an endothelial cell). To avoid confusion it might be wise to adhere to the old nomenclature (42).

DR. SCHWARTZ: On the basis of the measured size of stigmata and stomata it would appear highly unlikely that these account for the focal differences in permeability to albumin and fibrinogen in our data. If these were continuous with the subendothelial space we would have a completely leaky endothelium with no discrimination for macromolecular influx. I suspect that they are not involved, because on a numerical basis, they occur with approximately the same frequency in both blue and white areas although these show very significant differences in permeability.

DR. LEE: I would like to pass on to you another piece of information. When cholesterol was fed to swine in as early as 3 days not only the rate of ^{3}H thymidine incorporation and mitotic index of smooth muscle cells in the abdominal aorta were significantly increased, but also these parameters for endothelial cells were increased almost two-fold as compared to those in the control animals. By electron microscopy there was no evidence of redundancy and no obvious increase in the number of endothelial cells as far as we can ascertain. These observations indicate that the turnover rate of endothelial cells is increased three days after cholesterol feeding in these swine. The endothelial cell monolayer acts as a semipermeable barrier between smooth muscle cells and the bloodstream. Any alterations such as could be associated with increased rate of loss and replacement of endothelial cells might alter permeability. Thus, the quantity of blood constituents reaching underlying smooth muscle cells could be increased. Professor Shimamoto of Japan showed some time ago that when he injected cholesterol into a rabbit, that two hours after the injection, he found contraction of endothelial cells and gaps between them. These contractions and gaps will enhance infiltration of high-molecular weight constituents of the plasma such as β-lipoprotein into the subendothelial space and medial layers. Thus, increased lipid accumulation in the intima or medial cells may be attributed at least in part to these phenomena.

Gaps Due to Endothelial Cell Renewal

DR. BJORKERUD: Just a very short comment to finish this story about stigmata. I agree with you, Colin, that the number is probably not increased but remember that there is a flap on them. If there is a patent canal, who knows in what direction the fluid is streaming.

Are there normally Canals through the Endothelium?

DR. SCHWARTZ: I agree with Soren Bjorkerud. However, the question of the relationship of stigmata to stomata I find somewhat confusing. If stomata are stigmata in which the flap-like valves have opened then we might expect to find a significant interrelationship of the numbers of both stigmata and stomata in the areas of differing permeability. This is not the case.

DR. LINDNER: I am concerned about all this discussion about gaps and flaps or some widening of space between endothelial cells, including the picture of endothelial cell contraction that we have seen. The findings are just too dependent on the method, especially the scanning electron microscopy. The impression that spaces or gaps may develop from time to time, thereby allowing an increased influx of serum contents must be thoroughly confirmed before we build a whole system or hypothesis upon it. Dr. Olga Stein spoke about the differences and similarities between the endothelial cells of capillaries and aorta. We have heard just now about turnover rates after a short term cholesterol diet. We know that the turnover rate of capillary endothelium is higher than that of the aorta, for example. Capillary endothelium derives not only from the endothelial cells but from monocytes, hematogenic cells, as well. Now the problem in the aorta is whether or not the hematogenic cells take part in renewal or only endothelial cells or whether, as some persons think, the smooth muscle cells are involved also?

DR. OLGA STEIN: Well, I am sorry I never studied the turnover of endothelial cells and I really have no personal information of whether the endothelial cell can come only from an endothelial cell or whether it can be derived from other cells. I would like to guess that they would rather be derived from another endothelial cell, but my guess is no better than any other.

DR. RODBARD: The relationships between endothelium and medial musculature may also be viewed from a mechano-physiological aspect.

Vascular Dynamics - Rate of Flow

Discussion of these relationships at this Conference have thus far been concerned primarily with infiltrations of blood-borne materials into the vessel wall. Schretzenmayr (334) pointed out forty years ago that when the blood flow rate through the femoral artery increased, the artery responded immediately by an increment in its caliber. Potter (263) showed that cerebral vessels also dilated acutely when flow increased. During and after exercise of a muscle, the flow through its vessels increases, and the supplying artery dilates acutely in proportion to the magnitude of the increase in flow (200). When flow returns to normal, the vessel constricts to its previous caliber. Various workers have considered that the fore-going effect might be due to metabolites produced locally, or to hormones produced at a distance.

However, the same adjustment of vascular caliber to blood flow rate has been demonstrated on opening of an arteriovenous fistula. The change in caliber of the supplying vessels therefore cannot be attributed to metabolites or to hormones. Instead, the observed effects must be attributed to the flow rate itself. This accords with our hypothesis that vascular caliber adapts quickly to flow rate, presumably by means of drag receptors. It is pertinent to consider the relationship of flow rate to the caliber of blood vessels. From the mechanistic point of view, a change in flow rate can have an effect only on the endothelium. Flow per se cannot affect the media or adventitia. The endothelium is affected only by the streamlines immediately adjacent to the wall, the boundary layer. When the flow rate through a vessel increases, the change in the hydro-dynamic drag of the stream may be sensed by the endothelial cells.

Dr. Una Smith's (353) scanning electron micrographs (Fig.142) exhibit endothelial papillary excrescences. These resemble bristles which can bend with the velocity of the stream, thereby acting as flow receptors. When this concept is documented, we will be able to account for acute changes in vascular caliber as a result of an acute change in flow. The potential effects of drag on endothelial cells are indicated in Fig.143. Flow, indicated by the arrow, tends to deform the lining cells. Since each cell is firmly fixed to its basement membrane, it can not move. However, the cell can transfer the information of an increased flow to the subendothelial smooth muscle. This appears to induce relaxation of the medial muscle layers. With dilatation of the vessel, the flow velocity decreases and the drag on the vessel lining returns to the normal (set-point) value. On the other hand, if peripheral conductance (flow-rate/pressure gradient) decreases, the drag forces on the cell will be reduced, and the vessel will constrict. These considerations pose the physiological question of how such changes in caliber will affect not on the surface area of the vessel wall but also other factors that can modify the handling of cholesterol, albumin, Evans blue, or other circulating substances.

This approach suggests that the forces of the stream may play a role in the acute adjustment of the vessel caliber to the amount of blood flowing through it, and through such means determine vascular growth or even a reduction in caliber. The factors that affect vascular caliber must include a consideration of hydraulic interrelationships between endothelial cells and subendothelial muscle cells. These forces may also modify the tendency to infiltration of the vessel wall with circulating materials. Further elucidation of this factor will bring closer an understanding of the forces that determine vascular structure.

DR. WOLF: Dr. Rodbard's comments concerning hydrodynamics,

the physical properties of flow, reminded me of a question that I raised in the summary session of the Chicago meetings. We know what changes occur in the appearance of the lining of the vessels with cholesterol feeding. Do we have any evidence concerning hemodynamic effects of a ten-fold increase in serum cholesterol, such as may occur with cholesterol feeding? Is there a change in the physical properties of flow? I think it would be important to know if there is an effect on the hydrodynamics, particularly if there are sensors in the lining of blood vessel walls as Dr. Rodbard suggests.

DR. RODBARD: My discussion calls attention primarily to receptors in the vessel wall. In addition to the well established vascular receptors such as those in the carotid sinus and in the hypothalamus, there is now evidence for other varieties of sensory receptors in the arterial tree. Our studies have indicated that receptors may also be present in the endothelial cells. If receptors were present on the free surface of the endothelial cells, these cells would be sensitive to the flow patterns of the blood stream. The stream normally generates a force (drag) which tends to pull the lining cells with it. In a laminar stream the drag on the endothelial cells varies with the cube root of the flow rate, and with the viscosity of the blood (Fig.144). When flow becomes turbulent, drag increases with the square of the average velocity.

Possible Endothelial Sensors of Flow Dynamics

Thus far, attention of investigators has been focused on biochemical and pathological aspects of the vessel wall. I believe it is time to explore the potential role of mechanical factors, as well as the potential receptors that can cause the cells of the vascular wall to respond to these physical forces. When more attention is given to these essentially neglected factors, we shall be able to construct a more adequate analysis of the bio-physical-biochemical interactions that determine normal structure, and which undoubtedly play a significant role in pathological change.

DR. WEBER: I want to discuss some of our work on the vessel wall intimal surface in the early phases of cholesterol diet in rabbits. In the normal rabbit we have seen by scanning electron microscope features of the intima quite similar to the ones Dr. Bjorkerud showed us this morning. In Fig.145 one sees chiefly folds and grooves among the folds, microvillous projections and so on (404, 405, 406, 407). After a short period of hypercholesterolic diet (fifteen days) the surface is deeply changed, chiefly near the origin of the collateral branches (408, 409, 410) (Fig.146). Wondering how these findings could be interpreted, we have tried, by ultrathin sectioning of aortic vessels, to study if some ultra-structural property of the

RABBIT

Polysaccharide Endothelial Coating

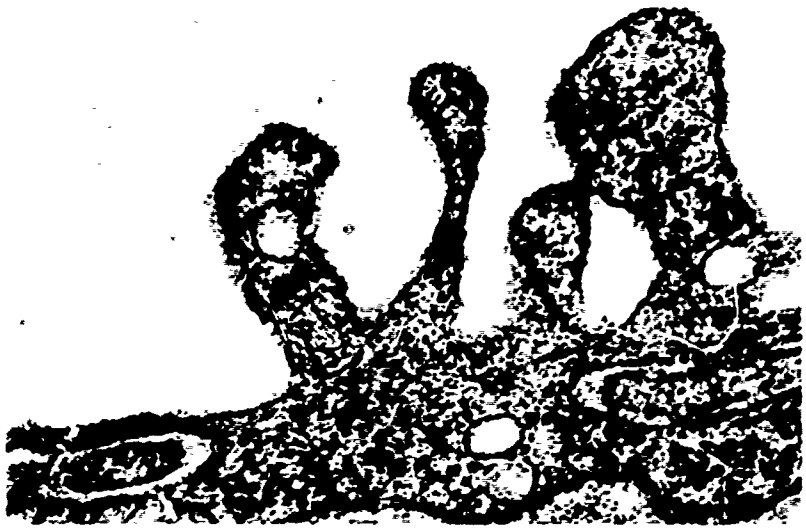


Figure 142: Endothelial projections. Scanning electron microscopy x 67,500. Courtesy of U. Smith.

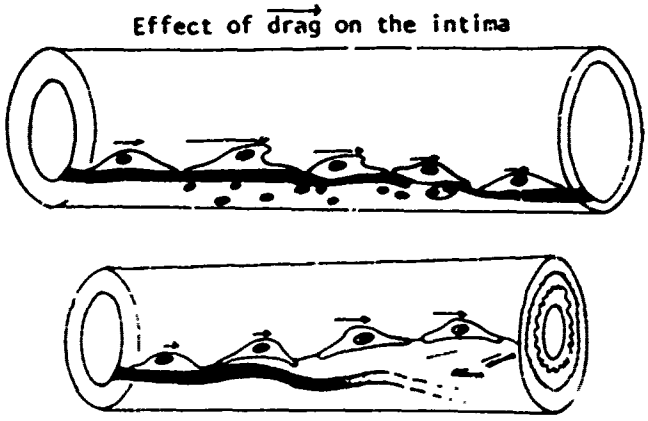


Figure 143: Effect of drag on intima. The potential role of hydrodynamic drag in the determination of vascular caliber (hypothesis).

VASCULAR ENLARGEMENT

In the upper drawing the first arrow (at left) indicates a normal (set-point) drag acting on an endothelial cell. At the second cell, the elongated arrow indicates an increased drag. This induces an acute dilatation which widens the lumen, reducing velocity and drag (third arrow). If the increased drag is persistent, the lumen is reorganized around a larger lumen (at fourth cell), and the local drag returns to normal values (fifth arrow).

VASCULAR DECLINE

In the lower drawing, a reduced rate of flow results in a subnormal drag force (short arrow over cell at left). This induces contractions of the medial smooth muscle (second cell), decreasing the vessel lumen, and thereby increasing the velocity and drag (third arrow) to normal values. The vessel is then reorganized around the reduced lumen (fourth arrow).

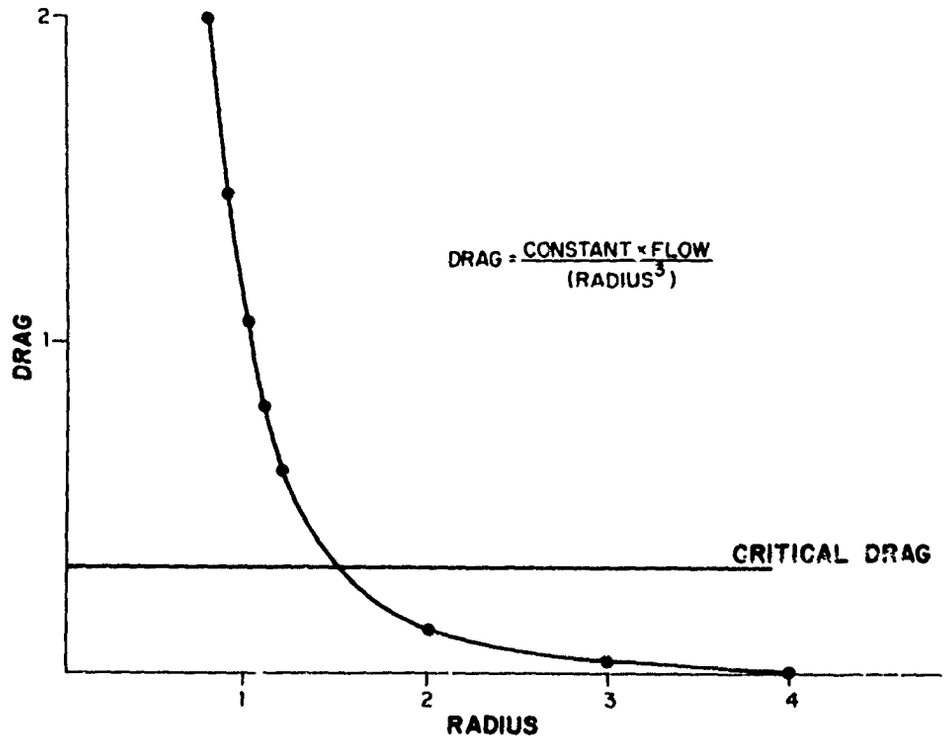


Figure 144: Relationship between drag and radius. Horizontal axis is the radius of the vessel. Vertical axis is the hydrodynamic drag. The set-point for the endothelial cells is shown as Critical Drag. If the critical set-point value is exceeded (as at drag = 1, radius = 1), the radius of the vessel is induced to enlarge about 1.5 times until velocity and drag are reduced to the set point. If the vascular radius is too large for the flow, and drag is less than the set point (as at radius = 2), the radius becomes reduced until the stream velocity is sufficient to return drag to the set-point value for the endothelial cells. If the change in caliber is persistent, the structure of the vessel is anatomically changed.

Figure 145: Rabbit Aorta. The intimal surface as it looks in adult rabbits. x 1000

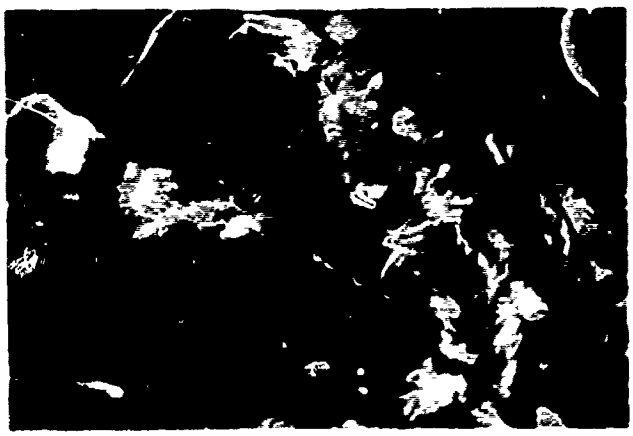


Figure 146: Rabbit on hypercholesterolic diet. Aorta After 15 days, a wrinkling of the intimal surface is evident near the origin of a collateral branch SEM x 1000

endothelial surface could be responsible for the changes noted by scanning electron microscopic examination. Looking for a polysaccharide containing coat whose presence and possible defensive functions at this level had been hypothesized by John French at the Lindau Conference (109), we made use at first of Ruthenium red and then of the Concanavalin A method (that had been proposed in the meantime by W. Bernhard and S. Avrameas, 1971 (27) in Paris). By this highly specific method we have been able to demonstrate on the normal aortic endothelial cells and in the plasmalemmal pinocytotic vesicles the presence of an ultrathin polysaccharide-containing-coat (or S. Benditt's "Glycocalyx").

After fifteen days of hypercholesterolic diet, this reactive coat showed a huge increase in thickness (Fig.147) (later on it may disappear and a plasmatic coat be deposited on the "denuded" cellular surface). Therefore we propose that the change of the intimal surface that we have seen by scanning electron microscope near the origin of the collateral branches may, in part at least, be explained by the increased thickness of the surface coat (or by the plasmatic deposition in the points where it has disappeared) (411, 412). It may be remembered here that surface glycoproteins coat thickness may look increased in "transformed" cells (342, 400) or, in a cyclic manner, when the DNA-synthesis is augmented (313).

In the newborn rabbit there is no clearly demonstrable coat on the endothelial surface. After fifteen days, in suckling rabbits, signs of a positive, but not yet of a strong reaction appear (Fig. 148) (413, 414).

After this short presentation of some of our results at the borderline among "normal" and "pathologic" let me shortly summarize what is known about the Concanavalin A method. The Concanavalin A method has been proposed as highly specific (27, 391). Its results depend on the fact that the Concanavalin A (which is a phytoagglutinin extracted from Canavalia ensiformis, Jack bean) has two active sites both of which can react with sugar or glycoproteins which contain branched terminal non-reducing alfa-D-gluco-pyranosyl, alfa-D-manno-pyranosyl or beta-D-fructo-pyranosyl residues. Concanavalin A is fixed only at one of its two active sites. The free active site operates as an acceptor of another sugar secondarily added to the system. The method makes use of horseradish peroxidase: the catalytic activity of the peroxidase is revealed by the diamino-benzidene method by Graham and Karnovsky. The reaction can be inhibited by addition in excess of a sugar which competes both with the enzyme and the sugar present in the cell for the reactive groups of Concanavalin A. The specificity of the action of Concanavalin A has been subjected to some criticism years ago because some authors (132) doubted that the glycoproteins would be the only substances to which Concanavalin A may react. And

Figure 147: Rabbit on hypercholesterolic diet. Aorta. In other areas, a film-like deposition hides almost completely the picture of the intimal folds. SEM × 1000

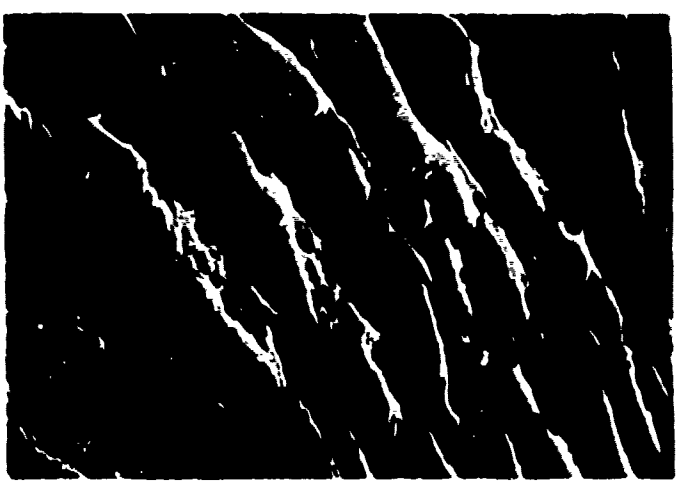


Figure 148: Rabbits subjected to combined treatment with hypercholesterolic diet and i.v. Tween 80. Aorta. After 15 days, the initimal surface looks normal even near the origin of a collateral branch. SEM × 1000

they suggested that even beta-lipoproteins could be evidenced by this method. At any rate, Cook (68) in his monograph on "Lysosomes" considered the method to be a "potentially extremely powerful tool" for staining the surface glycoproteins and, in fact, it seems a very useful method and is judged as "immunologically specific" by Roth (319). Anyhow, I think that the Ruthenium red method should not be discarded; in fact, I think that as the results are practically superposable, the two methods can be used "alternatively" (219).

The presence of surface glycoproteins over the endothelial cell though obvious from a general biologic point of view (423) may be of some interest for people concerned as we are with the problems of vessel wall permeability. The question that may be posed is how much the presence of a surface, Concanavalin-A-reactive, polysaccharide containing coat may be important in moderating or regulating the permeability of the endothelial cells (or other properties of their surface).

Endothelial Regeneration

DR. POOLE: I have been asked by the Committee to say a few words about the regeneration of aortic tissues in fabric grafts of the aorta in baboons which was particularly concerned with the behavior of the endothelial and smooth muscle cells (260). I want to preface my remarks by describing briefly an earlier study on regeneration of aortic endothelium in the rabbit's aorta (259).

RABBIT

We removed the endothelium mechanically from the distal 2 cm. of the aorta in just over a hundred rabbits which were killed and examined at intervals of from half-an-hour to 18 months after operation. We found that endothelium gradually grew over the denuded area from the margins of the surviving endothelium at both ends until a complete new endothelial lining was formed. The rate of regeneration was slow compared with that of capillary endothelium; it took about a year before the new endothelial lining was complete.

Fig.149 shows the regenerating endothelium at various stages in three specimens. The fact is that the newly formed endothelium is just what one would expect if the new endothelial cells are derived from pre-existing endothelial cells and not at all what one would expect if they were derived from blood-borne precursors. In any case, there can be no serious doubt about the origin of the new endothelial lining: numerous mitoses can be seen in endothelial cells just behind the advancing front. There is one further point I want to make about the appearance of the endothelium in these experiments. Dr. Olga Stein asked what silver lines looked like when examined in section in the electron microscope. We examined this fifteen years ago (108, 258) since there was, at that time, an apparent discrepancy between the light and electron microscopical

Figure 149: Regenerating endothelium in the lower abdominal aorta in 3 rabbits. The darkly stained area is the part not covered by endothelium. From left to right: 3 weeks, 3 months and 9 months after operation. x 3.

Figure 150: Endothelial cell junction in regenerating endothelium as seen in section in the electron microscope. From an organising mural thrombus of the rat's aorta 5 days after injury. x 52,000.

Endothelial Cell Junctions

appearances of endothelial cell junctions. On the one hand, examination of endothelium en face after silver staining appeared to show bands of material approximately 1 micron in thickness at the cell boundaries. This appearance had long ago led Julius Arnold (15) to postulate that there was a cement substance between the cells and this view was generally accepted up to the late 1950s. On the other hand, electron micrographs showed endothelial cells to be very closely applied, with a gap between the cell membranes no greater than 20 nm.

How could these seemingly discordant observations be reconciled? We examined sections through silver lines in the electron microscope and found that the whole surfaces of the cells including the surfaces in the junctions, were covered by small silver grains. The "cement" was an optical illusion; because of the complex interdigitating junctions, several layers of silver grains were seen superimposed in these regions, giving the misleading impression that a band of "cement" lay between adjacent cells.

In this connection I was very interested in what Dr. Russell Ross said about the much simpler junctions to be seen between newly regenerated endothelial cells; this has been my experience also (Figs.150, 151). Such junctions between newly-formed cells might well not show silver lines when viewed en face. This probably explains why in our experiments on regenerating rabbit aortic endothelium we often saw at the advancing front of the endothelium what appeared to be endothelial nuclei with no "cement" lines marking the cell boundaries (Fig.152).

When we came to study the behavior of endothelium in fabric grafts of the lower abdominal aorta in baboons, the position turned out to be more complicated. Our first observations (107) showed, rather to our surprise, that the graft was completely lined by endothelium only ten weeks after operation. The area involved was much the same relative to the size of the baboon as compared with the rabbit but absolutely, of course, it was much greater and since the cell size in the two species is not greatly different the absolute area would seem to be of some importance.

BABOON

There are probably several reasons for the difference in the two experimental situations. There may be a species difference. However, when we looked at specimens obtained from baboons killed at shorter intervals after operation (106) another factor became apparent. At a very early stage, small vascular channels form in the interstices of the woven fabric which connect with the lumen of the graft. From the mouths of these vessels which are quite numerous, endothelium spreads over the inner surface of the graft forming islands which enlarge and coalesce. So the endothelium is

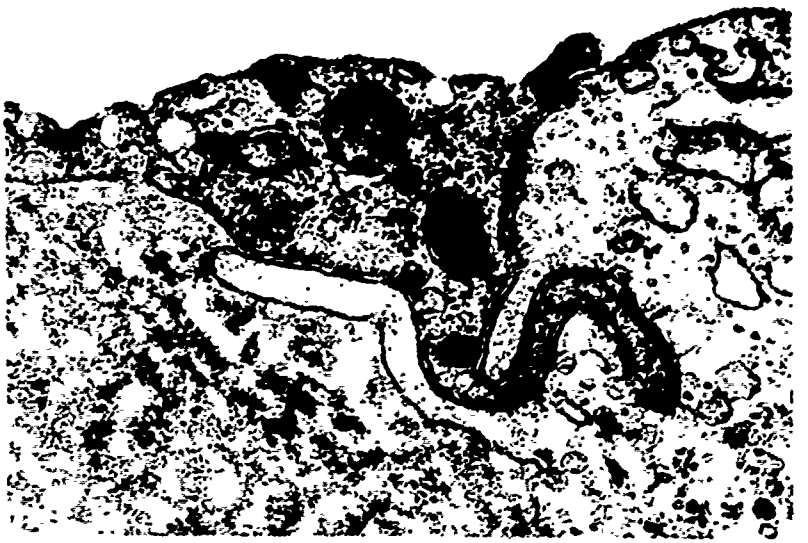


Figure 151: As in Fig. 150, 48 days after injury. x 41,000.

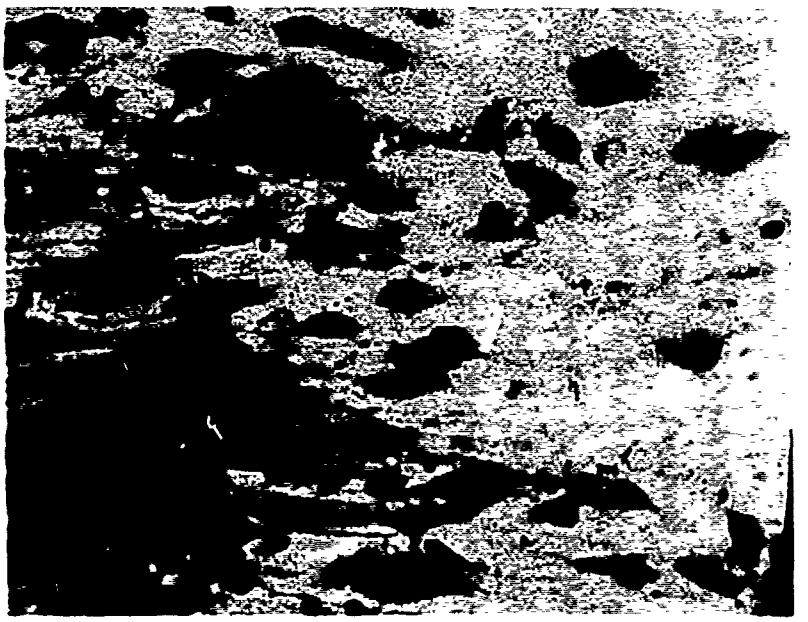


Figure 152: Edge of regenerating endothelium in the rabbit's aorta. Hautchen preparation stained by silver nitrate and haematoxylin. x 250.

advancing on many fronts instead of only on two as in our earlier experiments on rabbits (Fig.153).

I now want to turn to the behavior of smooth muscle cells in these two sets of experiments. The observations on the rabbit aorta provided an example of what now seems to be a general phenomenon: removal of endothelium from the inside of a large blood vessel leads to the proliferation of underlying smooth muscle cells. By the time the regenerative process was complete a layer of smooth muscle cells with collagen and elastic laminae between them had formed between the new endothelium and the original internal elastic lamina. This layer was in places substantially thicker than the tunica media (Fig.154). When the new endothelial lining was complete, however, this layer did not appear to increase further in thickness. Likewise in fabric grafts of the aorta a substantial layer of tissue, usually referred to as the pseudointima, formed between the endothelium and the tube of cloth. Again, the cells present are smooth muscle cells, and the layer seemed not to increase further in thickness once the endothelial lining was complete. RABBIT

These two sets of experiments answered certain questions but raised some new ones. It was established that the endothelium lining large blood vessels was capable of substantial regeneration and this raised the question as to whether or not endothelial cells in the uninjured aorta divide from time to time or (as has been claimed) are perennial or very nearly so (9). The fact that they undergo division was established by making autoradiographs of Hautchen preparations of aortic endothelium after injecting tritium-labelled thymidine into rats (257). There was also the question of the origin of newly-formed smooth muscle cells in thickenings of the tunica intima. The obvious explanation was that they were derived by ordinary cell division from neighboring smooth muscle cells.

Endothelialization of Grafts

RAT

However, the publication of a suggestion that they might be derived from blood borne precursors (168) drew attention to the fact that no concrete evidence for a local origin could be provided and it was felt that this needed to be investigated. In a study on the organization of mural thrombi in the rat's aorta (256) mitoses were found in smooth muscle cells adjacent to the injured region, the numbers of mitoses being substantially increased when the animals were treated with colcemid. It would be difficult to suppose that newly formed smooth muscle cells have an entirely different origin in other mammalian species, or in other types of arterial injury.

DR. WERTHESSEN: I would like to make a stronger case than did Dr. Poole for the species difference. Dr. Howard French, in Boston who is a vascular surgeon, has been trying to build artificial

Figure 153: Fabric graft of a baboon's aorta 10 weeks after operation. Whole-thickness preparation viewed en face from within. Stained by silver nitrate and cleared in clove oil. The network of "cement" lines is seen with the pattern of the woven fabric out of focus in the background. The mouths of two small vascular channels are present. x 44.

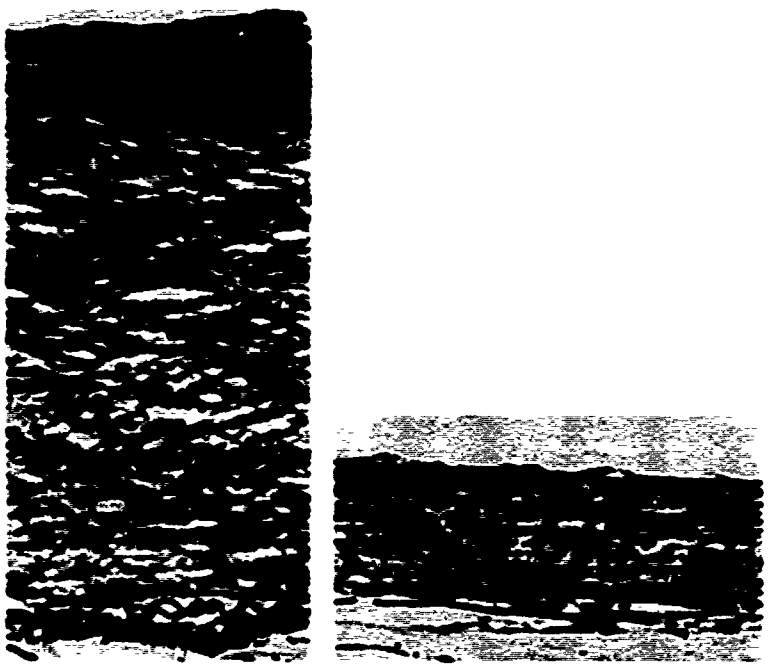


Figure 154: Histological sections showing thickened tunica intima after endothelial regeneration in the rabbit's abdominal aorta (left) compared with uninjured abdominal aorta from the same region in a rabbit of similar age (right). x 90.

arteries for surgery. He tried for years to get this done in the dog and he never had any success in developing an intima inside the pseudo-artery that he had induced to form beneath the skin. I arranged for him to try a baboon and he was delighted to find that in about that same six weeks period that we observed, he had a very nice intima that covered the entire lumen. The reaction did not occur in the dog within the same period of time.

Species Differences

BABOON

DOG

DR. POOLE: I quite agree with Dr. Werthessen that species differences almost certainly exist between different mammals in respect to rate of regeneration of aortic endothelium. Indeed, it would be most surprising if this were not the case. I was anxious, however, to point out that the two sets of experiments that I was describing were not strictly comparable and that therefore our experimental findings do not, by themselves, provide convincing evidence for a species difference.

DR. STRONG: In editing a paper one of my colleagues in the department referred to some early German work on injury of the arterial wall and smooth muscle thickening. I got the impression from his review that not only did the thickening of smooth muscle stop at a point in time but it actually became less with additional time. Is that correct, Dr. Poole?

DR. POOLE: Yes, that is correct as far as my own very limited observations go. I have examined the pseudointima in two fabric grafts of the aorta in dogs 18 months and 2 years after operation respectively. In both cases the number of smooth muscle cells per unit volume was much less than at earlier stages. The space between them contained large amounts of collagen.

DR. ROBERTSON: I will review very briefly studies carried out during the last two and one half years in our laboratory on the role of circulating vasoactive agents on vascular endothelial and smooth muscle cells (300, 301). As summarized in Table XXIII, the experimental design consisted of intracardiac or intraaortic injections of nanogram concentrations of a vasoactive agent in Ringer's solution to pathogen-free Sprague Dawley rats followed by simultaneous or delayed administration of either electron markers of different particle size, tritiated lipoprotein fractions or ^{125}I labelled serum albumin. The unavoidable ultrastructural artifacts usually found following routine fixation of large arteries were prevented by using exclusively in vivo perfusion with 0.1% isotonic buffered aldehyde solutions at physiological pressure ranges followed by prolonged post-fixation of the arterial segments immediately after cardiac arrest occurred. Specimens were then

Effects of Vasoactive Agents on Vascular Smooth Muscle

RAT

TABLE XXIII

Injection		Vol.	Aver. Time
I) Intracardiac (left ventricle) at mean systolic pressure.	1) Vasoactive agent or control solution	0.1 ml	12 sec.
	2) Ringer's solution with marker (HRP, Fe, C) or ^{3}H-LDL,-VLDL,-TG,-HS	0.5 ml	20 sec.
	3) Buffered Aldehyde	0.5 ml	20 sec.
II) Intraaortic via left carotid a. catheter implanted and injected with 50 μC of ^{3}H-7-α cholesterol 24 hrs. before.	1) Angiotensin II (0.06 ng in 0.036 ml R's x min. or control solution)		15 min.
	2) Simultaneous or delayed injection of markers as above		3-15 min.
	3) Buffered Aldehyde (immediately or after 24 hrs.)	0.5 ml	20 sec.

Samples from I) and II) processed for cytochemistry - EM- Autoradiography

Pathogen-free adult Sprague Dawley Rats (200-720 g)

TABLE XXIV

CHANGES IN ARTERIAL WALL PERMEABILITY TO ^{125}I ALBUMIN

INDUCED BY CIRCULATING VASOACTIVE AGENTS

	Total Injec. (x 10^{-9}g) (*)	Av. Time (sec.)	Subendothelial(**) Space	Arterial(***) Media
Norepinephrine	100-200	42	++	+
Serotonin	10-200	56	+++	+
Prostaglandin E_1	10-20	51	++	++
Angiotensin II	0.1-10	42	++++	+++
Control L-Isoleucine	2400-4800	84	-	-
Control Ringer's Sol.	2 ml	280	-	±

* In 0.1 Ringer's solution injected in left ventricle of a 200 g Sprague-Dawley Rat

** Intima and inner media

*** External media and adventitia

+ = 25% ++ = 25% ++ = 50% +++ = 75% labeled cells might be fixed as

carefully dissected and processed for cytochemical and electron microscopy-autoradiography.

Ultrastructural evidence of the passage of electron markers by temporary widening of intercellular junctions around desmosomes or tight junctions (302) could be found only under such experimental conditions. As shown in Table XXIV, intracardiac administration of serotonin, prostaglandin E_1 and angiotensin II induced significant uptake of ^{125}I labelled serum albumin by the intima and media and to a lesser extent by the external media and adventitia of the rat aorta. This very rapid response occurred within one minute or less after simultaneous injection of the marker and the vasoactive agent in the absence of significant increases in mean systolic blood pressure. Large volumes of L-Isoleucine or Ringer's solution alone, under identical laboratory conditions, induced no uptake of the label.

Because of the short-lived response of vascular cells to nanogram concentrations of angiotensin II, the term "trap door" was suggested for these temporary functional increases in arterial wall permeability (303).

Table XXV summarizes net percentual radio-activity compared to that of plasma in two layers of the rat aorta after simultaneous or delayed injection of tritiated high density lipoprotein (HDL) fraction with 0.1 ng of angiotensin.

Table XXVI shows similar results for low density (LDL) and Table XXVII for very low density lipoprotein (VLDL) fractions. In all three studies, highest labelled lipoprotein concentrations in both layers of the rat aorta occurred when the lipoprotein fraction was injected simultaneously with the octapeptide. If the injection was delayed for 60 seconds, however, there were significant and consistent differences in the rate of retention of each lipoprotein by the arterial wall. Why LDL and VLDL fractions were retained at higher concentrations by the aortic wall and the possible relationship between this phenomenon and the development of vascular disease is currently under investigation.

Finally, I would like to mention recent studies that have demonstrated the remarkable properties of nanogram concentrations of angiotensin II on DNA, RNA, and protein synthesis of vascular smooth muscle and myocardial fibers (179). This phenomenon has also occurred in cultures of vascular smooth muscle cells showing signifigant increases in mitotic activity and tritiated thymidine incorporation in less than 48 hours after exposure to the octapeptide (300). By contrast, catecholamines seem to inhibit smooth muscle and cardiac cell proliferation under similar laboratory conditions. The experimental and clinical implications of these

TABLE XXV

Incorporation of ^{3}H labeled High Density Lipoprotein Fraction within the Normal Arterial Wall

	A) Injected simultaneously with Angio II		B) Injected 60 sec. after Angio II		C) Injected 480 sec. after Angio II	
	I	M	I	M	I	M
HDL median % radioactivity from plasma	4.8	3.6	0.4	0.6	-	-
$\bar{s}$	1.47	0.84	0.12	0.22		

Left intraventricular injection 0.1 ng Angiotensin II in 0.2 ml Ringer's solution
I = Intimal and inner medial layers
M = External medial and adventitial layers

TABLE XXVI

Incorporation of ^{3}H labeled Low Density Lipoprotein

Fraction within the Normal Arterial Wall

	A) Injected simultaneously with Angio II		B) Injected 60 sec. after Angio II		C) Injected 480 sec. after Angio II	
	I	M	I	M	I	M
LDL median radioactivity from plasma	16.2	12.8	8.4	7.6	0.4	-
$\bar{s}$	4.64	3.42	1.49	1.83		

Left intraventricular injection 0.1 ng Angiotensin II in 0.2 ml Ringer's solution
I = Intimal and inner medial layers
M = External medial and adventitial layers

findings are yet to be evaluated, but indirect confirmation of the important role of the renin-angiotensin system on both vascular and cardiac muscle hyperplasia and hypertrophy preceding increase in blood pressure has recently been obtained in the spontaneous hypertensive rat (SHR) (304). In this experimental model, arterial medial hyperplasia and cardiac hypertrophy are found following significant but short-lived increases in renin levels well before sustained systemic hypertension can be detected.

These experimental results as well as others recently reported using Wistar rats (332) strongly suggest that arterial smooth muscle and cardiac muscle cells are able to respond with hyperplasia and/or hypertrophy to hypertensive stimuli before rather than after sustained increases in blood pressure levels. The relation of these findings to the onset and therapy of human hypertension are currently under intensive study.

DR. LINDNER: I am really very happy about Dr. Robertson's paper. Especially the result showing that smooth muscle cells can have reduplication and proliferation induced by several substances. That is very important for the whole story of heart disease, infarction included. Very interesting is that we could not find the same influence which we have seen actually in the older animals also like other groups especially like Hauss' group. We could not find it in the high proliferation stage of postnatal developing aorta (rat) that shows an increase of ^{3}H-thymidine labelled smooth muscle cells that means they are in the DNA-synthesis - (=S) phase just before reduplication with the first maximum of the labelling index on the seventh day after birth and the second but lower maximum of the labelling index on the end of (or shortly after) the third week after birth. But in contrast to your findings and our studies on older animals in this maturation one single injection of angiotensin, hypertensin or similar substances did not induce a higher proliferation of smooth muscle cells of aorta (as well as of heart muscle cells) in this maturation time of rats.

DR. ROBERTSON: You were using one single injection of angiotensin in hypertensive rats. Was there renal vascular hypertension? Were they Goldblatt kidneys?

DR. LINDNER: The blood pressure is increased by these substances followed immediately in the same time (that means shortly after a single injection) by an increase of the ^{3}H-thymidine labelling indices in smooth muscle cells of the aorta, but partly increasing and partly decreasing labelling indices of aorta endothelia and of heart muscle cells in young adult rats, in contrast to the findings in the first weeks after the birth. That was the reason to speak about these phenomena. The above mentioned increased smooth muscle cell turnover rates can be normalized in 1-2 days after a single injection.

TABLE XXVII

Incorporation of ^{3}H labeled Very Low Density Lipoprotein Fraction within the Normal Arterial Wall

	A) Injected simultaneously with Angio II		B) Injected 60 sec. after Angio II		C) Injected 480 sec. after Angio II	
	I	M	I	M	I	M
VLDL median % radioactivity from plasma	7.8	16.4	5.4	6.9	-	-
$\bar{s}$	1.24	5.46	1.82	2.13		

Left intraventricular injection of 0.1 ng Angiotensin II in 0.2 ml Ringer's solution
I = Intimal and inner medial layers
M = External medial and adventitial layers

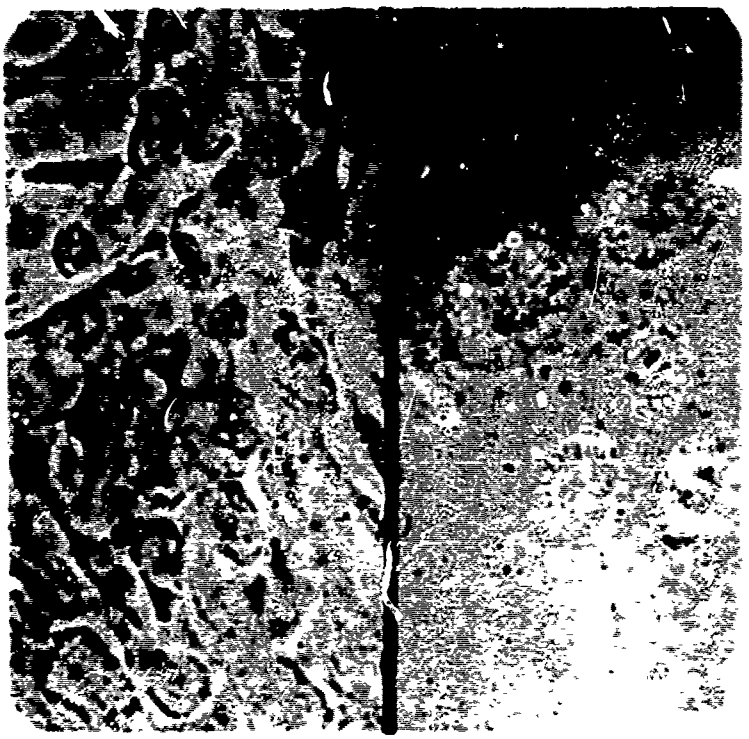


Figure 186: Effects of 20×10^{-12}g of angiotensin II on adult human arterial endothelial cell cultures (1) illustrates a controlled preparation and (2) morphological change following 60 minutes incubation in the octapeptide. Phase contrast microscopy x 650

DR. ROBERTSON: There could be a difference. Once we used only adult rats and they were normotensive. They were not hypertensive at the time of the study. Also if it is of any significance the spontaneous hypertensive rat developed the high uptake of thymidine preceding hypertension. So may be there is a difference there.

DR. RODBARD: I would like to ask Dr. Robertson about his first photograph. He indicated that pressor materials produced a specific wrinkling of the endothelial lining of vessels. What are the possibilities that the pressor materials produce the wrinkling as a result of vasoconstriction?

DR. ROBERTSON: I think that is a very good point, Dr. Rodbard. And I think that is the reason why I tried to present permeability data rather than morphology. We are very concerned with the fact that anoxia and simply just mistreating of the endothelial lining can produce what looks like morphological endothelial contraction. I think we have to be extremely cautious in making an interpretation of the ultrastructural evidence unless we have permeability studies to compare with.

DR. ADAMS: I wonder if Dr. Rodbard has any evidence that these villous projections are really some sort of receptor. It might be a little bit dangerous if we begin to think of things as receptors unless we know that they are.

Postulated Endothelial Receptors

DR. RODBARD: Projections have very recently been reported in a number of vascular sites, in the pulmonary artery in the canal of Schlemm, and at other sites where flow takes place. Thus far, the projections represent only anatomical findings. No means are yet available to test their functional activity. Such tests will depend on new techniques and ingenuity. The direction of the search for the possible functions of such projections will depend, as it does in all science, on hypotheses that are posited.

My laboratory has been examining factors that may determine the vascular caliber of the numerous sets of vessels in the body, not only of the blood vessels but also of the tubes of the lymphatics, the airways, of the biliary, enteric, genito-urinary systems and others. Our analysis suggests that the caliber of all of these may be determined by the hydrodynamic drag (shear stress) that acts on the lining cells.

From the point of view of structure, the projections would form excellent receptors. They resemble, in part, the bristles

that have been attached to the grids of vacuum tubes to measure flow rate. When the velocity and drag of the stream bend the bristle, the grid is moved and the gain of the tube is greatly modified. We have hypothesized that a similar receptor is present on endothelial cells (274, 339). We have been engaged in studies to test the drag concept (310). The concept appears to explain many of the complex relationships of blood vessel caliber. The discovery of the projections has been reported only in the past year. Thus far, our approach is a working hypothesis with a body of evidence that can provide a function for these new-found projections. However, such data do not constitute proof. I do believe that the presentation of a hypothesis together with the facts for its development is not dangerous. Such a sequence may be the way we can break through the complexities of the insuperable masses of data that await interpretation.

DR. A. P. SOMLYO: I would like to address my question to Dr. Robertson. I wonder whether an increased radio-active tracer content in the media is necessarily evidence of increased endothelial permeability caused by angiotensin. Did you do single pulse experiments and, if so, is it not possible that the increased tracer content in the media is due not to increased entry through the endothelium but to decreased clearance (exit) through the media? This could occur through contraction of smooth muscle increasing the tortuosity factor in diffusion.

DR. ROBERTSON: Dr. Somlyo's question is a very pertinent one. So far we have simply measured permeability after stimulation of arterial endothelial cells with vasoactive agents at subpressor levels using the experimental schedule previously described (302). Even the experiments that last less than 60 seconds between injection of the electron marker and in vivo fixation by perfusion at mean systolic pressure failed to categorically demonstrate whether our findings were due exclusively to increased permeability or decreased rate of clearance. Experiments applying Bevan's method for demonstration of vasoactive amines in the adventitial-medial boundary layer (30) are in progress. They combine ultra-rapid freezing of aortic samples on a specially designed stage with serial sectioning prior to quantitative measurements of tritiated lipoprotein fractions at different levels of the vascular wall.

DR. BURNSTOCK: Just one point of perhaps some interest to the question of whether the endothelial cells are contractile or not, relating to the kinds of filaments one sees in smooth muscle. As we heard from Andrew Somlyo earlier, the current view of smooth muscle is that there are three kinds of filaments - thick, thin and 100 angstrom or intermediate filaments. If you look at endothelial cells, you see only two kinds of filaments, the thin ones

and the 100 angstrom filaments, and not the thick ones. And at first thought one might wonder, well, this doesn't constitute any evidence that they might have a contractile system. On the other hand it is of interest that in the developing smooth muscle that we were looking at earlier, there are smooth muscle fibers early on which only have thin and 100 angstrom filaments but are quite efficiently contractile, spontaneously contracting. Later on when the muscle bundles form; the third thick kind of filament comes in. It is not inconsistent then that the endothelial cells may also be contractile with the same apparatus. How it works is very hard to imagine.

DR. A. P. SOMLYO: The fact that in cultured smooth muscle one does not observe thick filaments does not necessarily mean that they were not present in the contracting living fibers. There are two possibilities that could account for the observation. First, it is possible that in a multi-layered culture of smooth muscle cells some of the fibers actively contract and simultaneously impart motion to other, adjacent fibers that are not themselves contractile. The absence of thick filaments in this second type of passively moved fiber would not be surprising. Second, it is possible that in single contracting fibers the preservation of thick filaments for electron microscopy is not satisfactory, possibly because of swelling of the fibers during preparation. As usual, negative evidence about the organization of myosin does not readily lend itself to positive implications.

DR. ROSS: It so happens that one can demonstrate the thick filaments in smooth muscle cultures. All one has to do is tripsinize the cells and fix them at the point of tripsinization and lo and behold they are all there.

DR. BURNSTOCK. We have shown all three kinds of filaments in cultured muscle. But we see this later at about the time that these bundles are forming and they are fully differentiated cells. But the earlier stages when they are not fully differentiated they do still contract. And we only see two kinds of filaments in those and since the conditions are identical I think it is reasonable to suppose that they don't have thick filaments at that stage.

DR. A. P. SOMLYO: I would like to ask Dr. Robertson about his cultured endothelial cells. You showed contraction of cultured smooth muscle cells with angiotensin and I am wondering if you have any slides of the endothelial cells, or if you could perhaps describe their contraction.

DR. ROBERTSON: Fig.155 demonstrates morphological changes induced in adult human arterial endothelial cell cultures after one minute incubation in tissue culture medium containing 20 pica-

grams (10×10^{-12}g) of the octapeptide angiotensin II. The cells round up, becoming shorter and thicker in comparison to matching control cultures incubated with Roger's solution. This response is inhibited by ATPase and disappears after 10-12 in vitro passages.

DR. ROSS: I have forgotten who said it earlier in the day but someone talked about nexuses between endothelium and smooth muscle. I would like to know how good the evidence for that is; whether it is a very rare observation or whether it is a common observation.

DR. WOLF: And describe precisely to what type of attachment you refer.

DR. BJORKERUD: I am responsible for alluding in my lecture, to Huttner and coworkers (154) suggestion that gap junctions may be present between endothelium and smooth muscle cells. As far as I know there is no clear morphological evidence for gap junctions between these types of tissue. On the other hand, there is experimental evidence for transfer of information between the endothelium and the underlying smooth muscle tissue (417, 418) as also mentioned earlier.

DR. WERTHESSEN: Dr. Smith will summarize her studies of atherosclerosis from the biochemical point of view. After that we will consider Dr. Earl Benditt's "solution" to the problem. It is the most recently offered one and has an entirely different approach from any that we have tried in the past. He was to have attended the Conference but at the last minute was unable to come.

Chapter 4
DEVELOPMENT OF THE ATHEROMATOUS LESION

OVERVIEW

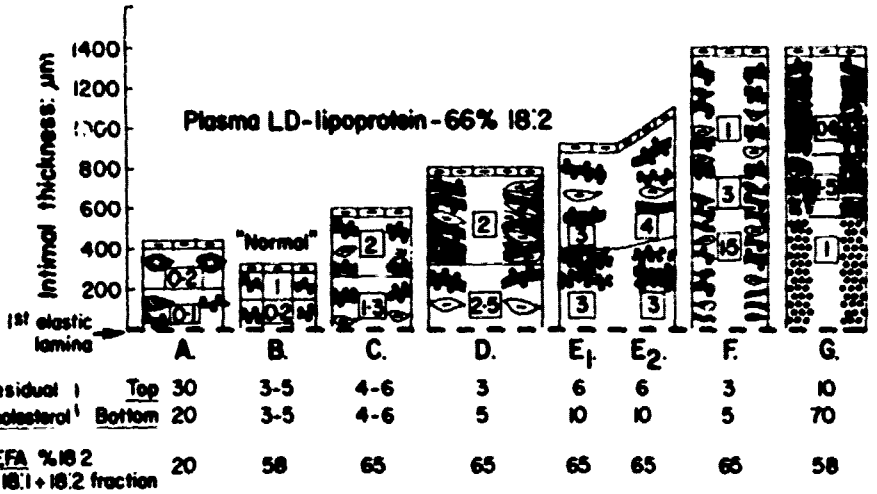
Dr. Elspeth Smith

I will try to outline the ideas on lesion development which I have evolved on the basis of quantitative chemical and immunochemical studies in human aorta; these findings may or may not apply to other vessels or other species.

The Relative Concentration of Lipids in Aortic Lesions

In the diagram (Fig.156) I have tried to relate morphological features with three key biochemical parameters: the concentration of intact plasma low density lipoprotein, which is expressed relative to its concentration in normal intima (designated as 1), the concentration of residual (electrophoretically immobile) cholesterol, and the percentage of linoleic acid (18:2) in the 18:1 + 18:2 fraction of the cholesterol ester fatty acids (CEFA). Adult normal intima with the diffuse intimal thickening which is invariably found in the third decade and upwards, is represented at B. Lipid stainable with sudan dyes occurs only as fine, extracellular perifibrous droplets. To the left of the normal intima (at A) is a juvenile-type fatty streak in which most of the lipid is within fat-filled cells. The cells contain a large amount of cholesterol ester with a highly characteristic fatty acid pattern in which oleic acid (18:1) is the major component; here it differs very markedly from the plasma lipoproteins and from normal intima in which linoleic acid is the major CEFA component (346). Therefore, it appears that these cells have not derived their cholesterol ester from plasma; there is much experimental evidence that they esterify cholesterol rapidly, and I think it is generally accepted that they are synthesizing their own cholesterol ester (262). In Berlin Dr. McGill gave us an excellent review of their epidemiological relationship to other forms of arteriosclerosis, but I am going to stick my neck right out and say that I think in the human aorta the juvenile-type fatty streak, consisting mainly of fat-filled cells, is not on the same pathway as the processes leading to large fibrous plaques. I have put them on the left side of normal to suggest that they are on a different pathway and will say no more about them.

The Significance of the Fatty Streak



		A.	B.	C.	D.	E_1.	E_2.	F.	G.
Residual Cholesterol[1]	Top	30	3-5	4-6	3	6	6	3	10
	Bottom	20	3-5	4-6	5	10	10	5	70
CEFA %18:2 in 18:1 + 18:2 fraction		20	58	65	65	65	65	65	58

Figure 156: THE RELATIONSHIP BETWEEN MORPHOLOGICAL AND BIOCHEMICAL FEATURES IN HUMAN AORTIC INTIMA.

The relative concentration of intact LD-lipoprotein is shown within each layer of the intima; concentration in upper layer of normal intima = 1.

Residual cholesterol (not mobile in an electric field) is expressed in mg/100 mg lipid-extracted dry tissue.

A) Fatty streak - lipid mainly within fat-filled cells.

B) Normal intima.

C) Gelatinous thickening.

D) Gelatinous plaque with greyish, opaque centre.

E_1) Raised gelatinous plaque; "reticulation" of collagen in deep layers.

E_2) Gelatinous periphery of plaque.

F) White fibrous plaque, low in lipid.

G) White fibrous plaque with underlying atheroma lipid pool.

Fat-filled cells.

"Normal"

"Thick" } collagen bundles.

Perifibrous lipid droplets (extracellular).

The adult normal intima is bathed with plasma and contains a large amount of electrophoretically and immunologically intact plasma lipoprotein. In fact, on a crude volumetric basis the concentration is the same in the intimal and plasma compartments, and they appear to be in equilibrium; if the plasma lipoprotein is high the intimal lipoprotein is high, and if the plasma lipoprotein is low the intimal lipoprotein is low (347). Starting from this baseline (B) my present view of the evolution of large fibrous plaques goes from left to right in the diagram, through the "gelatinous lesion" which appears to be synonymous with the intimal edema of the German workers and the insudation lesion of the Canadian group (141).

The Evolution of the Arteriosclerotic Plaque

Macroscopically, these lesions appear as intimal thickenings or peripheral zones round plaques; they are characteristically translucent, yellowish or pinkish in color and gelatinous in texture. Microscopically, they invariably show a very loose structure, often with thick collagen bundles which may stain diffusely with sudan dyes. The figure shows the relative concentrations of intact lipoprotein in the upper and lower layers of normal intima and lesions. In the lower half of normal intima (B) the concentration is very low - about 0.2; in the small gelatinous thickenings (C) it is doubled in the upper layer and increased about six-fold in the lower layer; in the plaque peripheries (C2) it increases four-fold in the upper and fifteen-fold in the lower layer. Thus there is not only an apparent increase in endothelial permeability, but a great increase in the permeability, and perhaps also retentiveness of the intimal tissue (348). I postulate that the next stage is an opaque greyish-whiteish area with a gelatinous surround. Microscopically, in the opaque area (D) there is a much more compact structure. This seems to take two forms - more commonly there are a lot of rather closely packed collagen bundles with relatively few cells, and in the other form there are a great many cells, which I presume are smooth muscle cells, packed rather closely together with little collagen. This seems to fit in with some of the tissue culture data that Dr. Ross presented yesterday; one can imagine that where there is a compact mass of cells they were dividing rapidly and not laying down very much collagen, whereas in the other situation they have stopped dividing and are producing a lot of collagen. The lipoprotein concentration is about double the normal in the upper layers, and this may be exceeded by the concentration in the lower layer (D). One also finds lesions in which the top remains gelatinous but the deep layer appears to be "reticulating". Instead of the collagen bundles remaining linear they seem to be forming a network with the strands coated with fairly abundant perifibrous lipid; here, for the first time in this progression, there is an increase in the residual choles-

terol concentration (E1). Lipoprotein is not the only plasma constituent present in the gelatinous lesions; it is closely paralleled by the concentration of fibrinogen. Expressed as volumes of the plasma from which they are derived, in order to get over the difference in their absolute concentrations in plasma, there is approximately twice as much lipoprotein as fibrinogen in the upper layers, but in the lower layers there is a relatively greater increase in fibrinogen (349).

In the classical large white plaques, surprisingly in thirty or forty per cent there is little lipid throughout their whole depth (F) (246). However, in about sixty per cent of the lesions there is a great mass of lipid under the white cap (G). This lipid is of the plasma lipoprotein type with linoleic as the predominant fatty acid in the cholesterol esters (350, 351), but it differs from lipoprotein lipid in having a greatly increased proportion of free cholesterol, suggesting cholesterol esterase activity and a much higher ratio of cholesterol to phospholipid. The distribution of lipoprotein is rather complex (F and G) with the highest concentration in the cap/fat junction region. Presumably the lipid is somehow split off the lipoprotein, but we have no information on the mechanism involved. In the gelatinous lesions all plasma constituents are increased; there is twice as much albumin and about four times as much lipoprotein and fibrinogen as in normal intima. So there appears to be an increase in permeability, and possibly also in retention. I think that some of the questions we have to ask ourselves are the following: Does the process start because the endothelium becomes more permeable and then the plasma constituents somehow stimulate smooth muscle cell proliferation and collagen production? Or does something happen to the smooth muscle cells first so that they stop eliminating plasma constituents that enter at the normal rate? Or are both these things happening? I think another very important question is: What is the role of fibrinogen? Is it possible that laying down a fibrin matrix in the region of the smooth muscle cells stimulates their proliferation and collagen production? I do not seem to see platelets within the gelatinous intima but could some platelet factor enter from mural micro-thrombi and perhaps stimulate smooth muscle cell proliferation?

Gelatinous Lesions

DR. SCHWARTZ: I should like to ask Dr. Smith a question and perhaps even suggest that Dr. McGill, Dr. Daria Haust and Dr. Strong comment. Can any of the participants make any comment on the frequency and topography of the gelatinous lesion in aortas, and how these might relate to other lesions and their development? This is one question I would like to see clarified.

DR. SMITH: I think one must appreciate that I am picking out

the two extreme ends of the spectrum. If you look at a normal middle aged human aorta, there's a hell of a mess, frankly. But if you are lucky you can find small lesions very rich in fat filled cells and at the other extreme you can find a group of lesions of very varying degrees of development containing practically no fat filled cells. Now I think in order to understand any process one has to start at the ends and then build up the more complicated mixed lesions, of which there are a great many, when you understand the simplest ends of the process. So, I do want to make that clear. The fatty streak and these fibrous type lesions or proliferative type lesions which are virtually free of fat filled cells are the two ends of the spectrum and there is great mixing in the middle.

DR. MCGILL: We need information on the extent, prevalence and natural history of this gelatinous lesion. This is a difficult lesion to identify in gross specimens, and I think the problem for the future is to define it better, to learn to recognize it grossly and microscopically, and acquire data on the age at which it occurs. Such information would help to fit this concept into the natural history of atherosclerosis. I just don't recall seeing this lesion to any great extent in people in the critical stage of atherogenesis, which we think is around twenty to thirty years of age, when fibrous plaques being to appear.

One other comment, Dr. Smith. You are wise to say that this lesion is limited to the aorta, because there are differences between the coronary arteries and the aorta in regard to the appearance of fatty streaks, the fibromuscular intimal layer, and the behavior of lesions subsequently. There may be a different natural history for the aorta than for the coronary arteries.

Mucopolysaccharides in Gelatinous Lesions

DR. WISSLER: I raised the question in Berlin and I still think it is a good question, as to how much of the gelatinous lesion really is an accumulation of acid mucopolysaccharides or proteoglycans and not really insudation in the usual sense. Now this kind of lesion, in my experience, is very rare in adults and when we do see it we frequently think it is a stage in reversal, and I wonder how Dr. Smith would react to that. I think we see a lot of lesions and indeed the lesions that we have studied where we have purposely reversed the rather advanced plaque, is really loaded with very loose tissue which has a lot of acid mucopolysaccharide, and I think in a postmortem service you have to be very careful because the lesions found at autopsy in those who died of wasting diseases may have undergone marked changes in the final three to five weeks of life, I think. So I think we have to be prepared as we always are at the post mortem end of things for perhaps confusing progression for regression. It is very difficult to take apart, as I tried

to point out in the plenary session in Berlin, because as in the human you are just looking at an end stage and I still think that the experimental model when we can study it more thoroughly may give us a better idea of pathogenesis.

Now, I would like to ask Dr. Smith two questions. I had always thought that the lipid core generally speaking had a rather high oleic acid level at least in the analyses that I was acquainted with and that linoleic was somewhat lower, so I am a little surprised by that statement. May be there are some other people who have a better command of that chemistry than I do. I would like to ask, in general, in the more advanced lesions, what is the proportion of the lipid that is in the form of lipoprotein and what is the proportion that is present in some form other than lipoprotein?

DR. SMITH: To answer the question on mucopolysaccharides, there is depressingly little data available on this. In a study of about about ten of these lesions some years ago we found the concentration of total mucopolysaccharides to be slightly lower than in adjacent normal intima (Table XVIII). The calculation was made on a dry weight basis which may not be the correct basis on which to express it. So there is, in fact, at present no data to indicate that there is an increase in mucopolysaccharides there. It is interesting that in the fat filled cell fatty streak there is a small increase, but in these lesions there was a very small decrease (352).

Now on the question of possible post-mortem changes, in fact we find no difference in frequency between bodies we have obtained two hours after death and bodies we have obtained twenty four hours after death. Furthermore, in the gelatinous lesions there is invariably intimal thickening and proliferation of collagen and smooth muscle cells. Although post-mortem changes are very worrying I do not really think that they account for this.

On the question of regression, and I think this would fit in with one of Dr. McGill's comments, I don't think that I see these in the twenty to thirties age group. I see them mainly in the 35 to 50 age group, most frequently in the younger coronary cases, and in the hypertensive cases; in older subjects and the long term carcinomas and wasting disease one rarely finds this type of lesion. In these cases it is usually a rather small, compact, white fibrous type of lesion.

But I think this whole question of regression is one that should be kept in perspective. I have the feeling that one particular lesion may be in regression with the atherogenic process in the individual very much in progression. And when one compares the experi-

mental regression lesion with the large fibrous plaque with its thick tough cap and cholesterol clefts, they are very similar indeed. I would almost wonder, and I think this fits in very well with Bruce Taylor's concept, if once you've got a jolly good tough collagen cap over the lesion it may have had it, as it were, and it doesn't progress, and may be it regresses. But that does not mean that there is not another gelatinous lesion next door which is progressing at a rapid rate.

On the question of the CEFA composition in the large lesions, some of the early data was very misleading. This was probably the result of mixing together intima and media, and lesions of different types. In fatty streaks and nodules, in which the lipid is predominantly in fat-filled cells, oleic acid is the main CEFA component, but in the "atheroma" layer from advanced plaques linoleic is the predominant acid (346, 350, 351). The proportion of linoleic acid is somewhat lower than in plasma lipoprotein but it is significantly higher than in the fat filled cells (Table XXIX). It is frequently assumed that the "atheroma" lipid pool under large plaques comes from fat filled cells which have disintegrated. This would entail a massive transformation of the cholesterol esters, presumably by preferential hydrolysis of cholesterol oleate. This idea receives some support from the large increase in free cholesterol (Table XXIX) and the not infrequent finding of a thick cap containing numerous fat filled cells, some of which seem to be disintegrating into the atheroma lipid.

However, we have recently made very detailed analyses of different fractions isolated from such plaques by micro-dissection, and were unable to find any evidence of differential hydrolysis (351). I think that even where there are a lot of fat filled cells in the vicinity of plaques, most of the pool of atheroma lipid is not coming from disintegrating fat filled cells but is coming directly from the plasma lipoprotein. I can't remember if I have answered all of the questions or not.

DR. LINDNER: I absolutely agree with Dr. Wissler's remarks and I think you are not right to say we have not enough data about the mucopolysaccharide (or GAG) content in this lesion. Naturally, in earlier works people pooled together parts of the aorta that were so long that they could not differentiate between the several kinds of lesions but now we have not only histochemical data but data from biochemical analysis as well concerning synthesis breakdown, total content and turnover rates of glycosamino glycans and proteoglycans in the several lesions, early as well as late.

The age dependent increase of GAG and collagen contents is evident (Fig. 157) for collagen shown by the hydroxyproline content. The higher content of hexosamine indicates the presence of neutral

TABLE XXVIII

CHANGES IN THE CONCENTRATIONS OF INTACT LD--LIPOPROTEIN, CONNECTIVE TISSUE COMPONENTS AND 'DEPOSITED' CHOLESTEROL IN EARLY LESIONS

	Percentage of the normal level			
	fatty streaks		'Gelatinous' thickenings	
LD--lipoprotein	28%	(14)*	198%	(17)
Total GAG	116%	(19)	91%	(7)
Collagen	111%	(19)	117%	(7)
'Deposited' cholesterol	1319%	(14)	138%	(17)

*Number of pairs of samples

TABLE XXIX

Total and free cholesterol, and the relationship between linoleic acid (18:2) and oleic acid (18:1) in the cholesterol ester fatty acids (CEFA) from plasma lipoproteins, normal intima and lesions of different types.

	CEFA	TOTAL CHOLESTEROL	
	Percent 18:2 in 18:1-18:2 fraction.	Percent free.	Concentration: mg/100mg (1).
PLASMA: Sf 0 - 12 lipoproteins.	46	25	-
INTIMA:			
Normal, age 30-39 years.	53	14	1
Fat-filled cells:			
from fatty streaks.	20	21	20
from caps of large plaques.	30	26	16
Gelatinous lesions: lipid-poor.	65	17	2
lipid-rich.	65	21	16
"Atheroma" lipid under large plaques:			
fat-filled cells present in cap.	(2) 59 - 64	40 - 42	17 - 44
no fat-filled cells in cap.	56	52	44

(1) mg/100mg defatted dry tissue.
(2) The first number refers to the middle layer and the second to the lowest layer of the amorphous lipid.

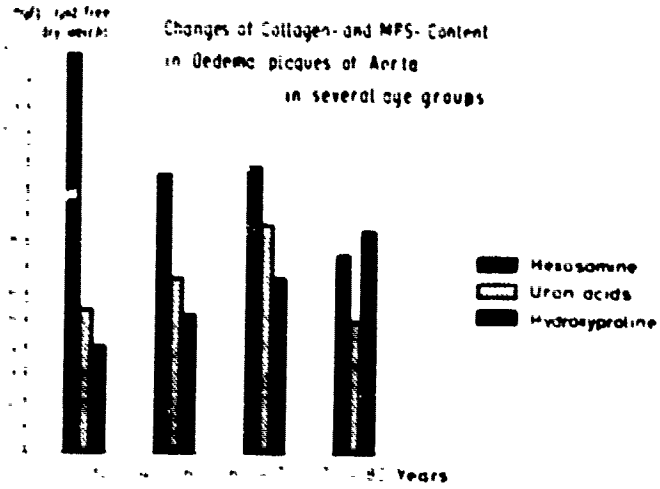


Figure 157

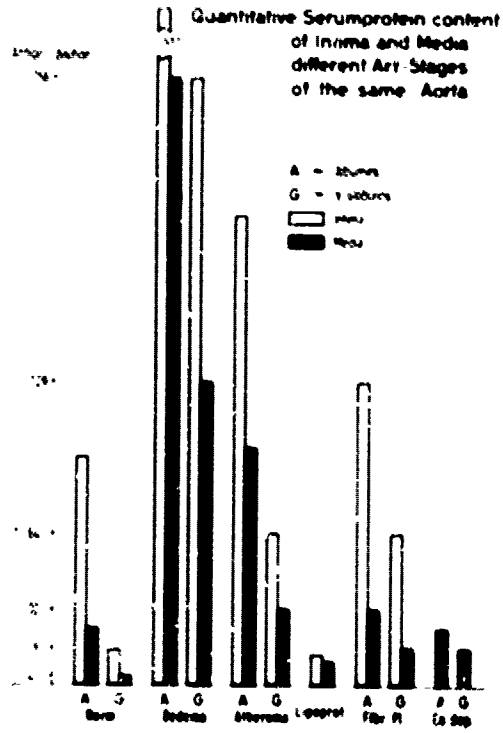


Figure 158

serum glycoproteins. The serum protein content is important for the physiological and pathological metabolism of the arterial wall. In the development of disturbed vascular metabolism preceding atherosclerosis we find a disturbed permeability and enhanced serum content. Quantitative immunological estimations of serum proteins in native tissue sections (carried out with the so-called ring-test-dilution method) are shown in Fig.158.

Summarizing the results in Fig.158 we can see: (1) The serum albumin content is higher in the intima than in the media, (2) higher than the γ-globulin content in every atherosclerotic stage, (3) the highest content of both serum proteins is in the edematous plaques. The serum protein as well as the hexosamine content decreases in atheromatous as compared to edematous plaques, but are higher than in adjacent unchanged parts of the same vessels.

So we have the highest content of these two serum proteins in the fresh edema plaques, which show simultaneously an increase synthesis of GAG and lesser of collagen. The S^{35}-incorporation assay on human material (Fig.159) shows that the vascular GAG synthesis decrease with age in all atherosclerotic stages and, most important, that the highest incorporation rates were recorded from edematous plaques.

Fig.160 shows that the total content of proteoglycans (as of GAG) and of collagen in young humans is lower in the mid-thoracic aorta than in more rostral or caudal segments. These local differences in content of GAG and collagen (demonstrated by uronic acid and hydroxyproline assays) must be kept in mind in evaluating experimental findings on various arterial sites in the body. The DNA content (as quantitative assay for the cell number) is also lower in the thoracic aorta as compared to the aortic arch and the abdominal aorta (see Fig.161). Thus the cell number seems to run in parallel to the total content of GAG and collagen in the human aorta. HUMAN

The total content of groundsubstance and the relationships between groundsubstance synthesis and degradation are shown in Fig.162. The total content of groundsubstance (and fibers) in a connective tissue is the sum of synthesis and breakdown. Both can be enhanced or inhibited at the same time or separately. In the first (upper) line the breakdown is enhanced, in the second (middle) line breakdown is normal and in the third (lower) line it is decreased. In the left row the synthesis is enhanced, in the right row decreased, compared with the normal situation (middle). The numbers mean temporal stages. In the first stage of atherosclerosis after injury, before morphological evidence for early lesions becomes visible, the breakdown of the groundsubstance (as well as collagen) may increase more rapidly than synthesis. In the end

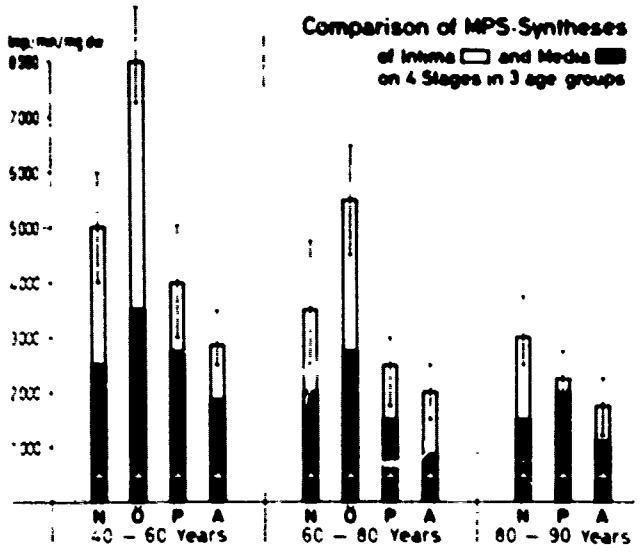


Figure 159: Summary of ^{35}S-sulfate incorporation assays on human material. N = normal arterial segment, O = edematous plaques, P = fibrous plaque, A = atheroma.

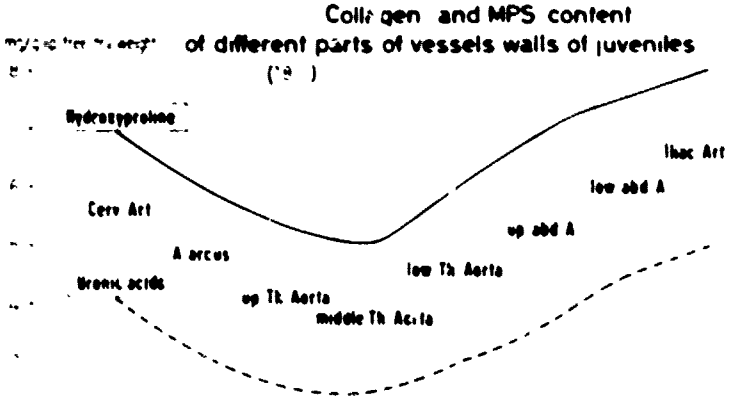


Figure 160: Quantity of collagen and mucopolysaccharide in segments of the arterial wall taken at different levels from children

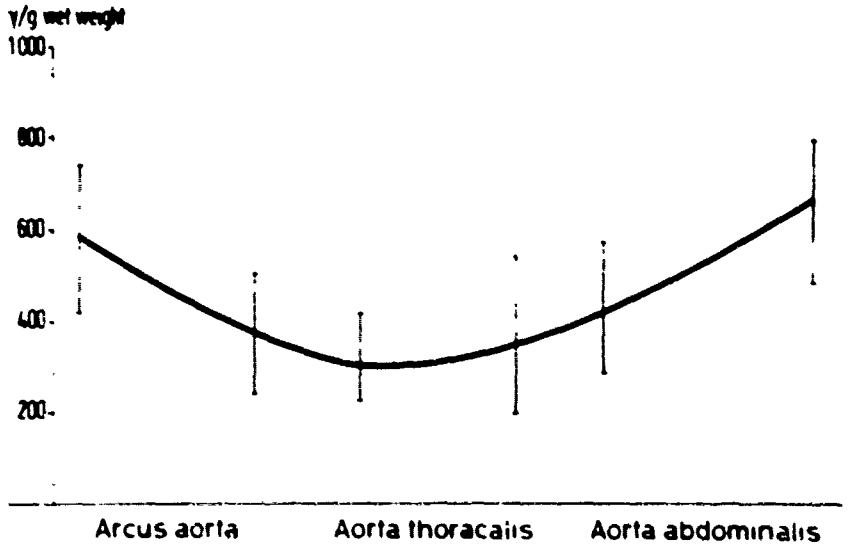


Figure 161: Quantity of DNA at different levels of the human aorta.

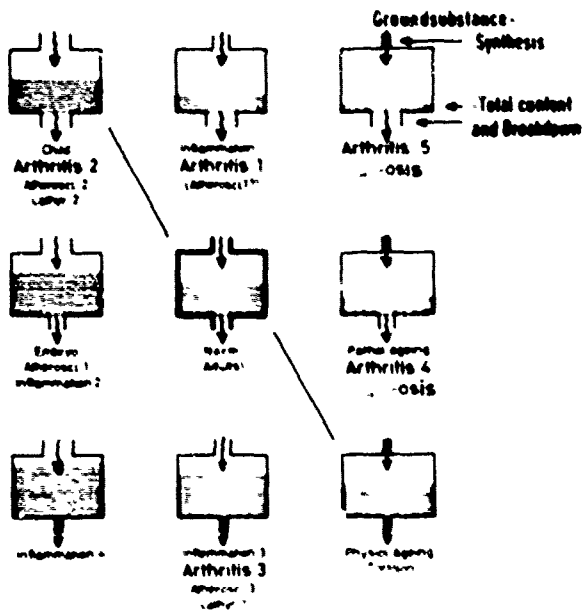


Figure 162: Schematic description of the relations between groundsubstance synthesis, degradation and total content in connective tissue in atherosclerosis in comparison to development and standard as to several other sicknesses of connective tissue and aging

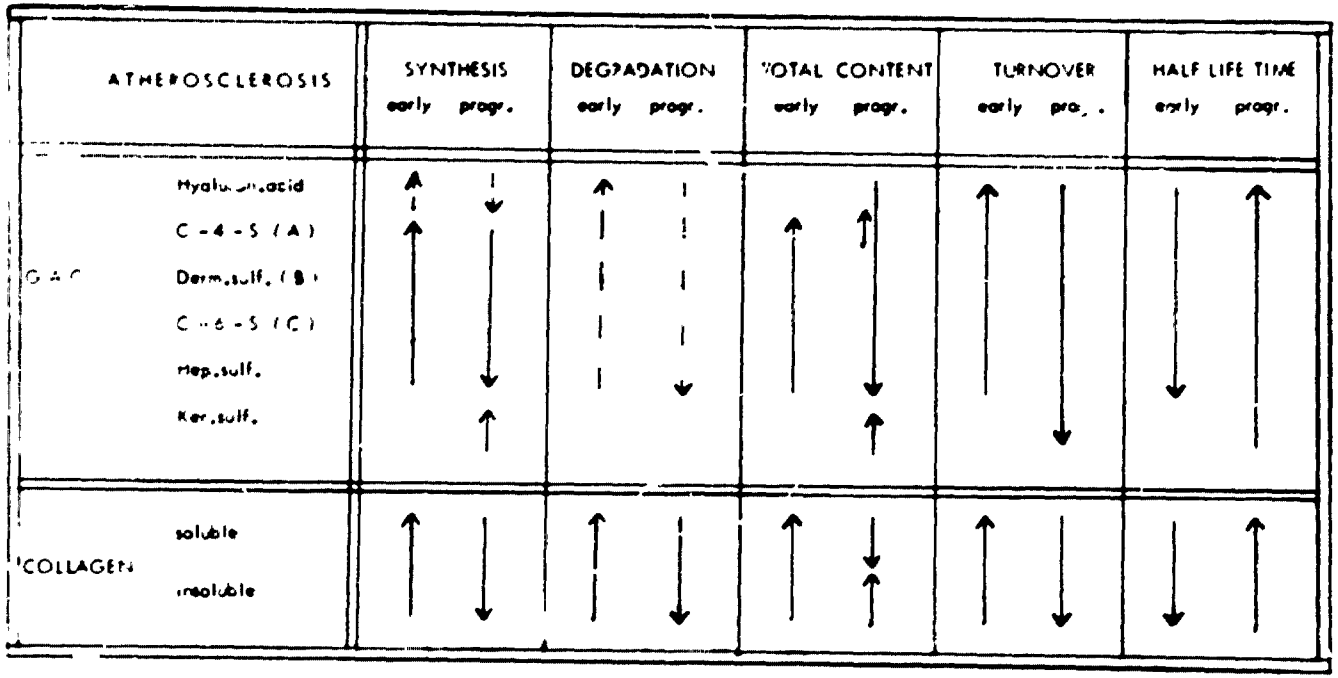

ATHEROSCLEROSIS
SYNTHESIS
early progr.
DEGRADATION
early progr.
TOTAL CONTENT
early progr.
TURNOVER
early progr.
HALF LIFE TIME
early progr.
GAG
Hyaluronic acid
C-4-S (A)
Derm.sulf. (B)
C-6-S (C)
Hep.sulf.
Ker.sulf.
COLLAGEN
soluble
insoluble

Figure 183

stages of atherosclerosis, the plaques, we find a further decreased total content of groundsubstance (as in the early stage of inflammation). When, on the other hand, synthesis increases more than breakdown the total content increases. Although turnover may decrease in later stages, since catabolism decreases more rapidly than anabolism. An increased total content of metachromatic groundsubstance results. With an increase in collagen content morphological disorganization occurs as well as necrosis and lipid and calcium deposition.

In understanding this process, therefore, we must distinguish between synthesis, degradation, total content, turnover and half life times, in early and progressive lesions and we have to fractionate the several patterns of mucopolysaccharides (GAG) especially the typical ones for the vascular connective tissue.

Fig.163 summarizes the process of lesion development as I see it on the basis of morphological and biochemical work. In summary it appears that lipid containing smooth muscle cells can not only replicate but can also synthesize and break down collagen and glycosamino glycans. Therefore when we see lipid droplets in smooth muscle cells, especially in light microscopic overstained cells, we can not conclude, as the old concept held, that the cell has nothing else to do than to die.

In early lesions of atherosclerosis both synthesis and degradation of MPS and collagen increase. So do their turnover rates but their half life times are shortened. As the atherosclerotic process continues anabolic and catabolic processes decrease. So also do the turnover rates and the total contents of the MPS - and the soluble collagen fractions. At the same time the total content of insoluble collagen and the half life times of the intercellular vascular connective tissue increase.

Concerning post mortem changes we can confidently say that they occur slowly since we have compared post mortem lesions with lesions freshly investigated after accidents or from amputated legs. The findings are quite similar.

DR. ROBERT: I would just like to refer briefly to Dr. Wissler's question because we also studied recently the type 1 to 3 lesions and could confirm Dr. Smith's findings that there is no apparent increase in acid mucopolysaccharides but we found a very significant increase of glycoproteins which can be extracted with one molar sodium chloride (244). So there are definitive biochemical changes even in the apparently normal-looking portions of the aorta at these stages of the atherosclerotic process.

Mucopolysaccharides and Glycoproteins

DR. STEIN: I have a question for Dr. Smith. The LP you referred to, is it LDL? And I see you refer always to albumin and fibrinogen and what about the HDL?

DR. SMITH: We have only rather limited data on HDL because we have had antibody trouble. But we do now have a small amount of information, and HDL is derived from about one-fifth the plasma volume of LDL. So, in fact, its retention is lying somewhere between fibrinogen and albumin. Obviously one really wants to study a whole range of plasma proteins of differing molecular weight but we have not got around to doing any of the others.

DR. KRITCHEVSKY: Perhaps the differences in the oleic-lineoleic ratios in the cholesterol ester fatty acids could be explained by assuming that the fatty streak, in which 12:2/18:1 is low, is a rapidly metabolizing tissue which is trying to "detoxify" the cholesterol by esterifying and storing it. While the trapping of additional serum should cause the ratio 18:2/18:1 to rise, it may not if the "detoxification" involves the synthesis of additional 18:1 cholesteryl ester.

DR. STRONG: Dr. Schwartz asked if Strong and McGill had anything to say about the topographical distribution of the gelatinous lesion, and I agree with Dr. McGill that we do not. I hope that Dr. Haust will respond to the question because she is well versed on that type of lesion. We do have considerable information about the topographical distribution of fatty streaks and fibrous plaques. It is very difficult to make any convincing argument about topographic similarities in the aorta because there are enough fatty streaks in the aorta to account for almost any more advanced lesion that would be superimposed on it.

Fatty Streaks in Coronary Arteries

In the coronary arteries and in the carotid arteries, the topographical case for a relationship between fatty streaks and fibrous plaques is so convincing that it is difficult to overlook. The fatty streaks occur predominantly in the same places that later become covered with fibrous plaques. One point of localization is the anterior descending left coronary artery just past the bifurcation and another is the carotid sinus. We have mapped out the distribution of lesions by centimeter from the ostium of coronary arteries, and the curves for prevalence of fatty streaks, fibrous plaques and complicated lesions are quite similar in topographic distribution. While there may be problems about quantitative rates of conversion, there is no doubt that fatty streaks and fibrous plaques occur in the same locations, at least in the coronary arteries and carotid arteries.

I am a bit worried about placing a lot of faith and confidence

in the pathogenetic importance of the gelatinous lesion when we are talking about a lesion that is said to occur first in persons, 30 to 40 years of age. In other words, we know that fibrous plaques become well developed by the end of the second and third decades. If these gelatinous lesions are not present before then, I do not understand how they can be the precursor to the fibrous plaque.

The Relation of Gelatinous Lesions to Fibrous Plaques

My final comment is on the fibrous plaques as described by Dr. Smith. I rarely see either of the varieties she described in pure form. I am referring to the fibrous plaque with no lipid and the fibrous plaque with a neat core of lipid in the base. I was amazed in trying to select "typical" fibrous plaques (the ones with the lipid core) to see how much lipid was in the thick coat covering the lipid core. There was a great deal of lipid near the surface as well as in the depth of most of the plaques. I do not believe that I have ever seen a fibrous plaque without any lipid in human lesions.

DR. HAUST: Dr. Strong wondered whether I would comment on the subject of insudation or gelatinous lesions in atherosclerosis. There are two parts to the problem: (1) Do the lesions occur at all, and (2) if they do, what is their distribution, incidence, etc.

The lesions do occur as was shown already in the thirties and forties by the German pathologists (220, 318) and later confirmed on this continent (141, 142, 143, 232). These lesions had been missed on gross inspection for a long time because they have no impressive color like the yellow streaks. They resemble pale grey blisters. The answer to the second question had never been seriously attempted and if I speak at all to this group on the subject it is with a plea in mind: perhaps we all should collaborate in an effort to investigate the distribution and incidence of these gelatinous elevations, to determine whether on the basis of their distribution and sites of predilection alone it would be possible to be convinced that they, indeed, are some forms of precursors of the well advanced atherosclerotic lesions. Other data do point to this association. Incidentally we see these lesions in younger subjects, under thirty years of age.

DR. SMITH: I was wrong in saying that they start so late. Looking at these with a biochemist's eye, I was thinking in terms of lesions which are large enough for chemical analysis. Yes, I would entirely go along with Dr. Haust that you see very small ones in the younger age groups. But from my point of view, I would not be able to analyze them.

Age Distribution of Gelatinous Lesions

Figure 164: Autoradiograph of human fibrofatty lesion following incubation in vitro with ^{14}C labeled oleic acid. The three cells shown show differing uptake and incorporation of the precursor into cholesterol ester and phospholipid.)

F - foam cell
M - monocyte
S spindle shaped cell

DR. McGILL: We need information about the quantitative aspects of the "gelatinous" lesion beginning at least in the teens and extending into the 30 and 40 year age group. This is the most difficult age group to study because mortality is low, most mortality is accidental, and it is difficult to get access to this material. However, we must have information - quantitative information - about this lesion in relation to other types in order to answer the question about its role in pathogenesis.

The Role of Glycosaminoglycans

DR. WIGHT: I would like to speak briefly of the role of glycosaminoglycans in lesion development. I think it is important that we pay particular attention to the changes in the individual types of glycosaminoglycans during lesions formation and not merely to whether there are total increases or decreases in these macromolecules during atherogenesis. Individual glycosaminoglycans differ in their ability to bind linoproteins (160) as well as in their ability to be antithrombogenic (129).

DR. LINDNER: I agree with Dr. Wight that it is very important for biochemical research on the several kinds of lesions in atherosclerotic vessels to fractionate the proteoglycans and glycosaminoglycans. One finds different patterns depending on the age of the lesion as well as on the chronological age of the donor. Several groups report an increased synthesis of acid sulphated mucopolysaccharides (MPS=GAG) especially of sulphated GAG (only a few report about unsulphated GAG) during the development of early atherosclerotic lesions.

RABBIT

Lipid Changes in Association with Circulating Antibodies

DR. ROBERT: We did quite extensive studies using ^{14}C-lysine incorporation into rabbits which were fed with cholesterol or received just Freund's adjuvant or were immunized with elastin and Freund's adjuvant (71, 283). We find a quite significant shift in the incorporation pattern in the different extractable and nonextractable fractions as obtained by our "chemical dissection" procedure. One of the most striking findings was the marked decrease of lysine incorporation in the structural glycoprotein fraction. This was observed both in those rabbits fed cholesterol and those which were immunized with elastin. There was a slight increase in the antielastin antibody titre in the cholesterol fed rabbits. We think that circulating antibodies might be important in this metabolic "shift" which can be shown by this technique.

DR. DAY: We have carried out a number of studies on the synthesis of lipid by human arteries obtained from renal transplant donors (398, 399). It was possible to compare the uptake and

incorporation of oleic acid into various lipid fractions in normal intima, fatty streak lesions and in fibro fatty lesions. In the normal intima the uptake of oleic acid and its incorporation into cholesterol ester is relatively low. In the adjacent fatty streak lesions however there is a marked increase in the amount of oleic acid incorporated into cholesterol ester. We also studied by autoradiography the cells responsible for the uptake and incorporation
HUMAN of oleic acid into lipid in the human lesion. Fig.164 shows an autoradiograph from a fibro fatty lesion, following incubation of the aorta with ^{14}C labelled oleic acid. Three cells are present. The uptake of ^{14}C labelled oleic acid and its incorporation into phospholipid and cholesterol ester is more marked in the lipid containing foam cell than in the adjacent monocyte or fibroblast.

We have done similar studies with ^{14}C labelled choline in order to look at phospholipid synthesis in cells from human intima, normal and atherosclerotic. There are shifts in the amount of label incorporated into phospholipid in the fatty streak and fibro fatty lesions compared with the normal intima. Most of the ^{14}C labelled choline taken up and incorporated into phospholipid occurred in the foam cells of the lesion (399).

DR. STEIN: I would like to remind the group about the paper of Zilversmit (434). I think he was the first to compare the incorporation of ^{32}P and ^{14}C acetate into atherosclerotic lesions in humans as compared to the normal part of the artery. He found a very marked increase in the lesions.

Lipid Incorporation in Lesions versus Normal Arterial Segments

DR. SCHWARTZ: I would like to ask a question of clarification of Dr. Day and perhaps the other participants. To what extent can the differences in incorporation using labelled precursors in normal and atheromatous or diseased tissue be due to different precursor diffusion into the lesion?

DR. DAY: This is very difficult to answer definitely. In the lesion it is possible that varying permeability to precursors may be partly responsible for the differences in uptake and incorporation observed. When one is dealing with an autoradiographic situation and looking at penetration or uptake by different cell types presumably one can assume that adjacent cells are exposed to similar concentrations of precursor. Differences between cell types may therefore be much more relevant than the grosser comparison between different lesions.

DR. BOWYER: I should like to add that the apparent rates of lipid synthesis in variously diseased tissues as measured by incorporation of labelled precursors, will also depend upon the

size of the existing precursor pool. We have measured the specific activity of the free fatty acid pool in segments of arteries perfused with ^{3}H oleic acid and find that it is 10 to 100 times less than the specific activity in the perfusate, because of dilution by the endogenous fatty acid in the arterial wall. Thus rates of arterial lipid turnover are 10 to 100 times higher than estimated from the perfusate specific activity, if it is assumed that exogenous and endogenous fatty acids are utilized at the same rates. In fact, a number of pools of lipids probably exist, each of which may utilize a separate precursor pool for synthesis. Thus the specific activity of a free fatty acid precursor pool for plasma membrane lipids may be similar to that in the perfusate, whilst the precursor pool for intracellular organelle lipids may have a much lower specific activity, being determined by rate of diffusion of precursor and intracellular dilution.

Lipid Turnover in Lesions

DR. DAY: I am aware of course of this point and while in the studies we did on human tissue we did not measure the intimal fatty acid pool, we have done so in our animal model studies and have previously presented data relating to the incorporation of fatty acids into intimal lipid based on the measured intimal fatty acid pool (78).

DR. BJORKERUD: There are additional factors which add to the difficulties encountered when arterial tissue is studied in in vitro systems. One such factor is the permeability of the tissue samples for e.g. substrates. Very little information is available on the permeability of human arterial tissue for different substrates. We tested the permeability of non-atherosclerotic human arterial intima and media samples for glucose. The penetration of this substance was rate-limiting for the rate of incorporation of glucose into lipids with tissue samples larger than 1.2 mg (d.w.). There is more information available on animal arterial tissue. Arnqvist presented recently a thesis with data on the size of the extracellular space of and the penetration rate of glucose into bovine arterial tissue (16).

DR. ROBERT: Just a quick answer to Dr. Schwartz's objection which in our experiments was avoided by cutting into tiny small slices all the aortas and measuring pool size and we could show, you see, that it is not an important factor in our experiments. But I agree with you that one has to do these control experiments before trying to interpret the results in terms of metabolic shift and that was done.

DR. McGILL: Dr. Day's slide with a column headed "Fatty Streak" reminds me of a caveat I think we should make in trying

to get information in the future that will answer this troublesome question about fatty streaks and their relation to other lesions.

Different Kinds of Fatty Streaks

There are several different kinds of fatty streaks and perhaps this much maligned lesion has a bad reputation because it comes in several different forms. The juvenile fatty streak in a teen-age person is quite different - not so much in gross appearance, but certainly in microscopic appearance - from that occurring in a middle-aged or elderly person. There may be three or four different kinds of fatty streaks, and they may be entirely different in origin and outcome. In the future when we use the term 'fatty streak' we must be specific. Is it the highly cellular lesion occurring in children with may cells filled with fat, or is it the lesion you see in middle age with lots of extracellular lipid clustered around connective tissue fibers?

DR. DAY: I apply the term fatty streak to a lesion primarily characterized by intracellular lipid present in foam cells.

I demonstrated a fibro fatty lesion as distinct from the fatty streak to illustrate the differing uptake of fatty acid by three types of cells present.

DR. ADAMS: I want not only to talk about the question of phagocytosis by smooth muscle but also to provoke some consideration of

Macrophages and the Phagocytosis of Lipid

where the phagocytes in various atheromatous and atherosclerotic lesions come from. I mentioned yesterday that many RE cells contain abundant catalase whereas smooth muscle does not. The reaction of sinusoidal histiocytes and blood monocytes for catalase is illustrated in Figs.165 and 166. When lipids, including atheroma-lipids, are injected subcutaneously, a fair proportion of the phagocytes in the lesion - presumably macrophages - can be stained for catalase (3) (Fig.167). In sections of atheromatous lesions of rabbit aorta there are no catalase-positive cells (Fig.168). I know that Dr. Poole (261) showed some years ago macrophages or monocytes passing through the endothelium into the wall of the vessel, but our data here would suggest that the main accumulation of cells are not of RE origin. It cannot be said that they are necessarily smooth muscle cells because this particular technique does not identify them as such. Likewise, the intimal cells in sections of human fatty streaks (Fig.169) and fibro fatty plaques are all catalase-negative (3). Again the intimal cells cannot be identified with the catalase method as necessarily being smooth muscle cells. Their butyry. cholinesterase reaction is in fact negative, whereas if they were normal smooth muscle cells they would contain this enzyme.

Benditt and Benditt (25) made the point from some very elegant

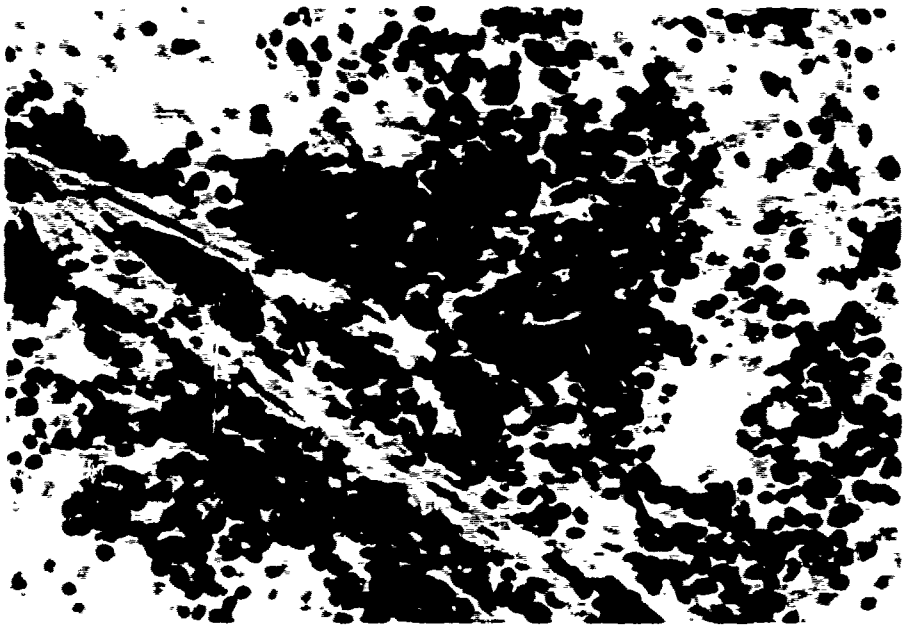


Figure 165: Catalase-positive sinusoidal and other histiocytes in rat spleen. Diaminobenzidine catalase reaction (cyanide-resistant; partly aminotriazole-sensitive); toluidine blue nuclear counterstain. x 450.

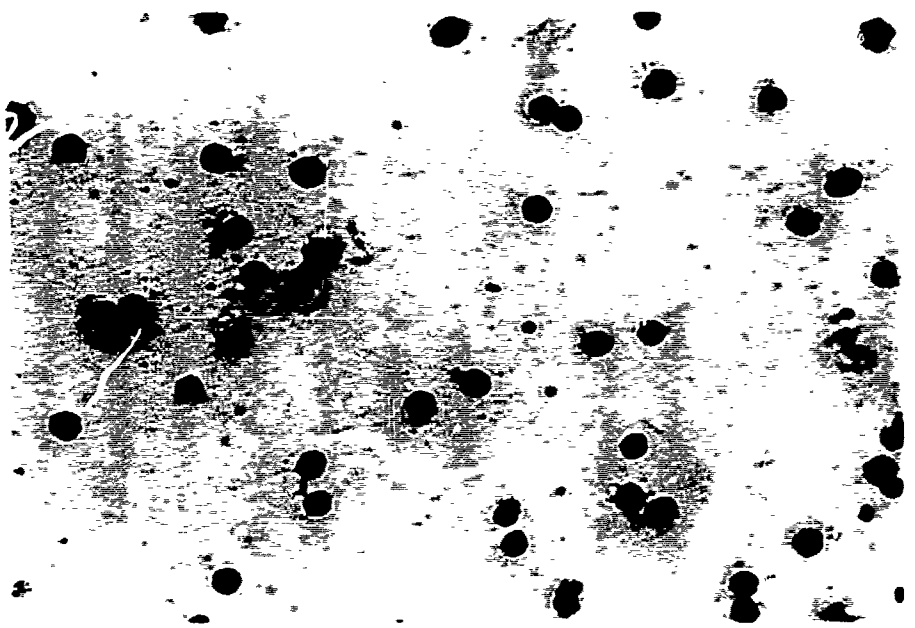


Figure 166: Rat exudative peritoneal macrophages after intraperitoneal injection of liquid paraffin. Note that about half in this field are stained for catalase. Same method as fig. 165. x 450.

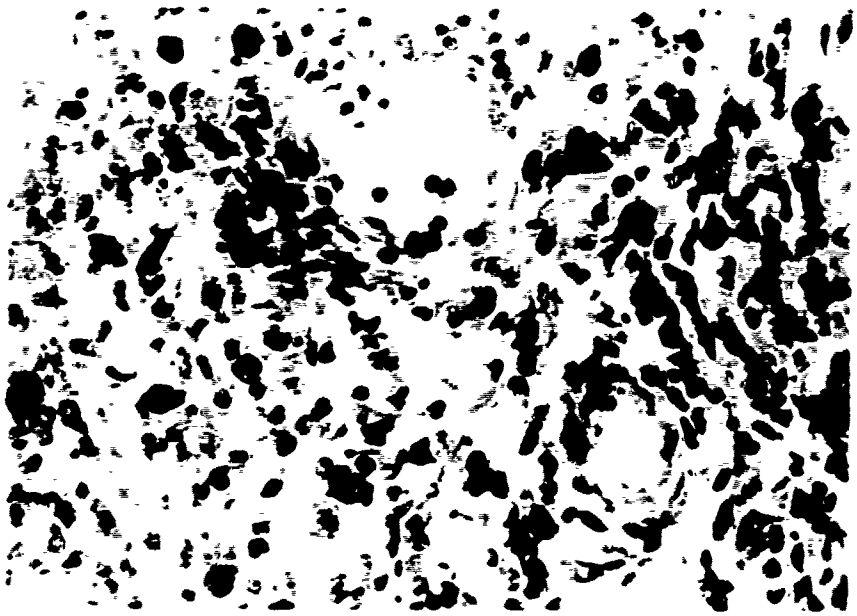


Figure 167: Catalase-positive macrophages around a subcutaneous implant of cholesterol oleate in the rat. Same method as fig. 165, x 400.

Figure 168: No catalase reaction in intimal cells in early rabbit aortic atheroma (4 weeks on 1% cholesterol diet). Same method as fig. 165, x 400.

work on isoenzyme studies that the lipophage smooth muscle is derived from a single cell or a single clone of cells. The Benditts suggested that this might indicate some change in the nature of the DNA of the cell induced by a chemical mutagen or virus. However, an alternative and simple explanation could be that only one or two smooth muscle cells enter the intima from the media and the intimal muscle cells are the product of the proliferation of that single cell.

I would now like to consider how quickly lipid can be resorbed from lesions. In the rabbit a cholesterol-induced fatty liver resolves in about two months (4, 148). When lipids are implanted under the skin they are cleared fairly rapidly. A total extract of atheroma lipids injected under the skin is cleared by macrophages in about nine weeks in the rat and 6-12 weeks in the rabbit (5). On the other hand, atheromatous lesions in the rabbit are at best slow to resolve. We found that aortic cholesterol in the rabbit goes up to 152% after one year's regression following a 3 month period of cholesterol feeding (4). The Rhesus monkey may partly resorb its coronary atheroma during 18 - 40 months of regression, but it seems at best to be a slow affair (14). I would like to impress on you that 10 mg. of lipid in a rabbit atheromatous lesion stays there for a year, but put the same amount of the same lipids under the skin and it goes relatively quickly.

RABBIT
RAT
RABBIT
MONKEY

One must conclude that the arterial wall intimal cells are very inefficient at lipid removal when compared with the macrophage. A number of possibilities could explain this difference. The first possibility is that smooth muscle has little or no phagocytic capacity. The second possibility is that arterial wall smooth-muscle may have a relatively low proliferative capacity and cannot keep up with the lipid that is being deposited in the tissue. Hence, extracellular lipid is allowed to accumulate in a quantity which is not seen where macrophages are present. The third possibility is that the smooth muscle cell is programmed to stay in the tissue and is not programmed to travel back to RE organs in the way that one would expect an RE macrophage to behave. In another context, the Schwann cell is an important phagocyte in the degenerating nerve; it seems to remain in situ and reuses the breaking down myelin lipids to make new myelin (268). The fourth possibility is that the endothelial barrier of an artery may be relatively resistant to the passage of macrophages. The major route for the macrophage to enter the tissues, as in inflammatory conditions, is more likely to be through the capillary and venular wall. Bjorkerud and Bondjers (39) showed that monocytes enter the aortic wall when the endothelium has been damaged, but against this Poole and Florey found macrophages traversing the apparently intact endothelium of the atheromatous aorta (261). A final consideration is that perineurium - analagous to an epithelium - around the peri-

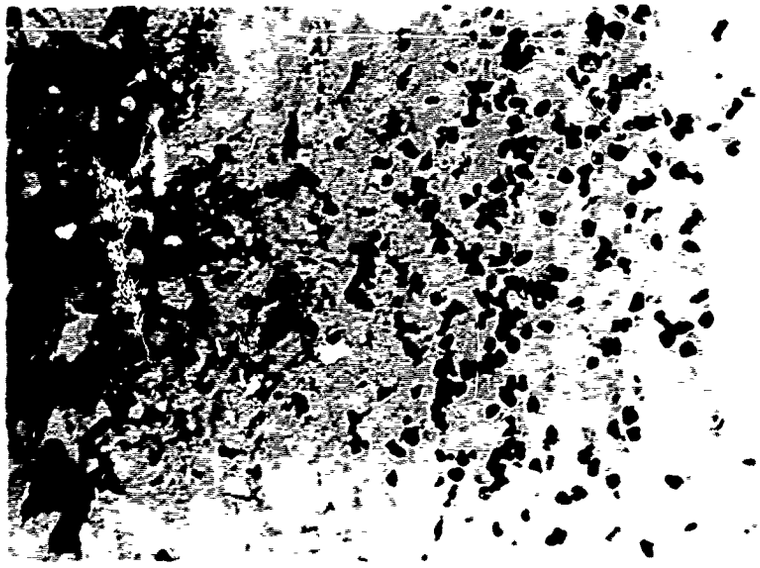


Figure 169: As for fig. 168, but aortic fatty streak in a woman aged 48, x 400.

Figure 170: Catalase-positive cells presumably macrophages - on inner aspect of endothelium of atheromatous rabbit aorta (12 weeks on 1% cholesterol diet) Frozen Hautchen preparation, same stains as fig. 165, x 400.

pheral nerve keeps macrophages out until the nerve is cut, and then the macrophages enter from the point of trauma where the perineurium has been breeched (28). Which of the various possibilities, if any, is responsible for the inefficient lipid handling in mature atherosclerotic lesions is uncertain. Nevertheless, some aspect of cellular lipid removal must be defective to allow so much extracellular lipid to accumulate.

DR. POOLE: Did you imply that none of the cells in rabbit atheromatous lesions are macrophages? If so, I think you should examine Hautchen preparations from these lesions, where nuclear morphology clearly identifies cells traversing the endothelium as macrophages (261). RABBIT

DR. ADAMS: No, I mean that the main accumulation of cells is not of reticulo-endothelial origin. I admit, however, that the endothelium is usually lost from arteries incubated in histochemical media. The suggestion to apply the catalase reaction to Hautchen preparations of endothelium is a very good idea.

Subsequent work has shown, in accord with Poole and Florey (261) a moderate number of catalase-rich cells (presumably macrophages) on both sides of Hautchen preparations of endothelium from atheromatous rabbit aorta (Fig.170). The presence of macrophages in endothelial preparations and their absence from the main bulk of intimal cells is not inconsistent with any of the foregoing comments, but it does leave unanswered the problem why the macrophage does not penetrate into the main mass of the lesion.

DR. HAUST: Dr. Poole indicated that more valid distinction (than by the above demonstration of catalase) is made between the macrophages, smooth muscle cells and endothelial cells by light microscopy on the basis of the nuclear shape. It became obvious in the past decade that this does not hold true, as by electron microscopy, many fat containing cells with rounded nuclei that otherwise were indistinguishable from those of macrophages on light microscopy, proved by other criteria to be smooth muscle cells (19, 114, 144).

I should like to conclude with two statements: Dr. Adams did not exclude in his presentation the possibility that macrophages were present in the lesions, but on the basis of his data stated objectively that the cells in the lesions are not catalase positive (which may imply they a priori do not belong to the catalase-positive cell type, or that they have lost this once-possessed characteristic). Secondly, one has to be quite careful in identifying any one cell type, particularly under pathological conditions, on the basis of the shape of the nucleus and on light microscopic examination alone.

EDITORIAL COMMENT

The discussion of Benditt's work focused on a paper (25) in which evidence for a monoclonal origin of human atherosclerotic plaques was presented. In the uninvolved portion of arteries both the A and B isoenzymes of glucose-6-phosphate dehydrogenase were found. In the fibrous plaques, however, only one or the other isoenzyme was present. The workers inferred therefrom a monoclonal origin of the cells in the plaque area.

Figure 171: Electron micrograph taken from a section of the thoracic aorta of a 19 year old black man who died as a result of an accident. It contains a cell which is clearly recognizable as a smooth muscle cell with lipid inclusion (SMC). There is another cell that is probably a monocyte or a macrophage without any lipid (M). The third cell (FC), a lipid laden foam cell, has none of the distinguishing features of the smooth smooth muscle cell.

DR. POOLE: I do not suggest that cells can be identified on the basis of nuclear morphology alone. However, the study of nuclear morphology does enable one to say with confidence that in the intimal lesions in the aorta of rabbits fed cholesterol three distinct types of cell contain fat droplets. Other evidence enables one to identify these three types as endothelial cells, macrophages and smooth muscle cells. RABBIT

DR. STRONG: I have some comments which are pertinent to the preceding discussion and which have a bearing on Dr. Benditt's theory. It seems to me that there are two main types of lipid-filled cells in human atherosclerotic lesions and in many experimental atherosclerotic lesions, certainly in the Rhesus monkey. There is no doubt at all that lipid-containing smooth muscle cells are present in human and experimental lesions. Fig.171 is an electron micrograph of a human aortic fatty streak from a 19 year old male. It contains one cell which is clearly recognizable as a smooth muscle cell with lipid inclusions. There is another cell that is probably a monocyte or macrophage without any lipid. And then there is another lipid-containing cell which has none of the distinguishing features of smooth muscle cells. This cell can be referred to as a "foam cell" and I believe that it is a macrophage or monocyte filled with phagocytosed lipid. Fig.171 A shows a lipid containing smooth muscle cell from the same fatty streak, and Fig.172 shows another foam cell from this lesion. Fig.173 demonstrates a lesion in a Rhesus monkey which has been fed a high cholesterol diet for twelve weeks. It shows an endothelial cell with no lipid, foam cells (which I think are macrophages filled with lipid), and a cell recognizable as a smooth muscle cell which also contains lipid droplets. Theories of pathogenesis must take the macrophage into account or show that the foam cells are really not macrophages at all but are unrecognizable smooth muscle cells. HUMAN MONKEY

Dr. Herbert Stary recently presented a paper in West Berlin giving his findings on the proliferation of arterial cells in response to cholesterol feeding in the Rhesus monkey, and he separated the cell types in accordance with those that I have just described. Using tritiated thymidine, he showed that there was no proliferation of the endothelial cells in response to feeding a high cholesterol diet. There was proliferation of intimal smooth muscle cells. There was also proliferation of foam cells. And there was proliferation of the medial smooth muscle cells. The intimal smooth muscle cells were proliferating at a faster rate than the medial smooth muscle cells. When groups of animals given the high cholesterol diet for twelve weeks were then returned to a normal diet for sixteen weeks there was a reversal of this increased proliferative activity. In an additional group of monkeys which were fed the high cholesterol diet for twelve weeks and then a normal diet for forty weeks, cell proliferation in all of these

Figure 171 A: Electron micrograph from a human thoracic aorta showing typical smooth muscle cell (SMC) containing many lipid droplets. Note the basement membrane, pinocytotic vesicles and myofilaments in the smooth muscle cell. In the upper right side of micrograph is a portion of an endothelial cell (E).

Figure 172: This micrograph shows a lipid-laden foam cell (FC) from a portion of the thoracic aorta of a human. Some of the lipid droplets have been dissolved out in the processing. Note numerous pseudopods along cell membranes.

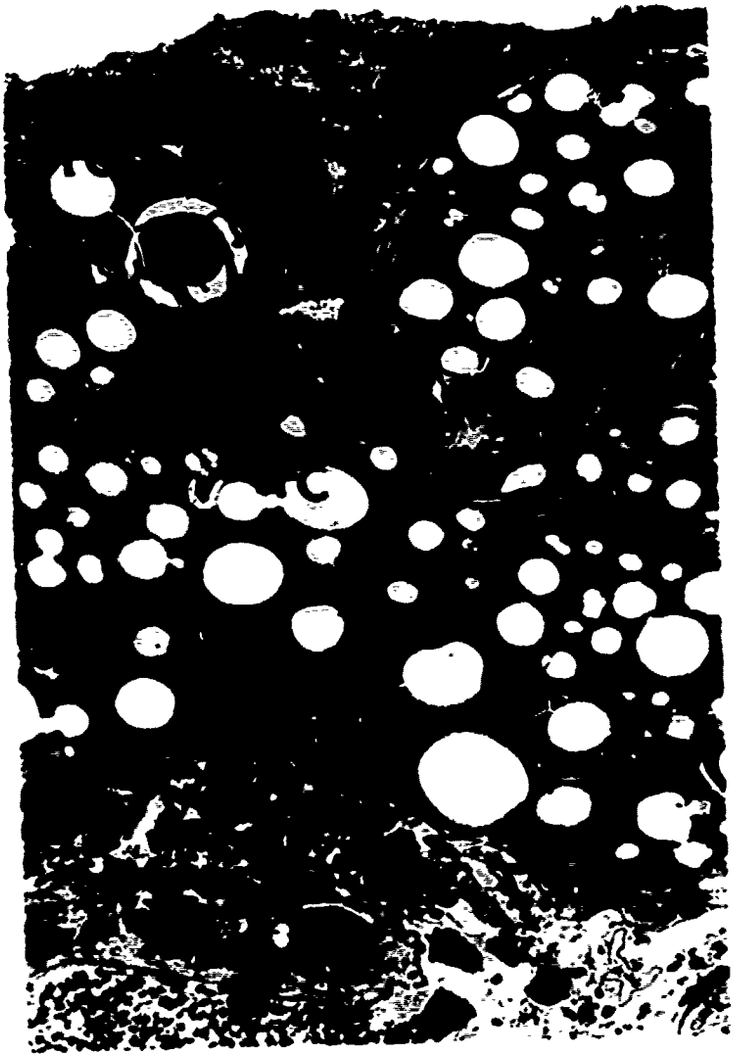


Figure 173: Electron micrograph showing a portion of aortic intima of a rhesus monkey fed a high cholesterol diet for 12 weeks. Adjacent to the endothelial cell (E) are three lipid-laden foam cells (FC). The pseudopods of these cells interdigitate to form a layered appearance. A portion of a smooth muscle cell (SMC) containing lipid droplets is seen in the lower section of the micrograph.

cell types was back to baseline levels.

I have a question for the audience. Obviously, the smooth muscle cell must be very important because of all the things it can synthesize, namely, collagen, elastin and GAG (glycosaminoglycans). Could macrophages under certain conditions manufacture these same substances?

DR. ROBERT: A very short answer. They can make GAG but none of the other macromolecules as far as I know. Tiny small amounts of collagen biosynthesis by hydroxyproline incorporation is demonstrable in any cell types. Theoretically any cell type can make any macromolecule. The question is how much will it make effectively in a given condition?

DR. LINDNER: I think actually macrophages can synthesize some polysaccharides and sulphated glycosaminoglycans, especially if there is phagocytosis of some material and then therefore, for example, hemosiderin or something inside, then we have a so-called Tragerstoff, or carrier substance composed histochemically of neutral and acid mucopolysaccharide-protein and lipoprotein molecules, a material which the macrophages cannot break down.

DR. HOFF: Fine-structural changes in the morphology of arterial intimal smooth muscle cells have been a common observation in animals following experimentally induced intimal thickening and atherosclerosis (149). Some of these alterations are considered to represent cell dedifferentiation (338). Wissler (424) has described these cells as multipotential mesenchymal cells. In the course of a study of human atherosclerotic lesions obtained within three hours post mortem, we have observed various ultra-structural alterations of smooth muscle cells in diffuse intimal thickening, fatty streaks, and fibro-muscular caps of fibrous plaques. One of the more subtle and perhaps earliest changes is a disorientation of myofibrils and a widening of the space between individual fibrils (Fig.174), possibly the result of local edema. A more characteristic alteration is the loss of myofibrils in the central portion of the cell and the presence in this area of abundant rough surfaced endoplasmic reticulum, mitochondria and Golgi zones (Figs.175, 176).

Human Modified Arterial Smooth Muscle Cells

These changes have been described in smooth muscle cells of experimental animals following mechanical injury (233), transplantation (18), diet-induced atherosclerosis (389), and cells grown in tissue culture (315). The basement membrane surrounding such cells is often discontinuous, swollen and frequently appears to be layered (Fig.175). Centrioles, suggesting subsequent mitosis, are seen in these cells with only minimal presence of myofibrils at

Figure 174: Electron micrograph of a modified smooth muscle cell in the fibromuscular cap of a human iliac artery fibrous plaque. Note the lack of orientation and spreading out of myofibrils in the cell's center, in contrast to a more oriented alignment of myofibrils on the cell's periphery. Foci of increased electron density can be seen in the areas of oriented myofibrils both at the cell membrane and within the myofibrils. Cisternae of endoplasmic reticulum and mitochondria are swollen, possibly the result of post-mortem changes. Basement membrane material surrounds part of the cell, and there are patches of material with similar structure and density close to the cell surface (arrow). x 12,800.

Figure 175: Electron micrograph of a longitudinal section of a modified smooth muscle cell in the fibromuscular cap of a human iliac artery fibrous plaque. Only remnants of oriented myofibrils can be seen at the cell's periphery. The remainder of this cell's cytoplasm contains rough surfaced endoplasmic reticulum, pinocytotic vesicles, some microtubules, and swollen mitochondria (possibly representing post-mortem changes). The basement membrane is present but discontinuous. Note the presence of multiple layers of basement membrane-like material running parallel to the cell surface. × 15,700.

Figure 176 Electron micrograph of a modified smooth muscle cell in the fibromuscular cap of a human iliac artery fibrous plaque. This cell is similar to that in Fig. 175 but cut in cross-section. Again, note the abundant rough surfaced endoplasmic reticulum, mitochondria, Golgi zones, dense bodies. Myofibrils cut in cross-section can be seen sparingly at the cell's periphery. Note the lack of a distinct basement membrane but the presence of some amorphous material surrounding most of the cell. This cell comes into close contact with a neighboring cell over a narrow span of cytoplasm (arrow). x 31,500

the cell's periphery (Fig.177). A common finding in smooth muscle cells of fatty streaks and fibromuscular caps of fibrous plaques is the appearance of vacuoles containing material of variable densities suggestive of secondary lysosomes (Fig.178). Some elongated cells considered to be smooth muscle, are almost entirely filled with such vacuoles (Fig.179). Such lysosomal bodies have been observed in smooth muscle cells in experimental atherosclerosis (247, 338) and in human atherosclerotic lesions (114, 141, 210). Ultra-centrifugation studies by Peters et al (254) have demonstrated two types of lysosomes in smooth muscle cells, a high density fraction containing high activities of hydrolytic enzymes and a lower density fraction also containing large amounts of cholesterol. It is conceivable that these observed electron dense vacuoles in the modified smooth muscle cells in human atherosclerotic lesions may represent both lysosomal fractions. Although the precise role of lysosomes in the atherosclerotic process is still unclear, it has been suggested that these organelles normally function as a disposal system for imbibed plasma constituents (254). Since arteries with lower acid hydrolase activity are more prone to atherosclerosis than arteries with high levels (221), it is possible that the secondary lysosomal bodies within smooth muscle cells of atherosclerotic lesions may represent incomplete digestion of engulfed material, in particular, lipid.

DR. McGILL: Dr. Benditt's concept would explain many puzzling things about atherosclerosis. Among them is the focal nature of a lesion. In dealing with hyperlipidemia, the strongest risk factor for clinical disease and for some types of lesions, we have always been puzzled by the fact that although the same blood bathes all the arteries, the lesions are focal. The concept that mosaicism exists in the smooth muscle cells of the arterial wall, or the concept that transformation takes place in an individual cell would, if true, explain the focal nature of the lesions. It might explain the focal nature of the juvenile fatty streaks which seem to represent lipid accumulation in smooth muscle cells in a particular period of life. It may explain the peculiar proliferative reaction that occurs in some fatty streaks under certain conditions and leads to fibrous plaques.

The Monoclonal Theory

DR. ROBERTSON: Regarding Benditt's observation on monoclonal cell populations, it may be worthwhile to briefly review tissue culture studies done in our laboratory utilizing morphologically identical populations of arterial intimal cells obtained from donors of the same sex and age with or without histological or gross atherosclerosis elsewhere (305). Cells from atheromatous arteries showed a striking increase in the rate of intracellular incorporation of tritiated homologous low density and very low density lipoproteins over similar cells from disease-free vessels. This seems

Figure 177: An electron micrograph of a modified smooth muscle cell in the thickened intima of a human abdominal aorta. Note the presence of two centrioles one in cross- the other in longitudinal section suggesting subsequent cell mitosis. Rough surfaced endoplasmic reticulum, mitochondria, and Golgi zones can be readily seen. A vacuole (large arrow) surrounded by two membranes contains cell debris and may represent an autophagic vacuole. A discontinuous basement membrane surrounds part of this cell. Note the close apposition of the cell membrane to that of a neighboring smooth muscle cell. x 19,000

Figure 178: Electron micrograph of modified smooth muscle cells from a human aortic fatty streak lesion. The upper cell has numerous myofibrils seen in cross-section. A group of swollen mitochondria, degradation products, and a lipid droplet are also present in the cytoplasm. The cell is surrounded by a basement membrane which is extraordinarily thick, particularly in the area adjacent to another cell. The lower cell, although surrounded in part by a basement membrane, shows almost no myofibrils. Swollen mitochondria, lipid droplets and secondary lysosomes (filled with different material of variable densities) can be seen in the cytoplasm. x 11 700.

Figure 179: An electron micrograph of an elongated cell presumed to be derived from smooth muscle in a human aortic fatty streak lesion. Note the numerous bodies with variable electron densities believed to be secondary lysosomes. Some rough surfaced endoplasmic reticulum and mitochondria are swollen. A basement membrane is absent around this cell. x 13,500.

to be a phenotypically expressed metabolic abnormality that is present in these cells even after several in vitro generations (Fig.180) Our data rules out neither spontaneous or viral induced transformation of human arterial cells occurring after birth as suggested by Benditt's results or a genotypically transmitted familial disorder expressed late in life. Experiments in progress, studying offsprings of patients with severe symptomatic atheroma, will attempt to answer this important question.

Xanthoma versus Atheroma

Regarding Dr. Colin Adams comments about xanthomatous cells, I would like to point out that we have carried out comparative measurements with cells from xanthomatous and normal skin as well as from the arterial wall in the same patient (306). We found that while both cell types incorporated autologous or homologous whole serum and particularly low density lipoprotein fractions very rapidly in vitro, only xanthoma cells showed morphological and quantitative evidence of regression of intracellular sterol deposits after short incubation in normolipemic sera. The arterial intima-cytes remained lipid-laden with eventual transformation into typical atherocytes or "foam" cells. These findings suggest that clinical reversibility of xanthomatous lesions may not be an accurate index of similar changes taking place in arterial atheromatous lesions.

DR. FISHER-DZOGA: I can't contribute any thing to this as far as the in vivo situation is concerned but we have some experience with primary cultures of mainly monkey and rabbit cells. We did grow out primary cultures and we convinced ourselves that they are smooth muscle cells in that they react positively with anti-actomyosin. Then we studied proliferation and I must say that proliferation is an increase or a second spurt of proliferation after these cultures have reach a stationary phase. They are six to eight weeks old when we use them for these proliferation studies, and they do accumulate lipids. In normal cultures we find anywhere between one to about 10% lipid incorporation, and it jumps up with the hyperlipemic serum, it jumps up to 36%. Normal LDL does not do anything. Hyperlipemic LDL gives a tremendous response. Not the normal concentrated LDL which was given in twice the concentration of this, so that we could compare it with diluted LDL.

MONKEY
RABBIT

DR. WERTHESSEN: Would you then conclude that you had a mixed population?

DR. FISHER-DZOGA: I should have mentioned that too. These are all cells from normal aorta and their response to hyperlipemic serum seems to be proliferation but it is spotty in each culture, and as I think Dr. Russell Ross mentioned, in his cultures he finds a homogeneous disposition.

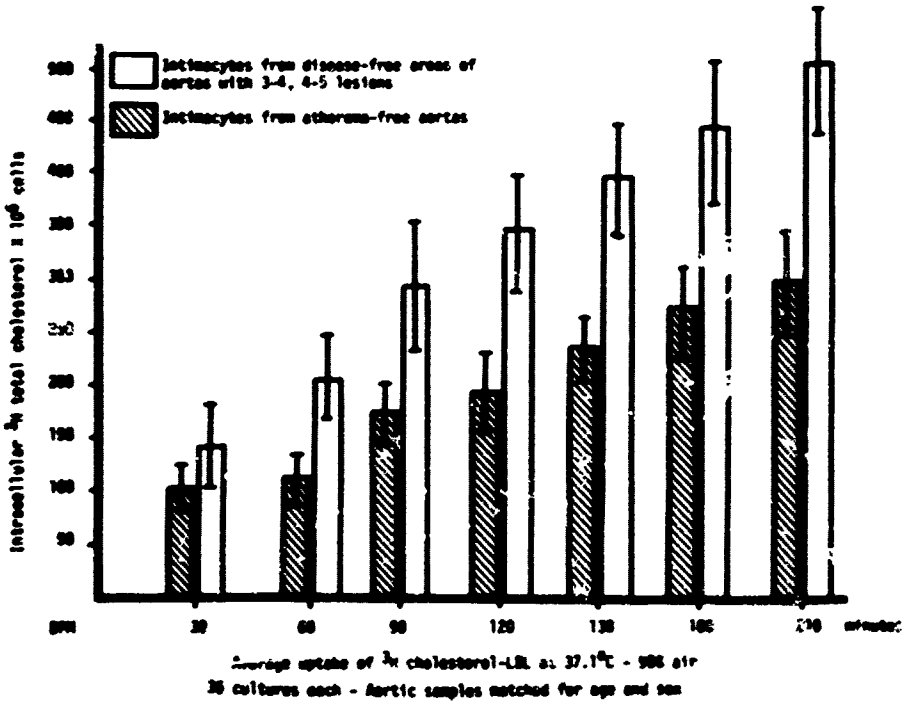


Figure 100: Summary of difference found in rate of tritiated low density lipoprotein cholesterol by human aortic intimal cells in short-term culture. The shadow bars indicate average uptake rates of intimal cells from atheroma-free aortas and the clear bars the rate of uptake by similar cells from disease-free areas of aortas with severe atherosclerosis elsewhere. The study was carried out with autopsy material from 76 male donors, matched for age, without clinical history of hyperlipemia, diabetes or hypertension.

DR. ROBERT: I would like to offer some speculations concerning the possibility of such a clonal mechanism. In order to have "clones" in lesions you must have several types of cells originally in the normal aorta. Here is one possibility, and this can be tied now to experiment we heard or read about. What we have to consider as a possibility for "clonal selection" is something which would exert a chemical or immunological "pressure" on cells to selectively proliferate. Such a mechanism could be for instance, that some of the smooth muscle cells do have receptors for some specific antibodies or autoantibodies and the others would not have receptors for these antibodies. This would originally be a very good difference between these two types or several types of cell populations. Fig.181 shows the finding that started all our speculations and experimental work on the immunological theory of arteriosclerosis. We screened several hundred so-called normal and arteriosclerotic people and all of them had low but reproducible amounts of anti-elastin antibodies. Those which really had strong lesions were in the lower titer class. Now these antibodies occur from age 20 and they disappear or get very low after age 70 only (Fig.182). The presence of these antibodies started a new experimental approach to the immunological theory of arteriosclerosis (294, 295). One problem was to explain how these antibodies really appear in the circulation and that is where we had to resort to a choice between an "instructive" or a "forbidden clone-type" of theory. Whatever the mechanism of production of these antibodies, we may assume, that some cells respond differently than others. Some cells, for example, which do have receptors could be repressed and the others could proliferate selectively.

The Monoclonal Theory & Antigenic Mechanisms

There are at least two possible mechanisms to explain how these antibodies are produced. One explanation could be that we have in the arterial wall an active degradation process producing the antigens continuously. At that time (294) we had to assume the presence of an elastase distinct from a pancreatic elastase. The best candidates were the platelets. And, as a matter of fact, we could isolate and purify a platelet proelastase which is activated to an elastase (196, 277). This enzyme could really responsible for the degradation of elastic tissues. But, as the "thrombogenic theory" was already not too popular at that time and as we had very good friends in the platelet field, we had to find another explanation. And this explanation, now that I see the work of Drs. Bjorkerud and Minick, starts to make sense to me. Because really what we admitted at that time (278) was that the platelet would come, adhere to a "site" of the intima, inject its enzymes and go away. A "hit and run" theory in a way. In order to assign an importance to such a mechanism

Possible Role of Platelets

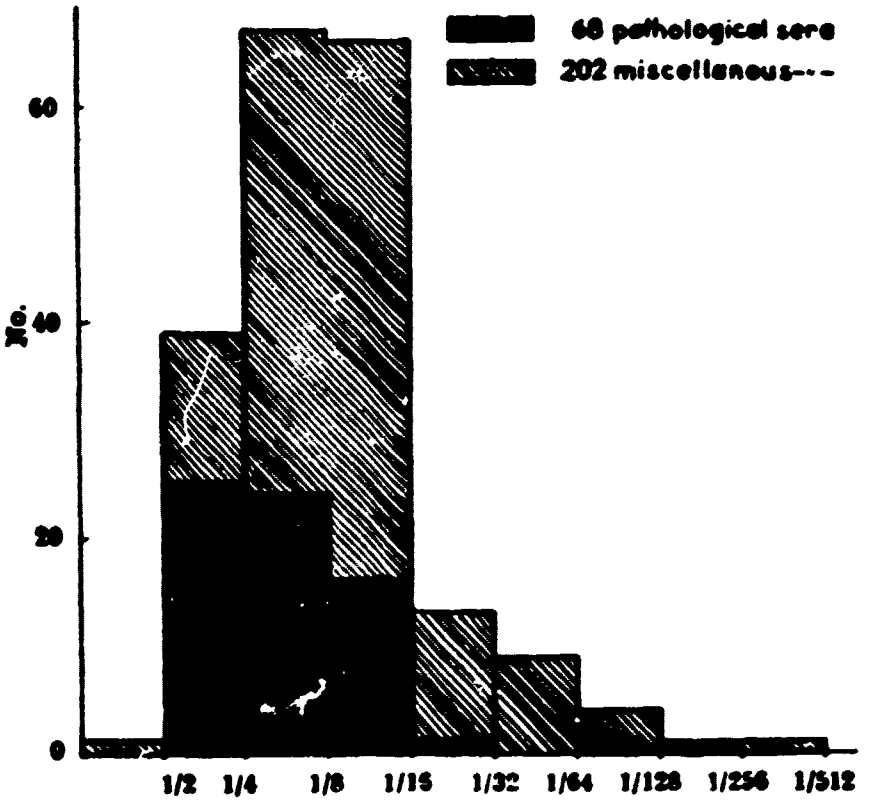


Figure 181: Anti-elastin antibodies in serum of atherosclerotic subjects compared to miscellaneous population.

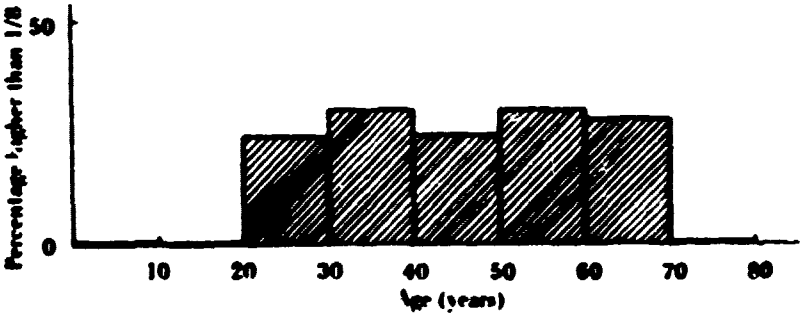


Figure 182: Relationship of anti-elastin antibody titre to age.

it should have a certain frequency. My question to Drs. Bjorkerud and Minick was whether it seems possible or not after the experiments that they have done to make a quantitative estimation of the frequency with which such an event could occur from age zero to age X. If this frequency would be high enough, then naturally this platelet elastase could contribute significantly to the degradation of elastin. The peptides produced could not be recognized as "self" and then the lymphocytes could produce antibodies which would react with those cells synthesizing elastin and produce a selective depression (or a selective proliferation) of certain cell types.

More recently we had to consider a different theory for reasons I really do not have time to go into. And that was really started by our aging studies (281). This other possibility is the "derepression" of "forbidden clones". Would "aging" produce antibodies toward "self" type of constituents such as elastin, and then these antibodies be adsorbed to the elastic fiber or to other constituents (mainly to cell constituents) and trigger the above mentioned selective proliferative mechanism. What makes it important to speculate along different lines is that we could never find a proportionality between the antigenicity of the aorta constituents and the frequency of lesions we could induce in rabbits. For instance, elastin which is a miserable antigen (it gives a titre of 1/8 to 1/1000 maximum by passive hemagglutination), induces nearly 100% lesions. The structural glycoprotein fraction of aorta which is a relatively good antigen can give passive hemagglutination titres up to 10,000 but induced only about 30% of lesions if we injected it with Freund's adjuvant into rabbits. And the 1M $CaCl_2$-soluble protein fraction of aorta which has at least twenty different constituents (by double diffusion) gives titres of up to 10^{-5} at least but gives maximally about 20% lesions (276). So we have an inverse relationship between immunogenicity and the frequency of the lesions produced. Therefore, the best explanation we could offer is a metabolic effect on some smooth muscle cells exhibiting a selective sensitivity to some types of antibodies such, for instance, as the anti-elastin antibodies.

DR. SINAPIUS: The most important layer and origin of smooth muscle cells of the vessel wall is the tunica media. Function and disturbance of function of these smooth muscle cells are of great interest. In his paper delivered at the Chicago Symposium in 1969 (6), Adams has pointed out that the accumulation of lipids (mainly cholesterol and cholesterol esters) in the intima depends on a disturbance of removal from the vessel wall through the media. According to Dr. Adams' observations qualitative and quantitative histo-

Peculiarity of Coronary Arteries

chemical evidence shows that enzymes concerned with arterial aerobic energy production decrease in the middle and inner-middle parts of the human tunica media with advancing age. In a previous publication (8) Adams has pointed to the support for this theory by the observation that lipid "is deposited in the inner, but not in the outer layers of the tunica media in atherosclerotic human arteries i.e., on the inner side of the mid-medial barrier."

This may be right in the aorta, which was investigated by Adams. On the contrary in the coronary arteries the deposition of lipids is not limited to the inner layers of the media, but extends very often throughout its whole thickness (Fig.183). In addition, Brundel as a member of our team has shown (50) that in the coronaries there is really no difference of enzyme activity in the layers of the normal tunica media.

Following these observations there seems to be no proven basis for a general theory in the sense of Adams', though he is surely right in the assumption that lack of removal of lipids is a very important mechanism of lipid accumulation in the intima.

I suppose that the removal of lipids in the coronaries and in other arteries of the muscular type depends on other conditions, perhaps on the elasticity of the vessel wall. By this way it would be possible to partly explain decreasing removal with advancing age.

DR. ADAMS: I think this is a very interesting paper that Professor Sinapius has read. I have only got a few comments to make. A particular factor is only one among many in atherogenesis, and I only put hypoxia as one among a number of factors. It is well established that in the human aorta that the middle and inner media get hypoxic (1, 429). This has been confirmed recently in the inner media of rabbit aorta by micro-electrode studies (240). The human coronary artery seems to develop a lesser degree of hypoxia (8), and, of course, this would be consistent with what Sinapius has been saying. I have one further comment to make which is in line with what Tibor Zemplenyi was showing yesterday. The packing of a cell with fat - such as we saw in some of Sinapius' pictures - is, of course a characteristic of hypoxic cells, although histological appearances do not necessarily prove that it is hypoxia.

The Consequences of Hypoxia

HUMAN

RABBIT

DR. GROSCHEL-STEWART: One of our medical students (333) found in blood samples of patients with thrombotic disease of the arteries (taken pre- and post-operatively) an antigen, that reacted with our antibody against smooth muscle actomyosin. We can only speculate about the origin of this antigen. It may come from

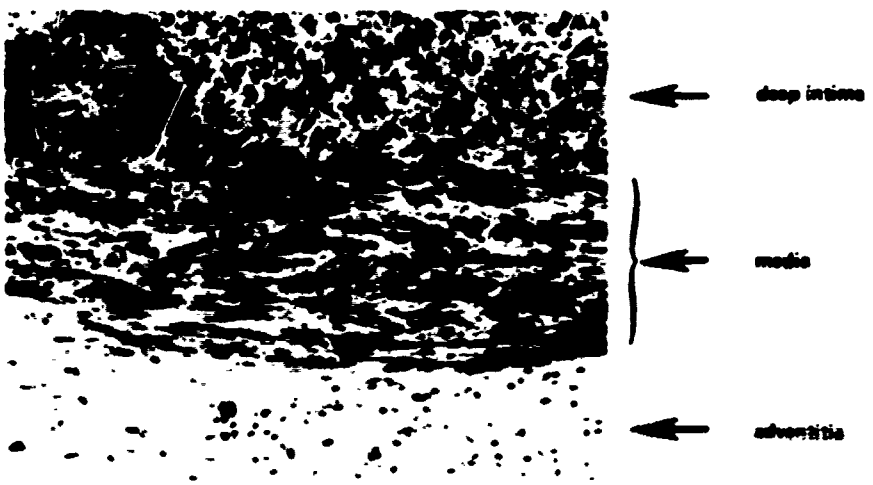


Figure 183: Intracellular lipid deposition in smooth muscle cells of the media, extending throughout the whole thickness of the media. Similar intracellular lipid in the adjacent deep intima (upper third of the figure). Feltrot and Mayer's haematum; x 270.

severed endothelial cells or thrombocytes, from the plaques described by Drs. Becker and Knieriem, or from the vascular smooth muscle cell itself. We do not know if these patients eventually formed auto-antibodies against these circulating antigen .

DR. KNIERIEM: I just want to make a comment on Dr. Benditt's paper (25) from the view of a general pathologist. This work should be repeated by other groups. I think will certainly be done. If the disease process of atherosclerosis would be really only focal, it might be true. But when we are studying atherosclerotic lesions in the human aorta as well as in the coronary arteries and measure them continuously on serial sections (110, 186, 217) we find the plaque-like lesions embedded within diffuse intimal thickenings which are progressing with age. By measuring human aortas and coronary arteries we could prove that diffuse thickening of the intima precedes later plaque formation. On the other hand, Dr. Benditt could not prove that diffuse intimal thickening is just caused by one clone of cells. Therefore, using common sense I don't believe that Benditt's theory will fit for all atherosclerotic processes.

DR. LEE: I am leaving for a moment Dr. Wissler's problem and am picking up for a moment Dr. Benditt's problem. Someone mentioned earlier that because of the diffuseness of the arteriosclerotic lesion, it is difficult to imagine that a monoclonal or single cell could produce such large diffuse lesions. I don't think Dr. Benditt claimed that the diffuse lesion is from a single cell. If I remember correctly there could be five or six different focal regions, each focal region being either A or B type, and when they coalesce later, you would probably see a mixture of A and B types. But in any case, we are interested enough in this problem so that I have arranged to get aortas from black females from Howard University in Washington, D.C., and also aortas from Nigeria, Africa - black females there in whom advanced lesions usually do not develop. For some reason their atherosclerotic lesions usually stop at a certain level and do not go on to the occlusive or infarct-producing lesions. We should be able to confirm or disprove this theory in the near future.

Now, from another standpoint, it is important to know, or at least interesting to know, whether the cells in the lesion are coming from the in situ intimal cells proliferating in that particular location or coming by migration from the media. Dr. Adams referred to this briefly a little while ago. It is not known at the moment. We are planning to do an experiment to answer this question, at least in experimental animals. We will label the fraction of arterial cells in the animal in vivo with ^{3}H thymidine with a pulse at the outset of the experiment. We are using swine as you know. We would wait for two or three months for a certain

number of lesions to develop in the aortas of these swine. After three months or so when we are certain the lesions are present we sacrifice the animal and autoradiographs of the aorta will be made. In the past, we designed a method to ascertain how many generations these cells had gone through by counting the number of grains. I will not go into details of this method, but if we study the labelled cells in the media we should be able to tell how many divisions these cells have gone through. If most of the cells in the lesion moved up from the media their number of grains or their generation should be similar to the cells in the media. But as Dr. Benditt says, if the cells within the lesion multiply many times to produce a lesion, then the number of grains should be totally different from those seen in the media. When we complete this experiment we should be able to know a little more about the movement of cells, and the location of proliferation.

DR. SCHWARTZ: I would like to ask a question relating to the concept of mono-clonal growth in this situation and address it to the group as a whole. Specifically, under what conditions, or with what techniques can one establish the mono-clonal nature of a cell population? Is indeed the demonstration of an isozyme an adequate demonstration of the mono-clonal nature of the cells? What are the statistics involved in mono-clonal growth? Does this imply that the cells are transformed and perhaps might behave like smooth muscle tumor cells? Does this concept have any implications in regard to the plating efficiency of cells in culture and the number of passages that might be obtained in tissue culture. I believe we need to know more about the definitive demonstration of mono-clonality in cell populations before one can accept this hypothesis as it stands at the moment.

Criteria for Mono-Clonal Origin

DR. ZEMPLENYI: I do not think that from the isoenzyme pattern only, without genetic considerations, one could answer these questions. The problems involved are more of a genetic than enzymologic nature. The fact that the gene for glucose-6-phosphate dehydrogenase is located on the X chromosome, and that a small proportion of black women are heterozygotes for the A and B isoenzymes, was very successfully utilized for the demonstration of the monoclonal origin of some uterine tumors and chronic granulocytic leukemia. Benditt's demonstration of the monoclonal origin of human atherosclerotic plaques is extremely interesting and provocative but is based on findings in three women only and evidently more data are needed. HUMAN

Additionally, I would like to underline the remark made by Dr. McGill, namely that in all consideration of the genesis of atherosclerosis one has to pay attention to the focal distribution of

the lesions and search for the causes of this distribution. Hemorrheologic factors play without doubt a great role in this regard and two participants of this symposium, Dr. Werthessen and Dr. Rodbard, were among the first investigators studying some of these factors. Recently an excellent mathematician and a physicist from the Jet Propulsion Laboratory joined the efforts of our group, lead by Dr. Blankenhorn, to elucidate some of the related factors. Dr. Lloyd Back from the JPL carried out a theoretical analysis of molecular oxygen and lipoprotein transport from blood to arterial wall. It was based upon conservation principles of diffusion and fluid dynamics for steady boundary layer laminar flow in arteries with irregular walls. In regions of decelerated flow and flow separation the analysis predicts decreased oxygen transport whereas in identical regions an increased lipoprotein deposition is predicted. Furthermore, a similar analysis dealing with the fate of platelets was also performed. In this connection we have to remember that platelets can contribute to the focal distribution of atherosclerosis because it was repeatedly demonstrated that they can damage the artery. One of the damaging agents could be elastase which was shown by Barbara Robert and coworkers to be contained in platelets (277).

My second comment or rather question deals with the previous discussion of Colin Adams in connection with the reticulo-endothelial system. It was shown by several investigators, especially by Jansco, that histamine is an activator of the RES (162). As far as arteries are concerned, the mast cells are the only source for histamine and they appear to have a focal distribution. Is it not possible that the absorption of lipids - which is according to Adams not the same in arteries as in other places - may be connected with the focal availability of histamine?

DR. BJORKERUD: I would like to make a comment to Dr. Robert's suggestion here. There is a relationship in this experimental model system between the absence of endothelium and, as it seems, a deficiency for the formation of elastic fibers (36). In non-atherosclerotic lesions induced with mechanical trauma well-developed elastic fibers and lamina are formed in the intimal thickening overlying the injured site. In the atherosclerotic type of lesions there are no large mature elastic fibers or lamellae but only small fragments of elastic material. We have been inclined to think that this may reflect a poor metabolic condition of the smooth muscle cells in the atherosclerotic tissue which could prevent the formation of mature large elastic fibers or, alternatively, that the conditions in the extracellular space of the atherosclerotic tissue prevented elastic fiber formation (185).

Deficiency of Elastin Formation in Atherosclerotic Sites

RABBIT

However, it is possible that increased breakdown of elastin by the mechanism proposed by Dr. Robert could be responsible for the deficiency of elastin fibers and lamellae in the atherosclerotic tissue.

DR. WISSLER: I think what Dr. Fisher-Dzoga was trying to demonstrate was, that under the condition of the primary explant being in a stationary phase, proliferation stimulated by low density lipoprotein application was due to clones of cells responding. We have recently carried this into the rabbit sub-culture in terms of what chemical changes are going on. Once again we have the same kind of phenomenon. We are studying cells that are grown in a confluent monolayer for two weeks. Dr. Chan who has been working with Caddy and myself has been doing this work, and I will report it very briefly. He also finds that with hyperlipemic serum he gets much more lipid uptake than with normal serum although there is some lipid visible in normal serum cultures also. These lipid droplets are present as droplets that probably still have some of the lipoprotein characteristics. After these cells are incubated with various kinds of serum or with fractions of serum he does analyses that include cholesterol ester, triglyceride, free fatty acid, diglyceride and phospholipid and so forth.

Special Characteristics of Hyper-lipemic Serum

The plan of these experiments is to incubate in concentrations of serum that go from 5% to 40% normal, normal serum and 5% to 10% hyperlipemic serum in order to get an overlap between the amount of lipid and cholesterol present in the cultures incubated with normal serum and with hyperlipemic serum. With hyperlipemic serum, one gets an accumulation of free cholesterol and cholesterol esters which one cannot match even with 40% normal serum. Any dilution of hyperlipemic serum leads to a rather remarkable and prompt accumulation of cholesterol esters in the cells that are essentially stationary and confluent in two weeks. This simply shows that the main lipids which are accumulating with very low concentrations of hyperlipemic serum are cholesterol and cholesterol esters. The phospholipid does not change very much at all. The triglyceride goes up intermediately. If one fractionates the serum and incubates with low density lipoprotein, very low density versus high density, then the main accumulation of cholesterol and cholesterol ester in these cells is either at twelve hours or at 36 hours. The low density lipoproteins from hyperlipemic serum seems to have an effect that cannot be duplicated by the low density lipoprotein from normal serum. The evidence at present suggests that there is a lot of movement on to the cell of cholesterol ester and out of the cell of free cholesterol and there must be breakdown of cholesterol ester to free cholesterol within the cell.

In summary, cells grown in either 2 or 20% normal serum have a similar lipid pattern but when very small quantities of hyperlipemic serum are added, there is a remarkable increase of both free cholesterol and of cholesterol esters; this despite the fact that the lipids are about the same in the medium with 20% normal serum as they are in 2% hyperlipemic serum. The lipids are liberated. The cells incubated in high density lipoproteins show a greater increase in triglycerides, and those in LDL have the highest increase in free cholesterol and cholesterol esters.

DR. WERTHESSEN: Are you implying that this effect is due to a special property of the cell?

DR. WISSLER: No, we think there is something triggering the accumulation of lipid in the low density lipoprotein molecule from hyperlipemic serum that is not present in the low density lipoprotein molecule from normal serum. This seems to be a similar phenomenon to the one Dr. Fisher-Dzoga reported in which something in hyperlipemic low density lipoprotein triggers proliferation that cannot be duplicated by normal serum. It might be what is stimulating mono-clonal proliferation if, indeed, that occurs as Dr. Benditt suggests.

DR. STEIN: This is a question to Dr. Fisher-Dzoga and Dr. Wissler. I admired the beautiful presentation and the slides of Dr. Fisher-Dzoga and was struck by the immense response to LDL in the hyperlipemic serum and the inability to show HDL in these cells. I wonder if this was not due to two factors. One of them is that in the hyperlipemic serum you usually get a tremendous increase of
RABBIT LDL and a decrease of HDL. Two, it is well known that in the rabbit the antigenic response to LDL is far greater than it is to HDL. This may be a technical problem.

DR. FISHER-DZOGA: In our hands HDL seemed to elicit good antibodies as demonstrated by immunoelectrophoresis but we should be able to get some more coated ones, possibly from Dr. Scanu who is making antibodies against different apoprotein, fractions three and four.

DR. DAOUD: I would like to comment on Dr. Wissler's study. This effect of hyperlipemic serum is a lasting effect because in one of our studies in tissue culture we took an explant from a hyperlipemic animal and grew it in normal serum. Even so after nine days these cells still synthesized DNA at a much higher rate than explants from normolipemic animals.

DR. WIGHT: I would like to direct this question to Dr. Wissler and Dr. Fisher-Dzoga. Is the uptake and/or accumulation of free and esterified cholesterol specific for smooth muscle cells in

culture or do other cell types in culture also accumulate these lipids?

DR. FISHER-DZOGA: Dr. Chan has some results with L cells and with skin fibroblasts. He found that smooth muscle cells take up more under comparable conditions.

DR. LEE: I would like to introduce you to some of the methods we have been using to produce atherosclerotic lesions. Yesterday we spent many hours discussing the endothelial cells and their function, and their possible relationship to the development of the lesion.

Endothelial Injury

We learned a technique from Dr. Spaet and his colleagues in New York City in which endothelial cells are denuded by introducing a Fogarty balloon catheter in the aorta or coronary artery and filling it with water or air, and then pulling this balloon up and down a few times. With this method we should be able to denude most of the endothelial cells, leaving little damage to the underlying structure. When this procedure was combined with high cholesterol diet, rather extensive atherosclerotic lesions developed within a few months. However, when we used regular commercial pellets, the lesions were minimal; no lipids were present in the lesions.

The following figures illustrate the results we have obtained with this technique. Fig.184 from a swine fed a high cholesterol diet for six months after balloon denudation shows you the kind of lesion obtained six months after this procedure. Thick necrotic calcific lesions covered the entire surface. These procedures have proved to be useful for production of lesions in various stages for regression or metabolic studies. This technique is advantageous over diet alone because we can produce lesions much faster and somewhat more uniformly. Fig.185 shows the lesion produced in coronary arteries. Only the left circumflex branch was ballooned and the swine was fed a high cholesterol diet. The lumen was found to be almost occluded with extensive atherosclerotic lesions, with calcification and necrosis. This type of lesion can be produced within three months and frequently these swine die suddenly. In previous studies, we found that the cause of sudden death of swine with revere atherosclerotic lesions was ventricular fibrillation and we think the ballooned swine are also dying from cardiac arrhythmias when they die suddenly.

FIG

Fig.186 shows extensive myocardial infarction of one of these ballooned swine. This model should be appropriate for studies where ischemic heart disease is needed and also would be an excellent model for coronary by-pass surgery.

I would like now to summarize briefly what I have presented

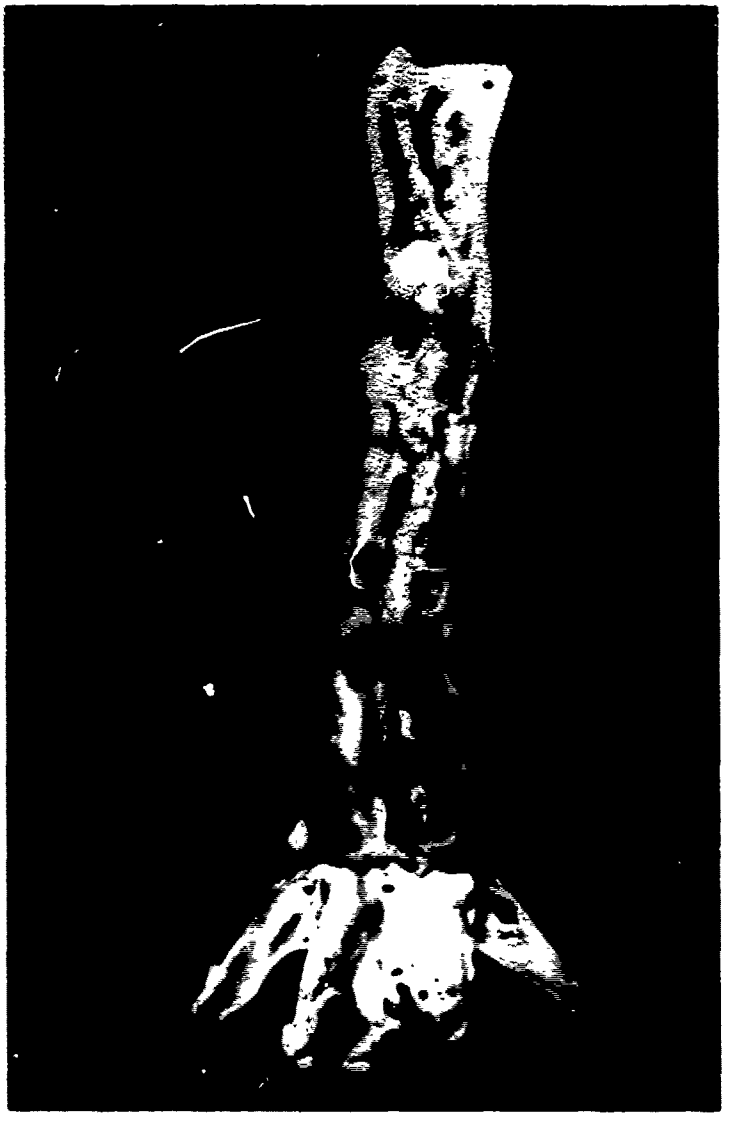


Figure 184: This abdominal aorta is from swine fed a high-cholesterol diet for 6 months after the ballooning. The thick shaggy lesion covers the entire surface.

Figure 185: Low power photomicrograph of the left coronary arteries. The artery on the right is the left circumflex coronary artery and the one on the left is the left anterior descending coronary artery. Only the left circumflex branch has been ballooned and the swine was fed a high cholesterol diet. The lumen is almost occluded with extensive atherosclerotic lesions with necrosis and calcification.

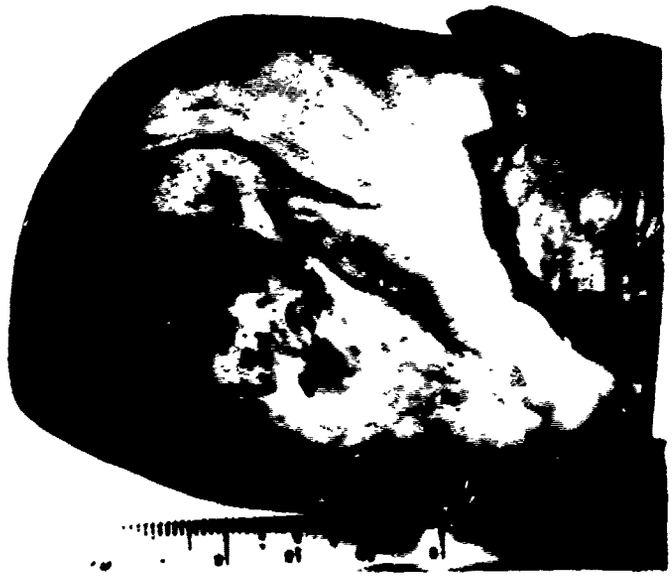


Figure 186: Anterior view of heart of swine fed a high cholesterol diet, and ballooned both left anterior descending and circumflex coronary arteries showing extensive acute myocardial infarction with aneurysmal dilatation.

in Berlin. In collaboration with Dr. Werthessen we have studied the necrogenic effect of a methanol extract of commercially available crystalline cholesterol. For the study of an acute effect of the methanol extract, 250 mg. per kg. body weight of the methanol extract was given to rabbits by gastric tubing. Within 18 hours focal necrosis and degeneration of aortic smooth muscle cells were evident (Fig.187) and the extent increased after repeated administration of the extract. In a longer term study a total dosage of one gm. over five weeks produced non-fatty proliferative lesions. It is interesting to note that unlike the usual cholesterol-induced lesions in rabbits these proliferative lesions did not contain foam cells but consisted mainly of proliferating modified smooth muscle cells and collagen. The control rabbits given equal quantities of pure cholesterol showed no such response. Therefore our findings suggest that the necrosis observed when cholesterol is used in dietary experiments cannot be attributed to it, but rather to contaminants, presumably oxidation products.

Injury from Oxidation Products of Cholesterol

DR. BOWYER: May I add to the record two other ways for removing endothelium. The first is with EDTA (341) which can be used in an isolated arterial segment and the second is by production of anaphylaxis (428).

DR. KNIERIEM: Finally, I would like to comment on the possibilities of studying proliferations of smooth muscle cells (115, 185, 187, 188, 425). This will be an additional comment to the remarks of Dr. Lee. As you know, Dr. Bjorkerud has developed a fancy device to produce two different types of mechanical injury of the aortic wall in rabbits (36, 37) which he called the longitudinal and the transverse injury. Referring to his illustrations I want to show one lesion from a longitudinal injury (Fig.188) and one of the diffuse intimal thickening after transverse injury. But I would like to restrict my comment to the ultrastructural properties of the proliferating smooth muscle cells in these lesions.

Endothelial Injury and Repair

Three weeks after transverse injury we could observe distinct layers of proliferated smooth muscle cells within the thickened intima (Fig.188a). Even seven and thirteen weeks later the different layers of proliferated cells are still recognizable (Fig. 188b and c). In some of the lesions the proliferated smooth muscle cells were covered by endothelial cells with a very edematous cytoplasm containing only a few mitochondria. The next figure (Fig.189) demonstrates how some lesions were uncovered by new endothelium. The irregularly arranged smooth muscle cells exhibit a very prominent endoplasmic reticulum. Between these prolifer-

Figure 187: This is a low power electron micrograph from the aorta of a rabbit killed 18 hours after administration by mouth of 250 mg/kg body weight of the methanol extract of crystalline cholesterol. Suggestions of fusiform densities and myofilaments are seen in places but the cytoplasmic boundary is vague. There are a number of dense bodies, focally distended perinuclear cistern and a collection of vesicles possibly the remnant of the Golgi or endoplasmic reticulum. The nucleus shows typical pyknotic degeneration. x 18,000

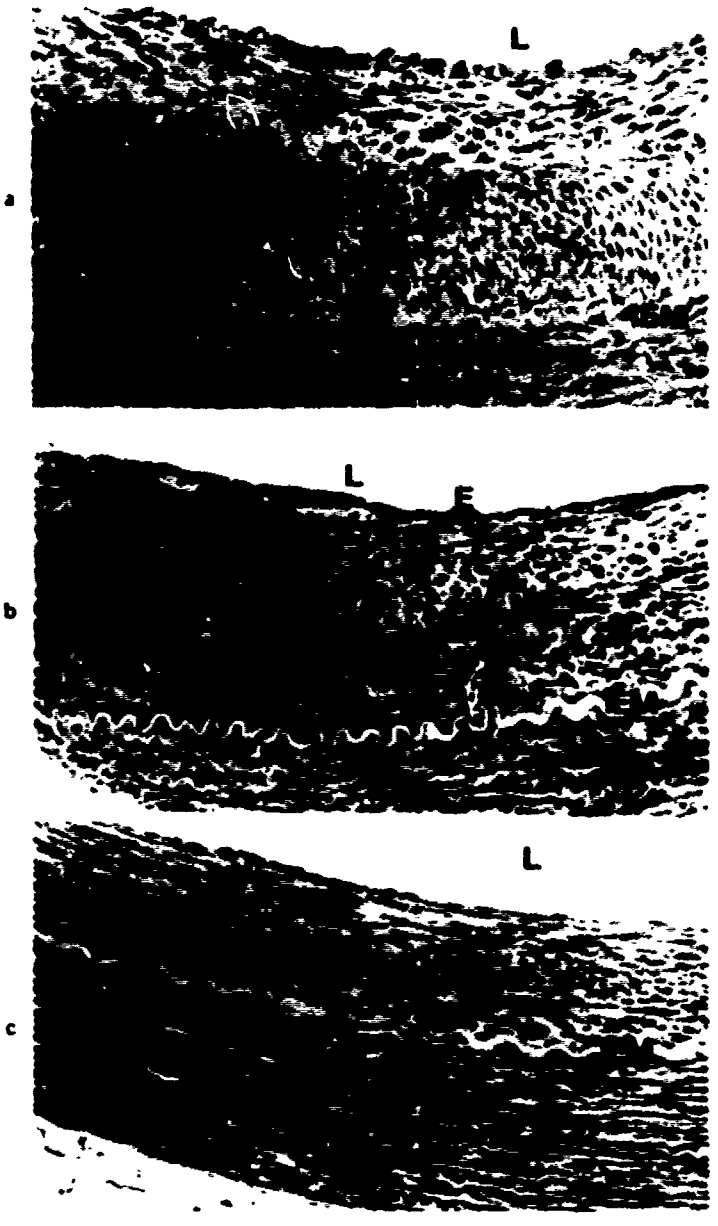


Figure 188: Semithin sections of intimal lesions following the induction of a superficial injury with large area after 3 weeks (a) 7 weeks (b) and 13 weeks (c). The intimal thickenings have 2 or 3 distinct layers of proliferated smooth muscle cells. L-Lumen, IEM - internal elastic membrane, M - media, x 230

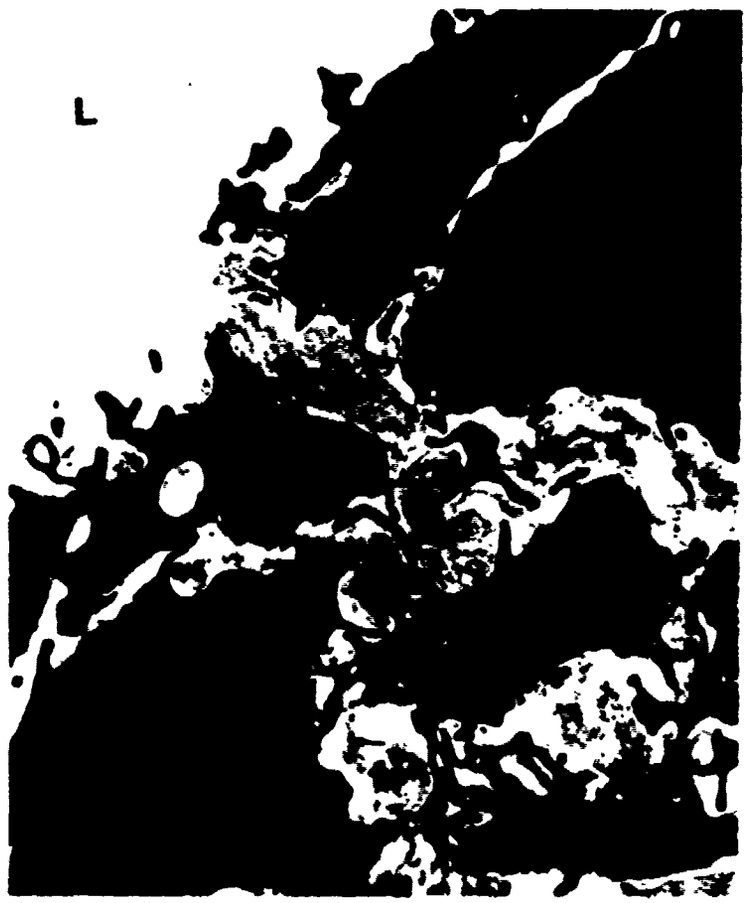


Figure 189: Superficial cell layer of a non-reendothelialized region of a 2-week old lesion. The proliferated smooth muscle cell have numerous vesicles (✓ ✓) at the outer cell membrane. Fibrin-like material (F) is present within the gaps and clefts between the smooth muscle cells of the surface lining. x 17,800.

ated cells you can see wide gaps by Dr. Bjorkerud's scanning electron microscopy. The next picture (Fig.190) demonstrates three and seven weeks after injury we found numerous proliferated smooth muscle cells oriented circumferentially, and large amounts of collagen fibers and groundsubstance within the extracellular space. At the basal layers of these lesions the smooth muscle cells were of a more oval or round shape and not as elongated as in the superficial layers. But still these cells contain increased rough endoplasmic reticulum, and within the extracellular space also immature elastin. Only a few scattered foam cells were found in these lesions of normolipemic rabbits. Some cells contain hemosiderin granules and other small lipid droplets (Fig.191). Several weeks later the lesions showed a decrease in cellularity, and thirteen weeks after injury we found much more immature elastic matrix and increased collagen fibers (Fig.192a). Finally small foci of microcalcifications occurred in the older lesions (Fig.192b).

DR. LINDNER: Dr. Lee, I chink the extent of the damage you are doing with the balloon technique includes some destruction of sub-endothelial structures. You showed a coronary artery with a small lumen and a greatly thickened wall composed partly of concentrically arranged collagen fibers. I would make the diagnosis of the final stage of arteritis without knowing what you had done.

Effects of Mechanical Injury

DR. LEE: I don't understand why you, Dr. Lindner, would like to consider it as the final stage of arteritis except in the broad sense that any reaction to injury is inflammation. The ballooned cornonary artery in Fig.185 showed an extensive atherosclerotic lesion leaving only a narrow slit-like lumen. The lesion consisted mainly of modified smooth muscle cells and foam cells with focal necrosis, hemorrhage and calcification. No more inflammatory cells were present than in lesions produced by diet alone.

DR. LINDNER: But you see if you have a really mechanical lesion on the vessel you naturally will find all types of hematogenic cells in the repair process of the vessel wall. You are seeing other connective tissues, including granulation tissue, after mechanical lesions. The process is like that seen after surgical lesions or inflammatory vascular disease rather than being characteristic of the atherosclerotic process.

DR. LEE: Well, may I point out another fact, Dr. Lindner. As I mentioned earlier, when a regular commercial mash diet was given to these swine after ballooning the artery, only minimal lesions developed without foam cells, hemorrhage or necrosis. In other words, unless a high-fat, high cholesterol diet was given concurrently with the ballooning the effect of the mechanical in-

Figure 190: Superficial and middle zones of a 3 week old intimal lesion. The endothelial cells (E) show signs of injury with pyknotic nuclei and budding at the surface. The underlying smooth muscle cells (smc) have numerous cytoplasmic processes. Within the extracellular space increased amounts of ground substance and collagen fibrils (c) x 17,100.

Figure 191: Proliferated cells of the intimal lesion containing lipid droplets (Li) and hemosiderin granules (g). 3 week old lesion after mechanical injury. $\times$ 14,800.

Figure 192: Intimal lesion 13 weeks after mechanical injury. a) Increased collagen fibrils (C) and irregular portions of elastic matrix (em) are present between the smooth muscle cells (smc). 12000 x. b) Granular microcalcifications (✓ ✓) surrounded by collagen fibers (C) and small elastin lamellae (em). x 5200.

sult was minimal. Thus it is difficult to imagine that the extensive lesion produced by the combined procedure is only the result of trauma.

DR. SMITH: I think really we are very much on the same lines. I do think that the point you raised about difference in different regions is of extreme importance and that one can only usefully approach this sort of work by direct comparison between normal intima and atherosclerotic lesions which are in the same vessel and as close together topographically as possible. Trying to compare different subjects and different ages one runs into trouble. Whatever the analysis one must try to do it in relation to its own standard within the same vessel.

DR. LINDNER: I think I said that this picture I showed particularly with incorporation rates, showed some plaques, and the normal was exactly that which you have spoken about from the same vessel walls, unchanged parts, and only then can you compare. I absolutely agree with you.

DR. WERTHESSEN: That is wonderful, to find two people in agreement.

DR. DAY: Mr. Chairman, if I can comment on the earlier paper of Dr. Lee and some of the remarks Dr. Knieriem and Dr. Bjorkerud made with regard to injury and the possible involvement of platelets in stimulating lipid metabolism by arterial smooth muscle cells. Dr. Mustard referred, in the Berlin meeting, to a model that was developed by Sean Moore in which continued injury to the intima of normal fed rabbits by indwelling polythene catheters was shown to give rise to lipid containing intimal lesions. Unlike the acute injury that Dr. Lee described, these lesions developed marked lipid accumulation (230). I do not propose to describe the lesions but I do want to show some of the subsequent studies (76) we carried out on the evolving lipid composition and metabolism of these lesions.

I would emphasize that these studies were carried out on normocholesterolemic rabbits. Microscopically the main lesion was a thrombotic lesion containing many platelets and with marked lipid infiltration. We also studied the composition of a second type of lesion which was present - a flat fibrous lesion - and then compared the composition of both the raised thrombotic lesion and the fibrous lesion with that of the adjacent normal intima. The free cholesterol, cholesterol ester and lipid phosphorus concentration of the three portions of the intima are shown in Fig.193 for the three time intervals studied. At two weeks both the free cholesterol and cholesterol ester, but not the lipid phosphorous concentrations of the raised lesions had increased markedly compared

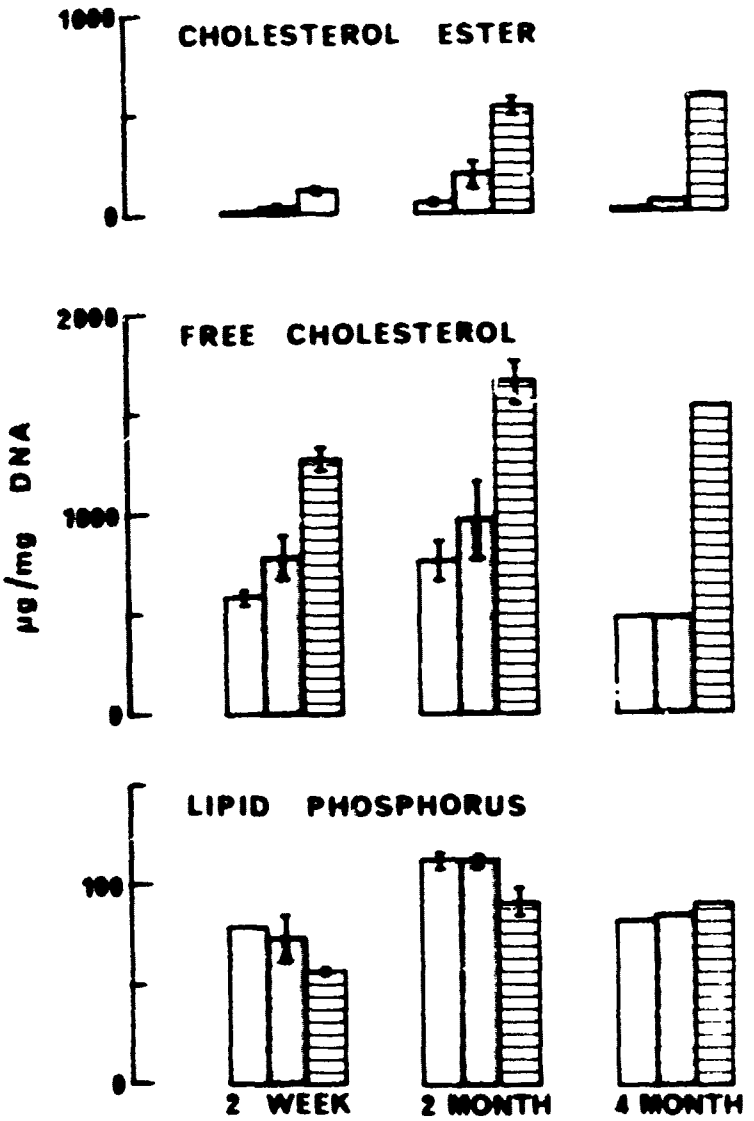


Figure 193: Lipid concentrations of normal intima (blank columns) and of fibrous (spotted columns) and raised lesions (horizontal line columns) at 2 weeks, 2 months and 4 months after insertion of polythene catheters. Means of batches with the S.E.M. where applicable are given. This figure is based on data from Day et al 1974b

with the normal intima. By four months the cholesterol ester content of the raised lesion was many times that of either the normal intima or of the fibrous lesion. The composition of the cholesterol esters present in the raised lesion were measured by gas liquid chromatography and shown to contain more oleic acid and less linoleic acid than those from either the normal intima or the fibrous lesion. Thus development of the lesion was associated with an increase in cholesterol esters differing in composition to that of the normal intima.

We also carried out metabolic studies and found that where ^{14}C-labelled oleic acid was incubated with the aortas in vitro that relatively higher amounts were incorporated into cholesterol ester in the raised lesion than in the corresponding normal intima or the fibrous lesion. It seemed that platelets which were present in large amounts in this lesion were associated with an increased diversion of precursors to cholesterol ester synthesis. The incorporation of ^{32}P phosphate into various phospholipids by the raised lesion when compared with the normal intima and the fibrous lesion indicated that the raised lesion showed a lower relative incorporation of ^{32}P phosphate into phosphatidyl inositol than did either the normal intima or the fibrous lesion. In this respect the lesions produced by the indwelling aortic catheter resembled atherosclerotic lesions produced by cholesterol feeding (237). The conclusion I wish to suggest is that changes in lipid metabolism characteristic of the atherosclerotic lesion produced by other agents may also be produced by stimulation of the intima by mural platelet thrombi.

DR. SCHWARTZ: I would like to make a comment. On the interpretation of the catheter lesions, and I think it is important to recognize that this is recurrent injury in which a variable amount of thrombosis is present. I think it is important to recognize that this is not a model for the study of the evolution of thrombus alone and that there is a very significant continuous mechanical injury involved in this as well. In other words, it is a complex model that involves both thrombosis and recurrent injury so I think that one has to be cautious.

DR. DAY: I think one would recognize that point. However it is a thrombotic lesion that is formed in situ and I think therefore is more likely to give information regarding platelet interactions than data obtained from injected thrombi.

DR. SCHWARTZ: I think it is important to emphasize that this thrombus is only one component of the lesions that are formed with this catheter technique and that there is a significant injury phenomenon which involves cells other than platelets. So, I am not really referring to my data on organizing thrombi. This was

not the intent. It was really to point out that this is a complex model which involves injury as well as thrombosis and one cannot extrapolate clearly to the changes which occur in organization of thrombus from this model.

DR. BOWYER: I am sure that Professor Day would agree that a foam cell is a foam cell is a foam cell (with apologies to Stein, G.!) It is not surprising, therefore, that we find certain derangements of lipid metabolism such as altered fatty acid, phospholipid and cholesteryl ester turnover in foam cells, however formed. The thing that interests me, however, for any model of atherogenesis, is whether there is a biochemical event, indicating a transformation of smooth muscle cells which can be detected before the establishment of a foam cell. Does Professor Day have any comment on that, please?

DR. DAY: Not directly. I think the importance of this sort of situation is that you can trace the events in the wall. I am perhaps not competent to do this from the microscopic point of view because I was not involved in this stage. But you can trace the events through an evolution which is essentially similar to the sort of evolution you see from the very early lesion with very little lipid involvement through to smooth muscle cells which are clearly identifiable as smooth muscle cells which become laden with lipid. We are, I think, in this situation dealing with a position where the lipid could possibly come from the serum but probably does not come from the serum because it is not hypercholesterolemic. So that there is not a large lipid load which would be involved in uptake of lipids from the serum. And the point that I would like to make is that we are dealing with a stimulated arterial wall, associated with smooth muscle proliferation, and that this goes on to lipid accumulation presumably by smooth muscle cell synthesis of the lipids that are involved.

DR. WERTHESSEN: May I interject a question here? To Dr. Day. Why do you assume that in a normal lipemic serum state that there is not a flux of lipid through the wall?

DR. DAY: I did not mean to convey that impression. The changes in lipid composition that are produced in the intima following the "catheter injury" are however quite gross. The accumulation of lipid is apparent by two weeks, the serum cholesterol is of course extremely low. It is possible that you might get a large influx from the serum under these circumstances but it is doubtful that it would explain the data, particularly where you have in addition the fatty acid composition of the lipids of the lesion differing from the serum and the normal intima.

DR. BOWYER: One of the most important ways in which choles-

eryl esters enter cells, as has been shown by Werb and Cohn for the macrophage (416) is by phagocytosis or pinocytosis. Has Dr. Wissler observed in his cultured smooth muscle cells soon after addition of low density lipoproteins an increase in the number of phagocytic vesicles? I wonder also whether any compounds which enhance phagocytosis, such as Fuftsin alter the lipid composition o the cells grown in normal serum without the addition of low density lipoprotein. We have now obtained evidence that certain forms of acute damage to the normal arterial wall will lead to an alteration in turnover of lipids, particularly phospholipids as revealed by incorporation of ^{3}H oleic acid or ^{32}P orthophosphate into a perfused segment for one hour. This is illustrated in Table XXX for an artery denuded of endothelium by treatment with trypsin in vitro and then immediately perfused. It can be seen that the damage has led to an altered lipid turnover (percentage of a lipid class labelled per hour by each precursor).

DR. WISSLER: The answer to the second question is no, we have not studied other kinds of substances that might stimulate phagocytosis. It is our impression that quite early in these cultures after low density lipoprotein is added that there is an increase in the pinocytotic vacules. If I could have been here the first day I would have shown a sequence of slides from the very early growth and stimulation of these cells through to very mature stages and late in lipoprotein addition. But I think there may be very well an increase in that machinery early after the addition of low density lipoprotein.

I would like to ask Dr. Smith how much of the total lipid is present in the form of lipoprotein, and how much in the form of lipid not associated at least immunologically with lipoprotein is present in these various stages. I think that is a very important point that needs to be clarified for the record.

I would like to point out one other thing and that is that the paper that probably impressed me the most in Berlin was the one of the Steins with Dr. Bierman on what is happening with time when lipoproteins are exposed to these cells in culture. It must be borne in mind that a fair amount of lipoprotein may pass through these cells and still get out into the media but it is not clear how much of the material could be identified immunologically as low density lipoprotein. For high density lipoprotein I guess about a third.

DR. SMITH: Dr. Wissler's question is most simply answered with a Table (Table XXXI). We can divide the tissue cholesterol into two fractions: (1) "Residual cholesterol" which is electrophoretically immobile and remains in the tissue sample after electrophoresis, and (2) "Lipoprotein-bound cholesterol" which

TABLE XXX

	^{3}H 18:1		^{32}P	
Lipid	Normal	Trypsin treated	Normal	Trypsin treated
LPC	0.049	0.080	0.124	0.467
SPH	0.006	0.023**	0.009	0.044
PC	0.223	0.283	0.452	0.297
PSI	0.072	0.686*	0.[illegible]3	1.946
Unknown	0.105	0.213	1.578	0.354
PI	0.233	1.340*	6.612	5.274
PE	0.082	0.249**	0.010	0.207*
FFA	0.437	0.491		
TG	0.230	0.318		
CE	0.204	0.038***		

*=significant difference P<0.05

**=significant difference P<0.01

***=significant difference P<0.001

LPC = Lysophosphatidyl choline; SPH = Sphingomyelin; PC = Phosphatidylcholine; PSI = Phosphatidylserine; PI = Phosphatidyl inositol; FFA = Free fatty acid; TG = Tri[illegible]ycerides; CE = Cholesteryl esters.

Percentage turnover per hour of lipids of normal rabbit aorta and aorta damaged by treatment with trypsin (0.2%) for 30 minutes, as revealed by perfusion at physiological pressure for 1 hour with Krebs bicarbonate buffer containing ^{3}H-oleic acid (0.5 [illegible]E/ml) and ^{32}P orthophosphate (0.944 [illegible] moles/ml).

$$\% \text{ turnover/hour} = \frac{\text{incorporation of lipid precursor into lipid class per hour}}{\text{Concn. of lipid class}} \times 100$$

Treatment	No. observations
Normal aorta	6
Trypsin	11

TABLE XXXI

The concentrations of "residual cholesterol" which is apparently not bound to intact lipoprotein, and of "lipoprotein-bound cholesterol" in normal intima and lesions.

	CONCENTRATION: mg/100mg defatted dry tissue.	
	"Residual cholesterol".	"Lipoprotein-bound cholesterol".
Normal intima.	3 - 4	2 - 4
Gelatinous thickenings.	4 - 5	4 - 8
Gelatinous peripheries of plaques.	4 - 8	8 - 16
"Atheroma lipid".	c.70	c. 2
Fatty streaks - predominantly fat-filled cells.	c.30	c. 1

migrates out of the tissue in an electric field, and in an antibody containing gel forms a rocket-shaped peak with area proportional to the lipoprotein concentration (347). The amount of LP-bound cholesterol is calculated from the peak area on the assumption that the relationship between peak area and cholesterol content is the same for the tissue lipoprotein and plasma lipoprotein. Preliminary preparative studies suggest that this assumption is valid. There is great variation depending on the subject's age, serum cholesterol level and blood pressure, but on average in normal intima cholesterol which is bound to intact lipoprotein accounts for 25-50% of the total cholesterol, and in gelatinous lesions for 40-75%. In one hypertensive, hypercholesterolemic subject the lipoprotein bound cholesterol in the gelatinous periphery of a large plaque was 30 mg/100 mg dry tissue.

In fatty streaks consisting mainly of fat-filled cells intact lipoprotein is invariably very low (348) and accounts for only a very small proportion of the total cholesterol. This is a most consistent finding, and its meaning is not clear to me. Presumably the fat-filled cells are destroying lipoprotein at a rapid rate, and one could postulate that this is some form of defense mechanism.

DR. WOLF: As an auditor of this fascinating conference may I try to put together what I think I have learned? The fresh insights that were brought into focus for me included the recognition that the basic mechanisms of smooth muscle contraction are essentially identical to those of skeletal muscle. Smooth muscle differs from skeletal mainly in structural components that allow for elasticity and for the maintenance of tension with little energy expenditure. In general the smooth muscle is more versatile than skeletal and in some ways, to pH changes for example, is also more vulnerable.

Summary Statement

In the inner portion of the arterial wall smooth muscle cells are arranged in a radial or circumferential way, while in the outer layers their arrangement is longitudinal.

There are two modes of contraction in vascular smooth muscle that appear to be related to different calcium activating systems. Phasic contractions are generated by an action potential (spike), while in tonic contractions a graded depolarization takes place without a spike discharge.

INNERVATION AND NEURAL MECHANISMS

Autonomic fibers course through muscle bundles making synaptic connections "en passage" with smooth muscle cells that are electro-

tonically connected with other neighboring smooth muscle cells. At branching sites coronary arterioles, like precapillary sphincters, are equipped with "intimal cushions". The structures are richly innervated, containing at least three different kinds of nerves.

In the heart norepinephrine is elaborated by small intensely fluorescent chromaffin cells that send processes to join perivascular plexuses but, unlike post ganglionic sympathetic neurons, these cells are not degranulated by 6-hydroxydopamine.

Arterial innervation includes a repertoire of chemoreceptors. Moreover the distribution of receptors to vasopressin, epinephrine or angiotensin may differ along the course of the same artery.

The products of mast cells located in the vessel wall, histamine, serotonin and heparin may gain access to smooth muscles through intravascular trabecular channels and thereby directly or indirectly exert their influence on the behavior of smooth muscle cells.

Important characteristics of smooth muscle cells were revealed by tissue culture experiments. Low density lipoprotein in the culture medium appeared to stimulate cell growth and proliferation. Blood platelets also appeared to provide a powerful proliferative factor, suggesting a pathogenic mechanism for atherosclerosis that may operate in the case of endothelial injury and platelet adherence. The proliferative substance occurring in platelets was found not to be dialysable. and therefore is probably neither serotonin nor histamine. Under anoxic conditions smooth muscle cultures accumulated lipid from serum.

LIPOPROTEIN METABOLISM

The capacity of smooth muscle cells to synthesize cholesterol appears to be relatively small. They are, however, normally in contact with serum lipoproteins that enter the arterial wall.

Their involvement in the atherosclerotic process is still unclear but it appears that an abnormal intimal accumulation of serum lipoproteins results in a gelatinous lesion which subsequently assumes a firmer, fibrotic character. As the smooth muscle cells ingest lipoproteins readily, they are able to catabolize them only to a very limited degree. The lipoprotein can, apparently, be "regurgitated" but in the process may leave behind some of its cholesterol, thereby accounting for the accumulation of intracellular cholesterol ester in arterial smooth muscles with age. An atherogenic diet appears to trigger an increase in cholesterol esterification, a process that was accelerated by hypoxia so that the smooth muscle cell took on the appearance of a foam cell.

CONNECTIVE TISSUE METABOLISM

The capability of smooth muscles to synthesize and secrete proteoglycans and collagen and elastin fibrils was emphasized.

The intricate process controlling the synthesis, arrangement and degradation of glycoprotein myofibrils, collagen and elastin and the cross linking process are gradually coming to light and their disturbed regulation offers an attractive candidate for a piece in the puzzle of the atherosclerotic process.

ENDOTHELIAL RELATIONSHIPS

The rate of replication of arterial endothelium is accelerated at branchings and in association with elevated arterial pressure. The rate of cell renewal is accelerated by injury and by cholesterol feeding but decreases with age. Indeed, the total arterial wall cell population appears to decrease with age except at the sites of atheroma.

The structure of the arterial endothelial lining in relation to its permeability to blood constituents, especially lipoproteins is still the subject of intense inquiry. Plasma constituents may enter the arterial wall to some extent under normal circumstances. In the presence of endothelial injury not only was permeability to lipid enhanced but the pattern of lipid distribution was altered. Temporary increased endothelial permeability to albumin, LDL and VLDL was observed after injection of very small amounts of angiotensin II. The agent also caused increased mitotic activity in smooth muscle cells. The increased cell proliferation antedated the appearance of hypertension in genetically hypertensive rats.

The normal endothelium was found capable of transporting lipoprotein molecules (HDL and LDL but very little in the way of chylomicrons) across the membrane via pinocytotic plasmalemmal vesicles. Other evidence points to a control mechanism for uptake of molecules into the arterial wall via gap junctions that provide a "metabolic coupling" between endothelial and smooth muscle cells. Irregular processes may protrude from capillary endothelial cells and modulate the process of transfer of molecules. Other evidence suggests that the capability of endothelial cells to contract is well established in venules but still uncertain in the lining of arteries.

Perhaps the papillary excrescences on the endothelial lining described by Dr. Una Smith may serve as intraluminal "drag" receptors that through a local neural adjustment bring about changes in arterial caliber in association with changes in flow. Therefore, a sort of velocity modulator mechanism is postulated causing

the underlying medial muscles to relax or contract. The possible effect of changes in arterial caliber on the transport across endothelium remains unexplored.

Another factor that potentially enters the equation of transendothelial transport is the "glycocalyx" coating of the endothelial surface that thickens under some circumstances and under others is replaced.

Following endothelial stripping smooth muscles proliferate and form a layer over the internal elastic lamina. This pseudointima thickens until the endothelial lining is restored. Thereafter, the number of smooth muscle cells slowly decreases. Species differences may be important with respect to the formation of a new intima. It occurs readily in baboons, not in dog.

The histogenesis of the smooth muscle cell remains an unsolved problem. So does its identity in advanced arteriosclerotic lesions in relation to endothelial cells and macrophages. Perhaps our most important need at this time, however, is an understanding of arterial fluid dynamics in relation to endothelial and smooth muscle cell behavior and permeability, especially as modified by branchings and by vasomotor activity.

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