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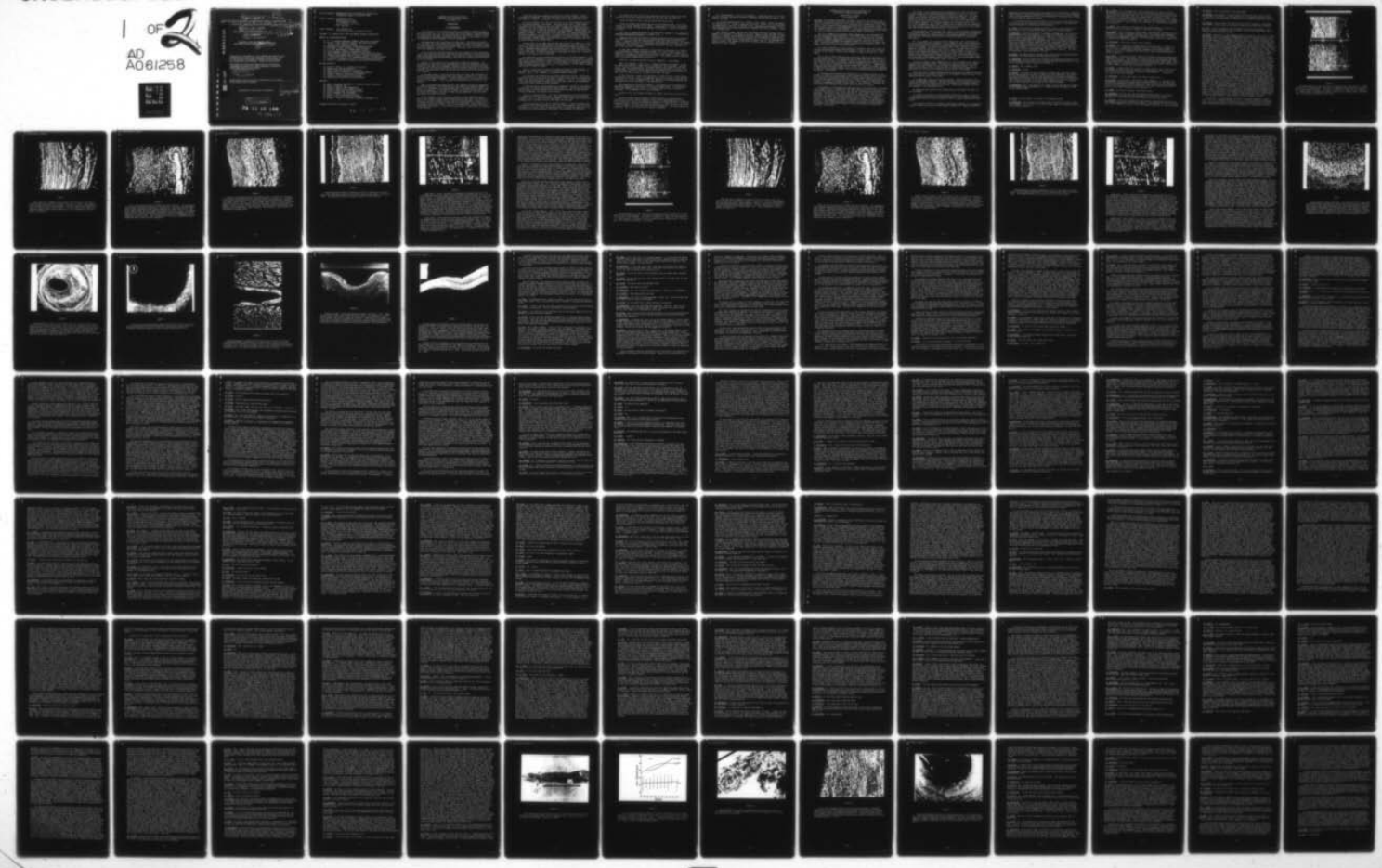

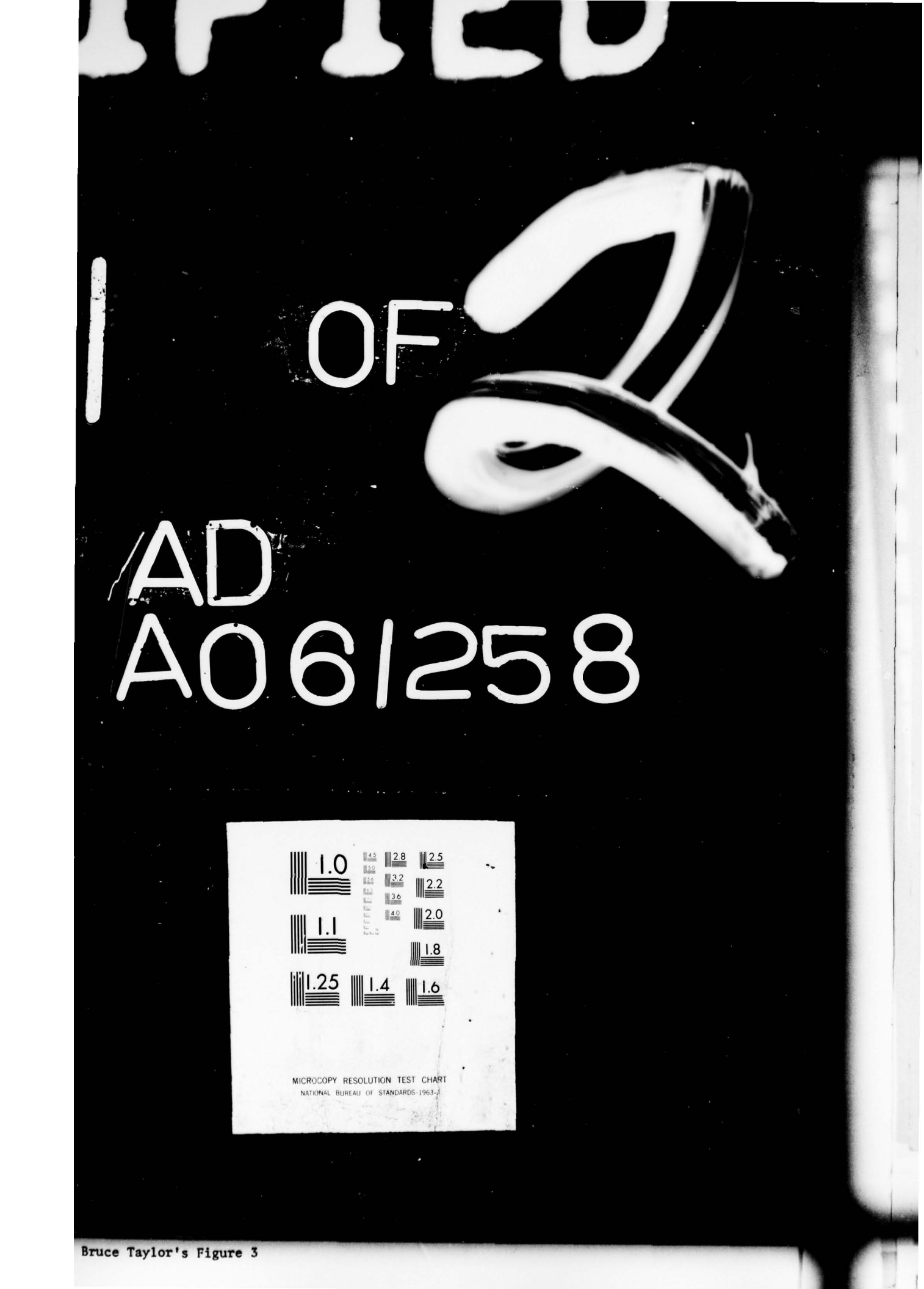

Bruce Taylor's Figure 3

(6) REPORT OF WORKSHOP ON

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STUDY AND REVIEW OF ANGIOTOXIC AND CARCINONGENIC STEROLS IN PROCESSED FOOD

Held at Boston, Massachusetts, 12-14 October 1977

(10) Stewart G. Wolf, Jr., M.D.

Totts Gap Medical Research Laboratories, Inc.

Bangor, Pa

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Prepared from a recording of the proceedings and revised by the participants who edited their individual contributions. Some of them supplied bibliographic references and photographs of the slides shown at the meeting. These have been included.

The workshop provided superb opportunity for intellectual interchange and resulted in several subsequent collaborative undertakings among the participants.

(12) 144 p.

Distribution of this report is unlimited

Title of Meeting: WORKSHOP FOR STUDY AND REVIEW OF ANGIOTOXIC AND CARCINOGENIC STEROLS IN PROCESSED FOODS

Place of Meeting: Massachusetts Room
Harvard Club of Boston
374 Commonwealth Avenue
Boston, Massachusetts 02215
Tel: 536-1260

Time of Meeting: 12-14 October 1977
(9-5 on 12 & 13 Oct; 9-12 noon on 14 Oct)

Chairman: Dr. Stewart Wolf, Totts Gap Medical Research Laboratories

Members of Collaborating Group:

Dr. C. J. W. Brooks (analytical chemist), University of Glasgow*
Dr. Sylvan G. Frank (physical chemist), Ohio State University
(in lieu of Dr. Albert H. Soloway)
Dr. Hideshige Imai (pathologist), Albany Medical College
Dr. Elizabeth H. Leduc (cytologist), Brown University
Dr. K. T. Lee (pathologist), Albany Medical College
Dr. Philip LeQuesne (chemist), Northeastern University
Dr. Charles Merritt (analytical chemist), Army Natick Laboratories
Dr. Vanayakam Subramanyam (organic chemist), Northeastern University
Dr. C. Bruce Taylor and Dr. S-K. Peng (pathologists), VA Hospital/Albany
Dr. John Watson (biochemist), University of California/San Francisco
Dr. Nicholas T. Werthessen (biologist), Brown University/ONR Boston

Invited Scientists:

Dr. Edward H. Ahrens, Jr., Rockefeller University
Dr. Konrad Bloch, Harvard University
Dr. David B. Clayson, University of Nebraska*
Dr. George Popjak, University of California/Los Angeles*
Dr. Terence J. Scallen, University of New Mexico
Dr. Elspeth B. Smith, University of Aberdeen
Dr. Leland Smith, University of Texas/Galveston

Observers:

Dr. Mark D. Altschule, The Francis A. Countway Library of Medicine
Dr. Edwin C. Gangloff, NIH*
Dr. Robert K. Jennings, ONR Headquarters
Dr. Vincent Lisanti, Tobacco Research Council
Dr. Francis W. Morthland, Army Research Office
Dr. Alan J. Sheppard, FDA, Washington
Dr. Shirley R. Tove, Army Research Office
Dr. Paul E. Tyler, Naval Medical R&D Command*
Dr. Richard L. Veech, St. Elizabeth's Hospital, Washington, D.C.

*Accepted invitation but obliged to cancel.

WORKSHOP FOR STUDY AND REVIEW OF
ANGIOTOXIC AND CARCINOGENIC STEROLS
12-14 October 1977

Introduction

N. T. Werthessen

The initiation of the collaborative study you are about to analyze occurred about seven years ago. Dr. Lee was running one of his molecular pathology meetings. Dr. Taylor and I were there. We were on the way to lunch after a lecture in which the Albany group and others had presented data that could (if one wished to do so) be interpreted as being improbable if due to cholesterol.

The Albany group had presented most of those data. Very briefly described, they had shown that, within three days after beginning cholesterol feeding to swine, the smooth muscle cells of the aorta had responded. The rate of thymidine incorporation had increased. Tissue culture studies, beautifully done, had shown that this was a true change in their modus vivendi.

In the discussion, Robinson added the fact that cultures of human lesions and normal tissue from the same aorta continually showed differences in lipid metabolism down through generations of subcultures. This data was not new; but Robinson emphasized that repeated observations had been made from many explants since he first reported his finding.

Benditt at that time had not yet produced his monoclonal hypothesis. But Robinson and I, years before, had suggested that a change in the genetic code was a possibility that had to be considered. Apparently no one had gotten excited about the suggestion until Benditt produced the kind of evidence acceptable in oncological research. Most certainly Taylor and Lee were not then looking at the problem from that viewpoint.

As you might expect, I took the position that the Albany data reinforced the tentative conclusion that Robinson and I had reached. Obviously if it were true, one could hardly blame cholesterol. A normal constituent of the cell cannot be expected to be a mutagen. I then told them of a meeting held in the 1950's in San Antonio.

When I was through describing the data, they agreed to give me an opportunity to test my hypothesis. That hypothesis was that spontaneous oxidation products in their U.S.P. cholesterol, plus those formed when it was mixed into the diets, were the active components. If my memory is correct, the observation that carried the day for me was provided by Taylor. He had long ago found that, like wine, aged cholesterol was better than fresh.

Let me indulge in a bit of personal history as to how I gained my hunch. While I was a graduate student under Gregory Pincus in the 1930's, the steroid hormones were isolated, structure proven, and their synthesis begun. This was also the period when Cook and Dodds isolated the first carcinogen. The hormones and the carcinogens could induce growth. Pincus and I attended Louis Fieser's course on phenanthrene derivatives. It was the only place where one could learn the chemistry of the steroids.

Fieser and Pincus got together and worked up a research program. Fieser's group made the compounds. I did the mouse work--twice a day, seven days a week, six months at a stretch. Fortunately, I could use it as one-half of my thesis.

The lasting benefit to me from this study was a life-long friendship with two of the greatest chemists I have known. The other chemist was Dr. Erwin Schwenk. The German Schering Corporation set up the American branch of Schering so that Schwenk, a Jew, could escape the Nazi holocaust. Schwenk built that branch of the corporation, ran it as president during the war, and became the leading producer of steroid hormones. I met him, too, while a graduate student. He gave Pincus the hormones we needed. When he retired in the late 1940's, he joined our group at the Worcester Foundation. At that time I was running the Cancer Research Laboratory. Our problem was the metabolism of the estrogens as it related to cancer. We had, at that moment, gained evidence that there was an enzyme in the blood of women that converted estrone to an inactive form.

Schwenk wasn't at all happy with our data and explained his doubts to us thoroughly. The prime one was that steroids, like cholesterol, were very labile. Thus, all we were doing could be nothing more than permitting spontaneous oxidation to occur. Experiments were designed to get around that point. They were successful. We then found that postmenopausal women lost the enzyme, but not if they had carcinoma of the breast. It took 10 more years to find what the enzyme did. It converts estrone to the inactive form of estradiol.

The other task we were tackling was the synthesis of ^{14}C-labeled hormones. The chemists could not introduce it into the molecule. They can now, of course. Schwenk read a paper from Schoenheimer's group which described the biosynthesis of cholesterol from labeled acetate by a rat's liver. He told me that, if I could biosynthetically label 100 grams of cholesterol using liver, he could make us all the labeled hormones the Worcester Foundation needed.

There is no need here to cover the biological findings of that research. It may amuse you, however, to know that the second time I met Dr. Bloch, we were on opposite sides in a hot argument at a Laurentian Hormone Conference.

The 100 grams were synthesized and several papers were written. Schwenk was especially kind to include me as an author in those dealing strictly with chemistry. That is why some people think I know sterol chemistry. I do not. But Schwenk and Fieser were always on hand to advise me how to do what we did in our measurements on the flow of the lipids and steroid hormones.

In 1956 the late Dr. Russell Holman and I organized a workshop in San Antonio. By that time our interest had shifted to atherosclerosis. Just as was notoriously done recently, one of my Trustees lent us her private airplane to bring Schwenk, Fieser, Kritchevsky, and Seifter to the session.

Seifter is a pharmacologist. His type of pharmacologists are firm believers in dosage-response curves in bioassays. They also check out the time needed for an agent to act. If they think that one response of their agent can be produced by something else, they check to see if the results are the same.

Seifter presented data on about a thousand rabbits. He had fed graded doses of cholesterol for varying lengths of time. The blood cholesterol levels were determined at all stages. Then, by various methods, he induced the same levels, but the cholesterol was synthesized by the rabbit. They had none in their diet.

The rabbits who built up their own cholesterol levels to as high as one gram percent did not get any lesions no matter how long they were kept that way.

Seifter finished his talk by asking Schwenk and Fieser, "Why?"

Fieser was younger than Schwenk and got to the blackboard first. He put the formulae of a dozen compounds on the board. "Among these," he stated (with Schwenk in complete accord), "is the active substance or substances. You have to find out which one."

This story is summarized briefly in our paper which appeared in the Archives of Pathology and Laboratory Medicine in November, 1976.

Schwenk tried to test the thesis by feeding cholesterol purified via dibromination every day. He failed and concluded that he had been wrong in agreeing with Fieser. I thought I knew why he failed when I spoke with Taylor and Lee. So, instead of purifying the cholesterol, we concentrated the contaminants of old and new U.S.P. cholesterol. But we had no assay system until one day Dr. Lee and I received a reprint from the late Dr. Shimamoto.

In that paper Shimamoto described a 4-hour effect of cholesterol on a rabbit's aorta observable by scanning electron microscopy. Dr. Imai, in Lee's group, knew Shimamoto's technique. The concentrate was given in that fashion; 24 hours later the rabbit died of hemorrhages through ruined arteries. That rabbit should be canonized. None have reacted so beautifully since then.

Naturally I set about isolating the active components. I got nowhere.

When I'm in England, I visit Dr. A. T. James and discuss my problems. He put Dr. Dunphy onto the task. Dunphy quickly separated out three fractions. Two were active. One ran with cholesterol. The other stayed at the origin of a thin-layer chromatogram. The middle fraction containing the hydroperoxides was inactive. The relative weights were such that we could assume that what was active was present in small quantity and also that it was there in at least two forms.

At this same time, as part of my ONR duties I visited the Army's Food Research Laboratories at Natick. There I was shown how they dried a mixture of milk and eggs. As politely as I could, I suggested that their methods were producing atherogens and carcinogens out of the cholesterol in the eggs and milk.

They hauled me over to meet Dr. Merritt. I was given a can of the dried milk and egg mix supplied to those on active duty in our Armed Forces. We extracted the lipids and separated out the sterols. Mr. Yeomans identified the 5,6-epoxide of cholesterol. It is supposed to be a skin carcinogen.

Needless to say, this finding increased our support from within our own institutions.

The next incident was my attending a Gordon Conference on atherosclerosis. Kritchevsky gave such a complete summary of the dietary experimentation that there was no discussion. That is not supposed to happen at a Gordon Conference. Kritchevsky, although he knew of our early data, had not mentioned it. Since he was an old friend, I felt it proper to rise and excoriate him for his negligence. Even that produced no discussion. But it did bring me to Dr. Watson's attention.

Not long afterwards, I was in his laboratory. I came home with a list of compounds. We bought some and Dr. Merritt's laboratory checked them for purity. They were not very good.

So I cnecked around Boston for a synthetic sterol chemist. Fieser and Schwenk were retired and ill. Soloway's name got on the list. He was one of my contractors on another project entirely. He brought in Drs. Subramanyam and LeQuesne. Not too long afterwards we were being supplied with compounds that even Dr. Merritt's laboratory considered pure.

That leaves Dr. Leduc. She got into this because ONR wants its Branch Office members to do research of relevance to the Navy. Funds can come from ONR. To avoid conflict of interest, we are obliged to have the work done under the sponsorship of a university scientist who is (a) interested and (b) of sufficient stature and reputation so as to insure that no "hanky panky" is going to go on. The mechanism employed is simple. The sponsor has title to the money, makes the reports, and is responsible for their content.

WORKSHOP FOR STUDY AND REVIEW OF ANGIOTOXIC AND CARCINOGENIC STEROLS IN PROCESSED FOODS

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DR. IMAI: This electron micrograph illustrates a portion of the thoracic aorta from a New Zealand White rabbit that was given a total of 1 g/kg of recently highly purified cholesterol divided into 25 to 100 mg per dose extended over a seven-week period. Such a picture is indistinguishable from that of an untreated control. In this perfusion-fixed aorta the endothelium is flat; the intima is quite narrow; and the internal elastica is nearly straight. In the underlying media the smooth muscle cells and the connective tissue form alternating layers. Next please.

This is an example of an extensive medial damage due to the concentrated impurities obtained from USP cholesterol. These smooth muscle cells are the usual and, thus presumably, viable smooth muscle cells. This portion of the cytoplasm is severely condensed suggesting cell death by pyknosis, and these are what I interpret as severely distorted and fragmented remains of nuclei with disrupted cytoplasm. These dense particles are further deteriorated and fragmented nuclei. The boundary of the cell is not clearly discernible.

As I shall illustrate in a later session, by looking at these cells under the electron microscope and counting certain numbers of cells, I can express the angiotoxicity of test agents in terms of the frequency of dead and dying cells. Next please.

This is from a rabbit that was given a total of 1 g/kg of the concentrated impurities divided into 25 to 100 mg/kg per dose extended over seven weeks just as in the cholesterol control. The internal elastica is this wavy line. This portion of the intima is about normal, and there is diffuse fibromuscular thickening as shown here. Such a lesion is devoid of foam cells and stainable lipids. I emphasize this absence of foam cells and stainable lipids because the lesion in the rabbit induced by conventional cholesterol feeding of 1 to 2 g/day is characterized by the abundance of foam cells and stainable lipids. There was no concurrent hypercholesterolemia in our rabbits. Next please.

In the process of bioassaying the angiotoxicity of oxidation products of cholesterol, we found that the pulmonary artery responded by grossly visible thickening in 72 hours after intravenous injections of the test agents suspended in saline. This particular rabbit was given two doses of 5 mg/kg of 25 hydroxycholesterol and one dose of 5 mg/kg of cholestane-3β,5α,6β-triol. The rabbit was anesthetized with 50 mg/kg of pentobarbital sodium 24 hours after the last injection. The heart and blood vessels were perfusion-fixed under initial pressure of 25 mm Hg.

The bronchus is in the middle; the pulmonary vein on the inner side is unchanged. The pulmonary artery is thickened and the lumen remains narrow, resisting the initial perfusion pressure of 25 mm Hg. The wall has a characteristic gelatinous white appearance. The slice of lung on the right side is from a rabbit that was given three doses of 5 mg/kg of cholesterol purified via dibromination. The bronchus in the middle, pulmonary vein, and the pulmonary artery. This pulmonary artery is similar to that of the saline-injected or non-treated control. The changes due to injection of the suspension of purified cholesterol are confined to the peripheral arterioles. These changes are embolic crystals of cholesterol, thrombus formation, and organization of the thrombi leading to fibrous thickening of the peripheral arterioles. Next please.

This grossly visible thickening of the pulmonary artery after the injection of saline suspension of oxidation products of cholesterol is due to a series of changes. At one end is this acute necrotizing angitis as shown here. The lumen remains narrow despite the perfusion pressure or perfusion fixation. The endothelium is changed but present. The intima and media are inflamed amd edematous. Nuclear debris are present throughout the wall. In contrast, the adventitia is only slightly edematous. The adjacent lymphatics are distended, which I think is due to the inflammatory edema. Thus it appears that the agents that are responsible for these changes of the pulmonary artery reach the wall from the main lumen rather than from the surrounding blood capillaries. Next please.

This micrograph is from the same lung. There is acute necrotizing inflammation of the superficial layer. In addition, this arterial wall has diffuse fibromuscular thickening and mound-like fibrous plaques. These changes happened in three days after the first intravenous injection of the oxidation products of cholesterol. Next please.

This is an example of an extensive medial damage due to the concentrated impurities obtained from USP cholesterol. In the summer of 1976 I took my sabbatical at the Tokyo Metropolitan Institute of Gerontology where a particular strain of rabbit was available. This strain is Japanese White Nippon Institute of Biological Sciences, or 2W-NIBS, and this is said to be at F-17. This particular strain of rabbit is especially susceptibe to this kind of arterial injury. From 10 to 40 mg/kg of the concentrate suspended in saline was injected daily for three days. Tissues were fixed in 24 hours after the last injection. Extensive damage to the media is readily demonstrable by light microscopy. Most of the smooth muscle cells are either gone or are dead and seen as nuclear debris. This sort of medial damage has not been produced in New Zealand Whites. Next please.

The speed of repair in such damaged aorta is slow as compared to the speed of response in the pulmonary artery. The rabbit was left alone after such a single chemical injury for 10 weeks on the stock diet of commercial pellets. This is again from the thoracic aorta. There are foci of hypocelluarity suggesting the persistent cell loss due to the previous cellular injury. Next please.

There are areas of diffuse fibromuscular thickening and such mound-like plaques. These were found in an average of three out of 24 consecutive cross sections of the descending thoracic aorta. Next please.

At a higher power this is the internal elastica. There is this much of the intimal fibromuscular thickening. There are some foci of hypocellularity, and some collections of bundles of muscle cells that are arranged in an abnormal fashion; that is, parallel to the long axis of the aorta. These bundles suggest proliferative response of the smooth muscle cell.

In summary of this outline of the angiotoxicity of cholesterol and these oxidation products:

(a) Purified cholesterol at unconventionally small doses, such as a total of 1 g/kg extended over seven weeks by gastric tubing or 15 to 30 mg/kg in three days by intravenous injection of saline suspension, does not induce significant arterial lesions.

(b) Oxidation products of cholesterol, individually combined, or as a naturally occurring mixture at the same or lower dosages as used in (a), induce significant

enhancement of arterial smooth muscle cell death, and in a particularly susceptible strain of rabbits, extensive necrosis of the aorta, in the 24- to 72-hour bioassay. A basic form of arteriosclerosis or intimal fibromuscular thickening can be induced by the same agent in seven to 10 weeks.

(c) The determination of relative potency of discrete oxidation products and the search for the most angiotoxic combination are in progress. Data from these current experiments will be given at a later session.

DR. AHRENS: Would this be an appropriate time to ask for a clear definition of what is meant by pure cholesterol and what is meant by oxidation products?

DR. WERTHESSEN: Purified cholesterol is defined as dibromo pure. Most of it was prepared by my wife as she learned to do it under the tutelage of Dr. Schwenk. We don't like it to be more than four to six weeks old. It's not as pure as Lew Engel's done by the Schoenheimer technique. I believe Dr. Merritt can show us that even the dibromo pure is not perfectly pure. As to the oxidation products: What he was showing you there, where he says "the concentrate" is the original concentrate I made by taking about 5 kilos of Bruce Taylor's wonderful yellow, rancid, aged cholesterol and/or fresh USP cholesterol and partially dissolve the stuff in hot methanol, let it cool and collect crystals, then run it down to a mother liquor, which on evaporation under nitrogen gave a crude oil. And that's the concentrate. Does that define it for you?

DR. AHRENS: Are there physical constants that could be used to characterize pure cholesterol that's been through the dibromination process?

DR. WERTHESSEN: We never employed any because my experience with Schwenk (he did all of that way back) was that if you go through this dibromination twice in a row, with his procedure, that's as pure as you can get it.

DR. MERRITT: May I comment, Nick?

DR. WERTHESSEN: Surely.

DR. MERRITT: It seems to me that although it probably hasn't been done before that in considering what you call, or specify as, pure cholesterol, you ought to give some consideration, after its gone through the purification procedures of dibromination or whatever, to make some specification as to how it's stored thereafter, because as you know it's a very labile compound and unless it's properly kept to avoid oxidation, you're going to have oxidation products.

DR. WERTHESSEN: Well I was talking to Dr. Ahrens on that point and he's supposed to know something about cholesterol, so I figured he'd know you kept it in the cold and under nitrogen.

DR. AHRENS: Did you keep it in solvent or in crystalline form?

DR. WERTHESSEN: For this work it was always kept as crystals but under nitrogen in the freezer. But it doesn't last that way long either as Dr. Smith can tell us later anytime he wants to.

DR. L. SMITH: If I could make just a brief comment about Dr. Ahrens' question. The physical constants of pure cholesterol are certainly available and I would not advise anybody to disregard their use. One can make the batch up and keep it under conditions, free from oxygen, and it may remain fairly acceptable. However, the batch should be purified to standards before one may trust its purity, and it ought to be kept free from air; otherwise, air is going to cause degradation. It depends how long the sample is kept and under what conditions as to how much deterioration may be involved.

DR. AHRENS: Am I correct in believing that mass spectrography is a relatively insensitive detector of impurity, perhaps at a level of 1% or higher?

DR. L. SMITH: Well one percent is easily found. One needs only a simple thin-layer chromatogram to show a one percent impurity, but we are talking about components present at less than one percent, and measurment of such levels has to be achieved by additional chromatographic methods. Mass spectrometry directly on a sample might not detect these impurities readily.

DR. WERTHESSEN: Do you want to add to that, Charlie?

DR. MERRITT: Oh, I just want to confirm what Dr. Smith has said. I think if you are looking for a criterion to establish the purity of the material, probably it is best to use a chromatographic procedure--probably liquid chromatographic procedure on silica gel or something like that would be able to show the presence of oxidation products.

DR. L. SMITH: I have tried to derive criteria that might be free from argument with respect to what is pure cholesterol. There are pitfalls in setting forth stringent conditions of testing. High resolution or high efficiency gas chromatography, thin-layer chromatography, and more recently available high pressure liquid column chromatography appear to be essential tools, but the melting point (Kofler, under the microscope) remains a highly sensitive measure of purity.

DR. AHRENS: I wonder whether any of you--I've never tried it--but I wonder if any of you have ever tried using your tongue as a chromatographic column. It's the most sensitive organ in our body for picking up trace materials. I wondered if that had ever been even attempted.

DR. WERTHESSEN: I don't think there's any flavor to these, Pete.

DR. L. SMITH: Use your nose. One can smell these components. If one makes pure cholesterol by bromination, debromination, recrystallization, etc. and then starts smelling the preparation every day, there will come a day when an odor is perceived. This is the first sign of serious autoxidation. One cannot readily detect autoxidation at this stage chemically, but one can smell it.

DR. AHRENS: Well that's the point I was trying to make.

DR. WERTHESSEN: We'll go into this with much more detail later, so you can see that what we were dealing with was contamination of the order of one part per thousand and it would have satisfied the melting points and other things.

DR. SHEPPARD: We who have grappled with high pressure chromatography have had more than simple problems with the oxidation products. The difficulty is that all of them do not have chromophores in them. If you are using UV detectors, you won't find them.

DR. MERRITT: Well, that depends on the wave length.

DR. SHEPPARD: Not entirely. Some of them you pick up and some of them you don't. You can use an RI detector, but unfortunately it's so temperature sensitive that it is necessary to have some means of stabilizing the surrounding temperature environment.

DR. SCALLEN: In the controls that were fed the purified cholesterol, even though there weren't lesions, did the cholesterol content of the arterial wall increase?

DR. IMAI: I have not determined the cholesterol content in these rabbits. All I have is the serum cholesterol values and these are all within normal limits and, if anything, these are on the low side.

DR. TAYLOR: Since this is a heterogeneous group of scientists I shall quickly review the methods of repair of damaged or degenerated portions of arterial walls. If the entire group has a similar concept of arterial repair, I believe the thinking of all participants should run along similar lines and, hopefully, theories and future plans and goals will develop more soundly and harmoniously. When there is severe damage or degeneration of the arterial media, an aneurysm will develop at this site. Rupture of the aneurysm does not usually develop because the extracellular collagen fibers and elastic membranes are only minimally damaged; in earlier studies all cells of a medial segment were killed by hypothermal injury. (Taylor, 1955 and Cox et al., 1963). Figure 1 (lower half) shows a hematoxylin and eosin stain of a normal rabbit's aorta; note that the intima consists of a single layer of endothelial cells lying directly over the convoluted internal elastic membrane. The upper half of Figure 1 is a Weigert's elastic tissue stain. It again shows the close proximity of the internal elastic membrane to the lumen of the vessel; there is no intimal thickening. Figure 2 shows a segment of rabbit's aorta one week after killing all medial cells with expanding carbon dioxide in a special instrument (temperature -52° C); note that extracellular collagen and elastica are essentially intact and also that in the lesions produced by applying hypothermal injury externally, at the external elastic membrane, the endothelium survived. At this time (one week) there were distinct bulging aneurysms at sites frozen, undoubtedly due to loss of medial smooth muscle cells. Figure 3 illustrates a florid proliferation of primitive mesenchymal (Langhans') cells between the endothelium and the severely damaged media. Note the calcified media and elastic fibers; this finding of calcification of damaged media was moderately common in rabbits' old, killed media. At this age (8 weeks) the primitive cells had just begun to differentiate and moderate amounts of thin elastic and collagen fibers could be demonstrated. At five to six weeks after aortic damage, even though smooth muscle cells were not yet fully developed, a rich network of new elastic membranes and collagen fibers had begun to develop (Fig. 4). Full maturation of elastic membranes and formation of a new internal elastic membrane in the intimal scar required five to six months. Primitive mesenchymal (Langhans') cells in the proliferated intimal scar required 10 to 12 weeks for differentiation into mature functional smooth muscle cells (Figs. 5 and 6 - lower half). At 12 weeks after hypothermal injury, aneurysmal dilatations at sites of injury had been corrected by the regenerated, functional vascular tissue in the intimal scars. All studies discussed above were done in rabbits and monkeys on a cholesterol-free diet; rabbits' serum cholesterol levels were 70 mg/dl or lower and rhesus monkeys' serum cholesterol levels averaged 154 mg/dl.

In addition, our group produced cold injury of arterial segments in rabbits (Kelly Jr. et al., 1952) and monkeys (Cox et al., 1963) after elevated serum cholesterol levels had been induced by adding cholesterol and neutral fats to the diets. Figure 6 (upper half) shows an aortic lesion produced in a rabbit when its serum

Dr. Bruce Taylor's Figure 1.

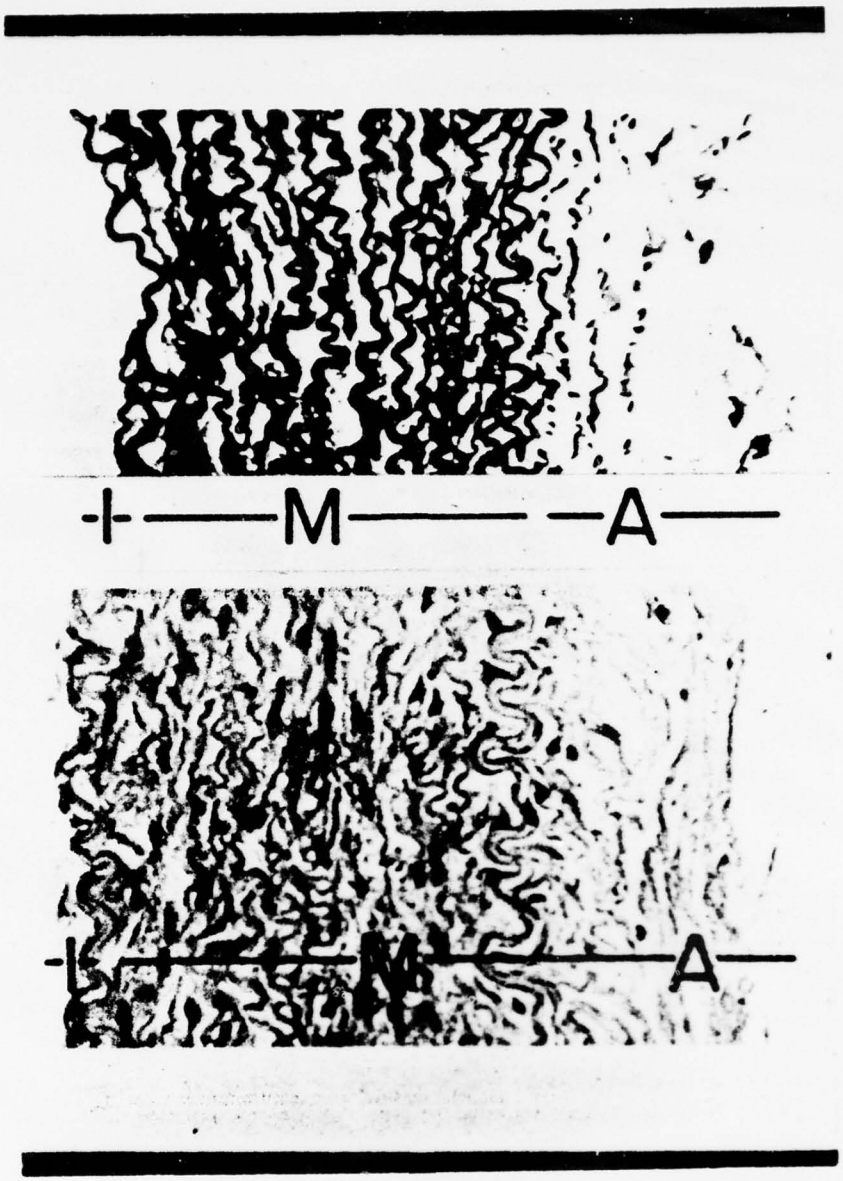


Figure 1

Photomicrographs of a cross section of a hematoxylin and eosin stain of a normal rabbit's aorta (lower portion). Note that the endothelium lies essentially on top of the internal elastic membrane. The intima is labeled "I"; media, "M"; and adventitia, "A". Upper portion is a Weigert's elastic tissue stain of a normal aorta. Note that there is no intimal thickening in this vessel.

Dr. Bruce Taylor's Figure 2

Figure 2

Cross section of a hematoxylin and eosin stain of an aortic wall from a rabbit one week after freezing. Intima at this time is intact. Smooth muscle cells of media are totally disintegrated. Granules of calcium have appeared in the matrix between medial elastic lamellas. Elastic lamellas are straight because of absence of viable smooth muscle cells. Rare inflammatory cells are present in adventitia.

Figure 3

Cross section of an aortic wall eight weeks after freezing. The regenerated intima closely resembles the media of a normal aorta. Most cells have the morphologic aspects, distribution and staining reactions of smooth muscle cells. The matrix between these cells is composed principally of elastic tissue. The damaged media contains scattered fibrocytes, collagenous fibrils and degenerated elastic lamellas in its inner half. The outer half of the media is a homogeneous mass of fragmented calcified tissue. The calcium deposits are partly surrounded by macrophages and show evidence of early resorption.

Dr. Bruce Taylor's Figure 4

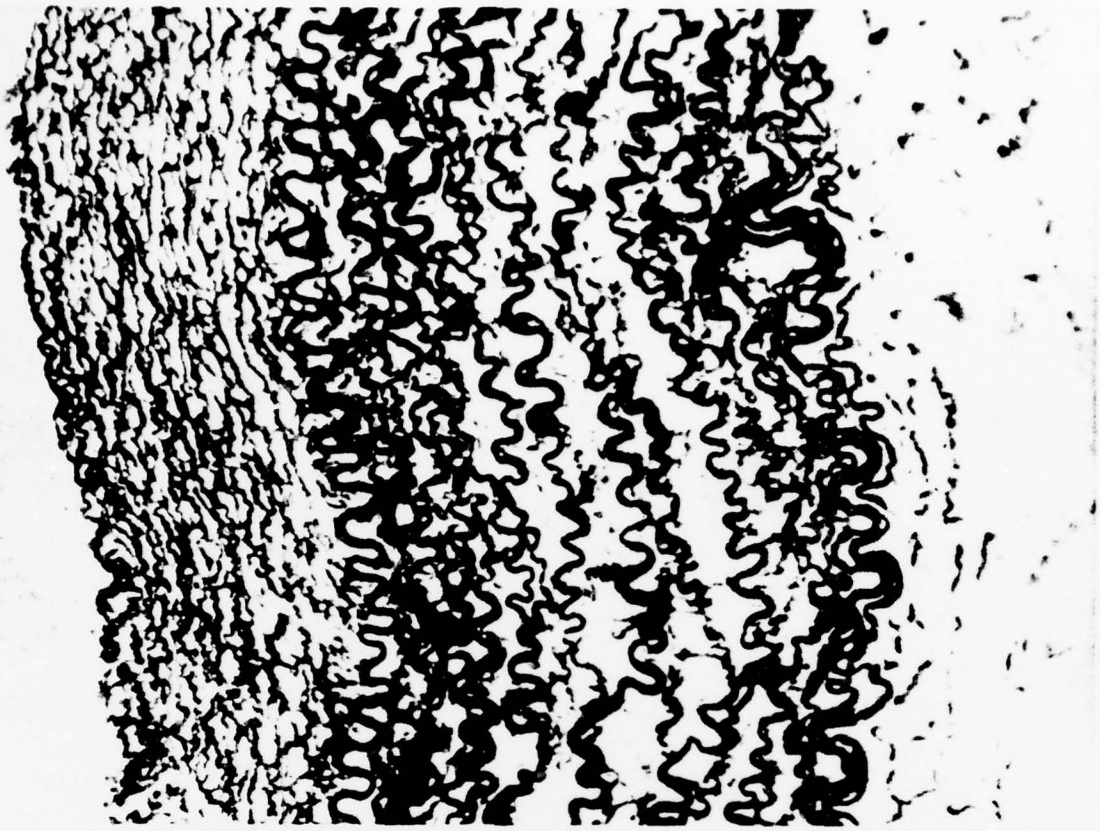


Figure 4

Elastic tissue (Weigert) stain of an aortic lesion, six weeks old, produced by freezing the abdominal aorta of a juvenile rabbit on a normal diet. The proliferated intima contains many thick fibrils which have become oriented to resemble thin medial elastic lamellas. At about 12 weeks elastic tissue in the proliferated intima had structure very similar to that of the medial coat of a normal aortic segment. Elastic lamellas of the media apart from the internal and external elastic membranes are discontinuous, fused, irregular, and distorted. Elastic tissue of the adventitia appears normal.

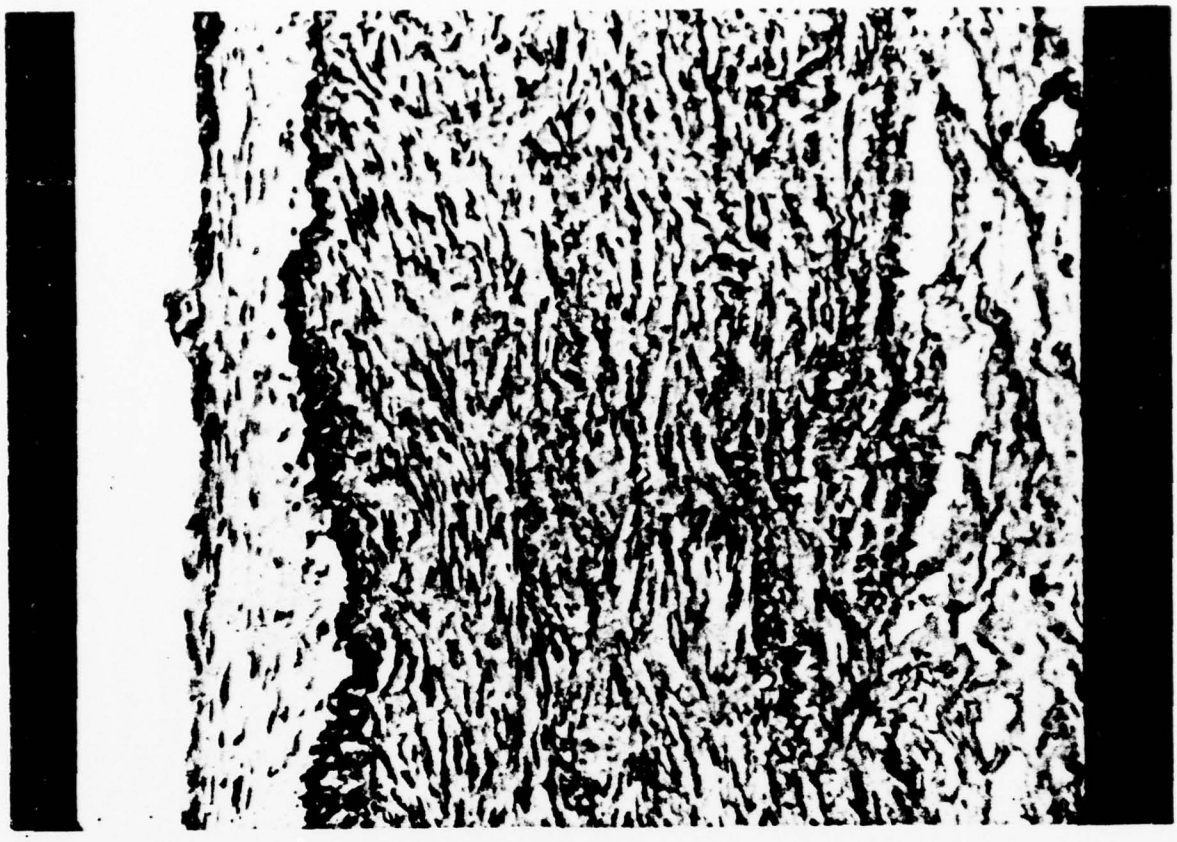


Figure 5

Photomicrograph of healed, hypothermal lesions (22 weeks old) in control monkey with mean serum cholesterol of 119 mg% (stained with Sudan IV and aniline blue). Note complete absence of stainable lipids in intima or media.

Dr. Bruce Taylor's Figure 6

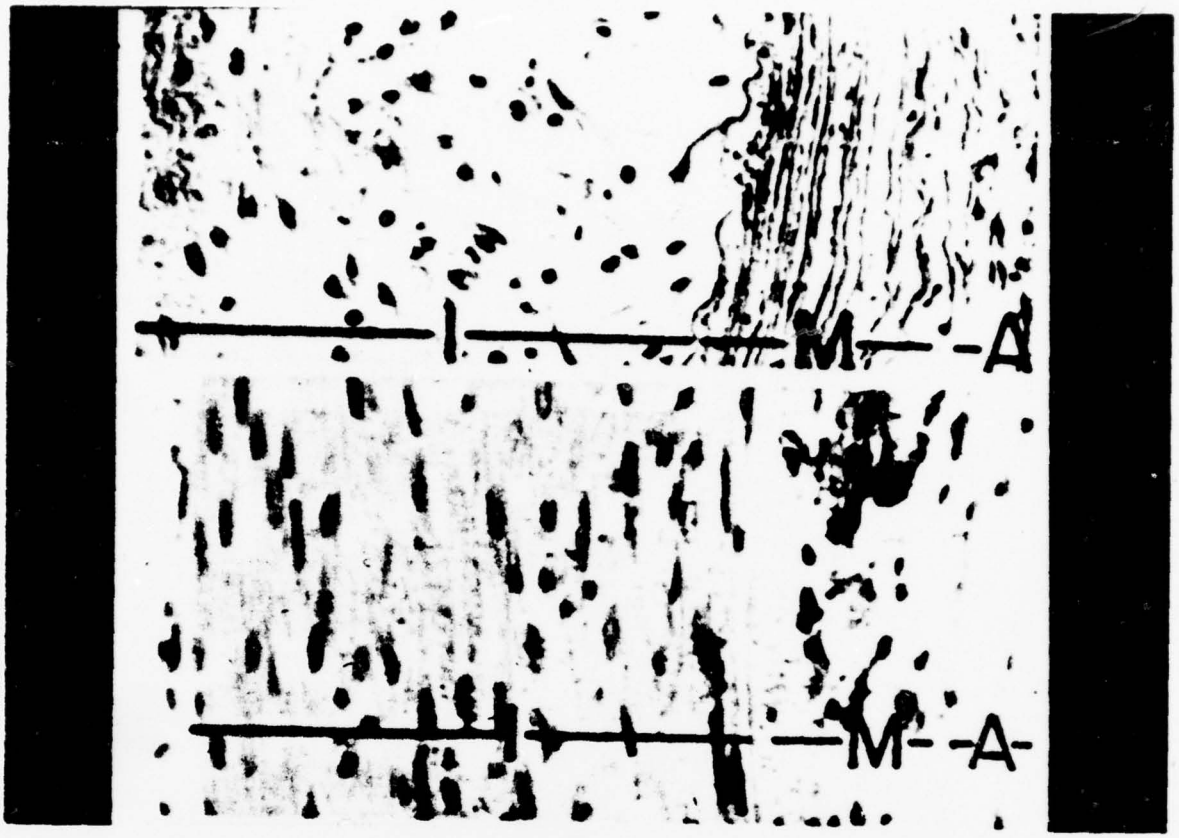


Figure 6

(Upper half). Lesion, 26 weeks old, produced by freezing the wall of the lower abdominal aorta of a rabbit with an average blood cholesterol level of 1,090 mg. per 100 cc. In hypercholesterolemic animals, lesions less than nine weeks old usually showed this "mucinous" degeneration of the proliferated intima. In this animal, 70% of the intima of the thoracic aorta was involved by atheromas, and there were no microscopically demonstrable spontaneous atheromas of the lower part of the abdominal aorta. The extent of the proliferated intima (I), the degenerate media (M), and the adventitia (A) are indicated. Note that the lipophage infiltration is restricted to the inner one-half of the proliferated intima. Comparison with Figure 6 (lower half) demonstrates the marked alteration in the intimal repair following freezing of the aortic wall. Fibroblasts are less numerous, and the newly-formed fibroelastic tissue has a peculiar loose reticular character resembling that found in "mucinous" degeneration of proliferated intima in human arteriosclerosis.

(Lower half). Cross section of a lesion, 12 weeks old, produced by freezing the abdominal aorta of a juvenile rabbit ingesting a vegetarian diet. The intima (I) at this stage is as thick as the original undamaged media. There is no localization of lipids in the lesion. It is composed principally of parallel smooth muscle cells lying between thick lamellas of elastic tissue. The badly damaged media (M) is largely occupied by masses of calcium, which are surrounded by macrophages and dense fibrous tissue. The adventitia (A) is composed of dense bundles of fused collagenous fibrils.

cholesterol was 1090 mg/dl; the lesion was allowed 26 weeks after freezing for its development. Note the large collection of lipid-laden macrophages in the deeper one-third of the intimal scar. The excellent regeneration of good functional scar tissue described earlier has been totally disrupted. Fibrocytes are much less frequent and no mature smooth muscle cells can be identified. The newly formed fibro-elastic tissue is of very poor quality and has the loose reticular character resembling that found in "mucinous" degeneration of proliferated intima in human arteriosclerosis. Figure 7 illustrates a lipid-laden intimal scar which developed three weeks after the common iliac artery had been injured, by freezing, in a rhesus monkey with a serum cholesterol level of 470 mg/dl. Figure 8 is a severe, constricting atheromatous lesion produced by freezing the common iliac artery of a rhesus monkey with a serum cholesterol level of 419 mg/dl; the damaged artery was removed for microscopic studies after 136 weeks had been allowed for it to form an intimal scar in the presence of a persistent hyperlipidemia of over 400 mg/dl. Note the abundant deposit of lipids in the extremely thick intimal scar which has reduced the lumen more than 80 percent.

Figure 9 illustrates an aortic lesion induced in the aorta of a rabbit fed only 1050 mg/kg of a concentrate of autoxidation products of cholesterol by gavage in doses of 100 mg/kg three times per week, and later reduced to 25 mg/kg three times per week, over a seven-week period (Imai et al., 1976). Serum levels of cholesterol, total protein, calcium, and phosphorous were normal throughout this seven-week period. This lesion consists of fibrous intimal scarring secondary to medial cellular damage produced by intestinal absorption of the isolated autoxidation products (extracted selectively from the unoxidized fraction of cholesterol with methanol). The intimal scars lie over the internal elastic membrane which is indicated by the arrows.

Figure 10 illustrates coronary medial death (lower segment of artery) with an overlying fibrous intimal scar. This lesion was produced by tube-feeding a total of 700 mg/kg of autoxidation products to a young adult rabbit over a period of 36 days; originally 100 mg/kg were fed three times per week, then the dose was reduced to 25 mg/kg three times per week (Imai et al., 1977). This animal's serum levels of cholesterol, total protein, calcium, and phosphorous were normal throughout the study. Had there been a hyperlipidemia in the rabbits illustrated in Figures 9 and 10, they undoubtedly would have developed fatty atheromatous deposits as illustrated in the hyperlipidemic rabbits and monkeys earlier (Fig. 6 upper portion). Figures 7 and 8. If one produces intimal scars in normocholesteremic rabbits (Ssolowjew, 1928) or normocholesteremic monkeys (Taylor et al., 1963) and allows healing for a period of two or more months then challenges these scars by inducing dietary hypercholesteremia, the scars are almost totally immune to atheromatous deposits. Interestingly, adjacent normal, unscarred arterial tissue develops florid atheromatosis (Figs. 11 and 12). The immunity of these vascular scars that have formed in normocholesteremic animals still remains an enigma which seems to contradict many concepts of atherogenesis.

I have a few closing comments. First I would like to point out that we have a colony of chickens in which half the hens can't lay eggs (Ho et al., 1974). When these non-layers are exposed to a photoperiod of 16 hours per day, their bodies synthesize large amounts of endogenous cholesterol but can't eliminate it by laying a daily egg (with a yolk containing 250 to 300 mg of cholesterol). One of the next experiments we must do is feed autoxidation products of cholesterol to these non-laying chickens with endogenous hypercholesterolemia as well as to a second group with diet-induced hypercholesteremia (using commercial U.S.P. grade cholesterol) and also a third group made hypercholesterolemic by feeding purified cholesterol free of autoxidation products. These three different studies should add considerable clarification to the problem of the role of autoxidation products in atherogenesis.

Dr. Bruce Taylor's Figure 1.

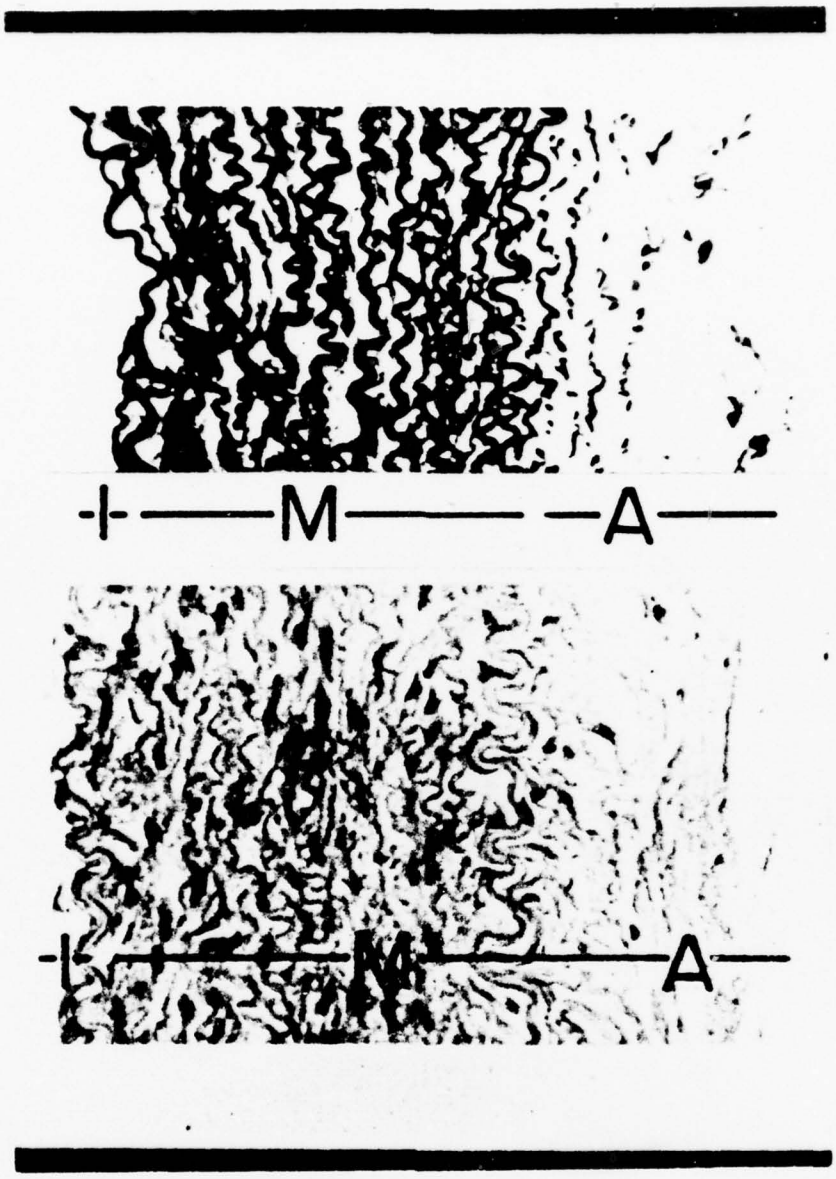


Figure 1

Photomicrographs of a cross section of a hematoxylin and eosin stain of a normal rabbit's aorta (lower portion). Note that the endothelium lies essentially on top of the internal elastic membrane. The intima is labeled "I"; media, "M"; and adventitia, "A". Upper portion is a Weigert's elastic tissue stain of a normal aorta. Note that there is no intimal thickening in this vessel.

Figure 2

Cross section of a hematoxylin and eosin stain of an aortic wall from a rabbit one week after freezing. Intima at this time is intact. Smooth muscle cells of media are totally disintegrated. Granules of calcium have appeared in the matrix between medial elastic lamellas. Elastic lamellas are straight because of absence of viable smooth muscle cells. Rare inflammatory cells are present in adventitia.

Figure 3

Cross section of an aortic wall eight weeks after freezing. The regenerated intima closely resembles the media of a normal aorta. Most cells have the morphologic aspects, distribution and staining reactions of smooth muscle cells. The matrix between these cells is composed principally of elastic tissue. The damaged media contains scattered fibrocytes, collagenous fibrils and degenerated elastic lamellas in its inner half. The outer half of the media is a homogeneous mass of fragmented calcified tissue. The calcium deposits are partly surrounded by macrophages and show evidence of early resorption.

r. Bruce Taylor's Figure 4

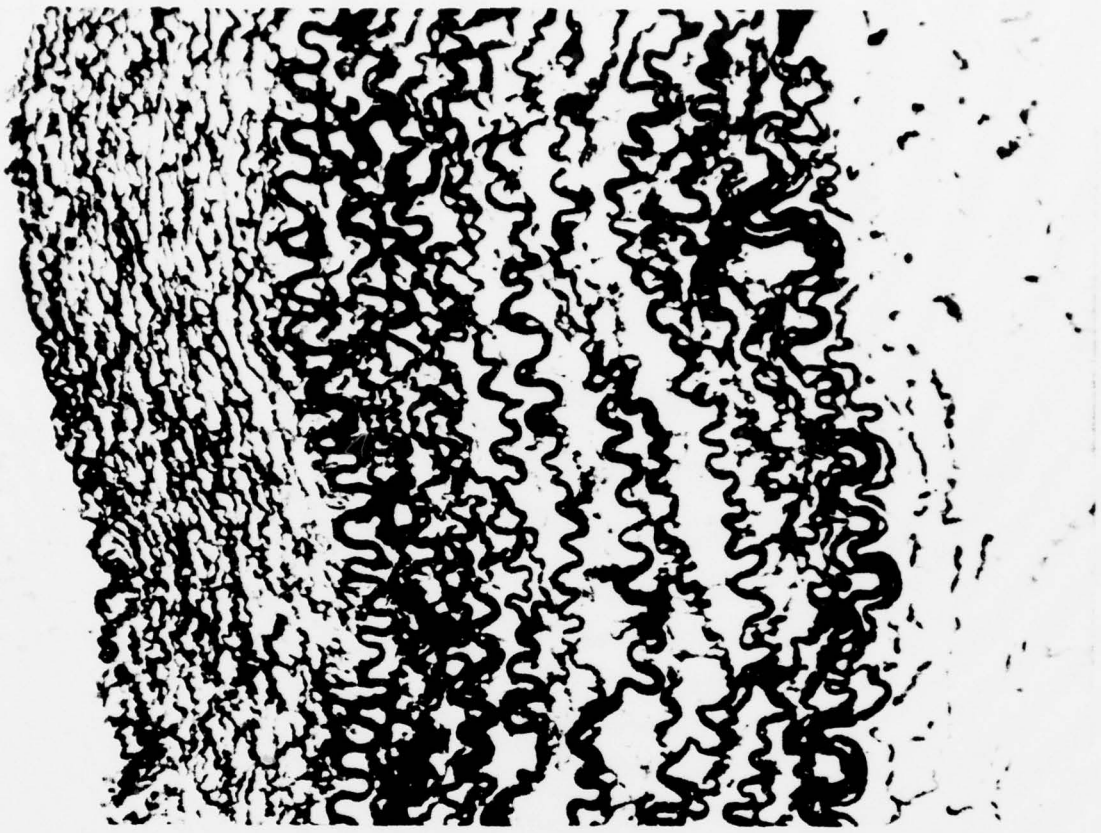


Figure 4

Elastic tissue (Weigert) stain of an aortic lesion, six weeks old, produced by freezing the abdominal aorta of a juvenile rabbit on a normal diet. The proliferated intima contains many thick fibrils which have become oriented to resemble thin medial elastic lamellas. At about 12 weeks elastic tissue in the proliferated intima had structure very similar to that of the medial coat of a normal aortic segment. Elastic lamellas of the media apart from the internal and external elastic membranes are discontinuous, fused, irregular, and distorted. Elastic tissue of the adventitia appears normal.

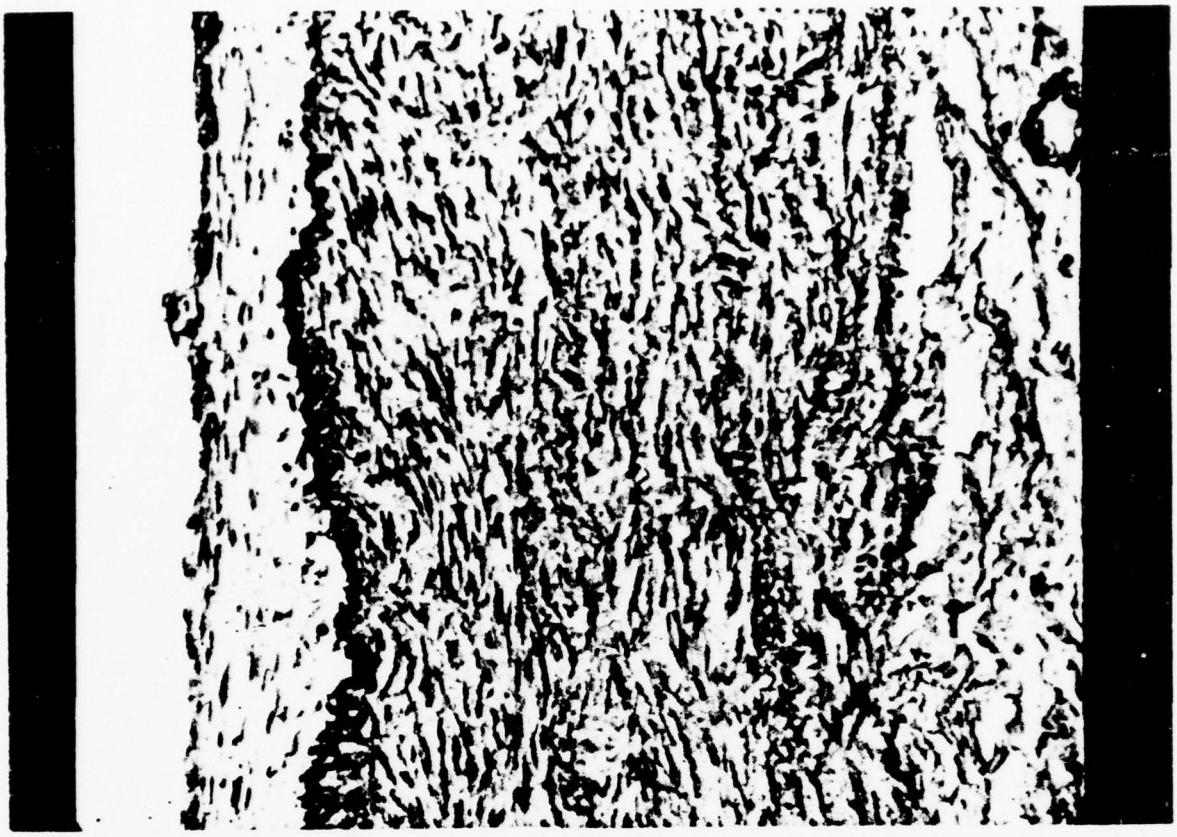
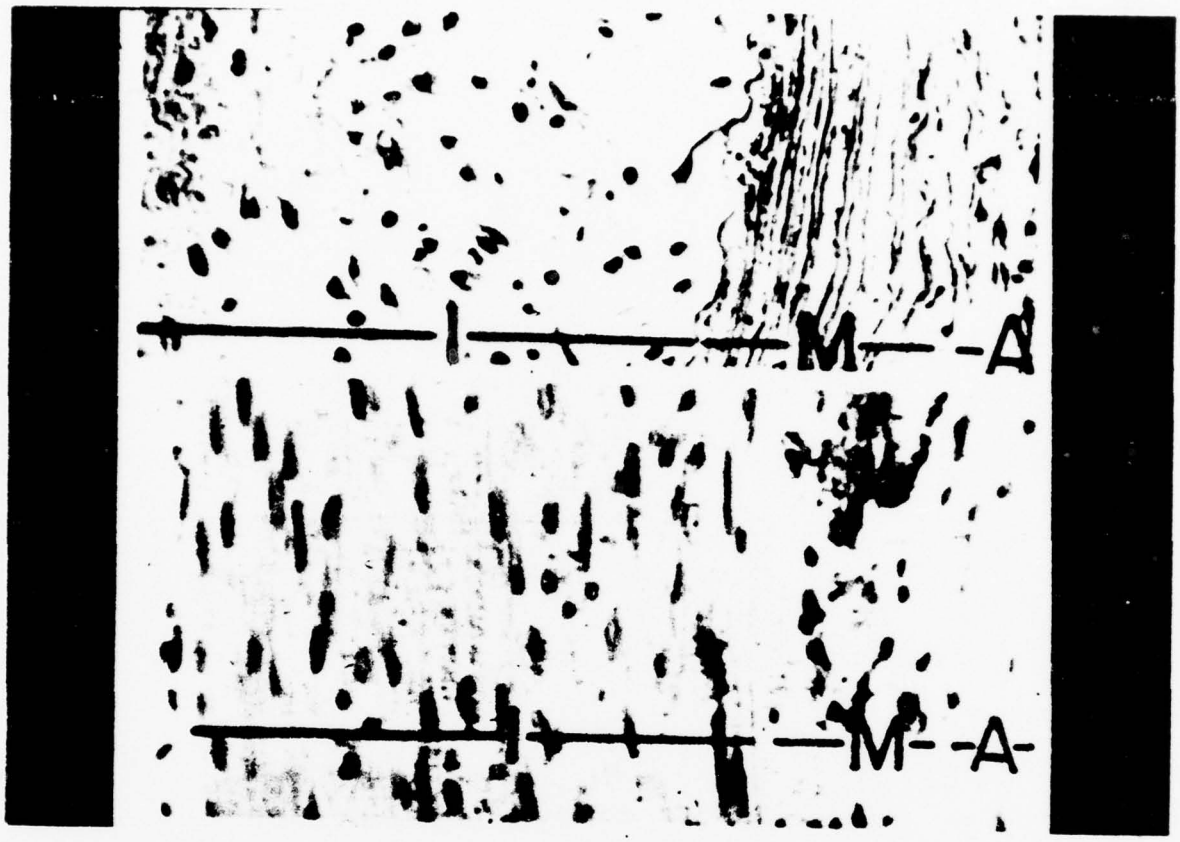


Figure 5

Photomicrograph of healed, hypothermal lesions (22 weeks old) in control monkey with mean serum cholesterol of 119 mg% (stained with Sudan IV and aniline blue). Note complete absence of stainable lipids in intima or media.

r. Bruce Taylor's Figure 6



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Dr. Taylor's Figure 7

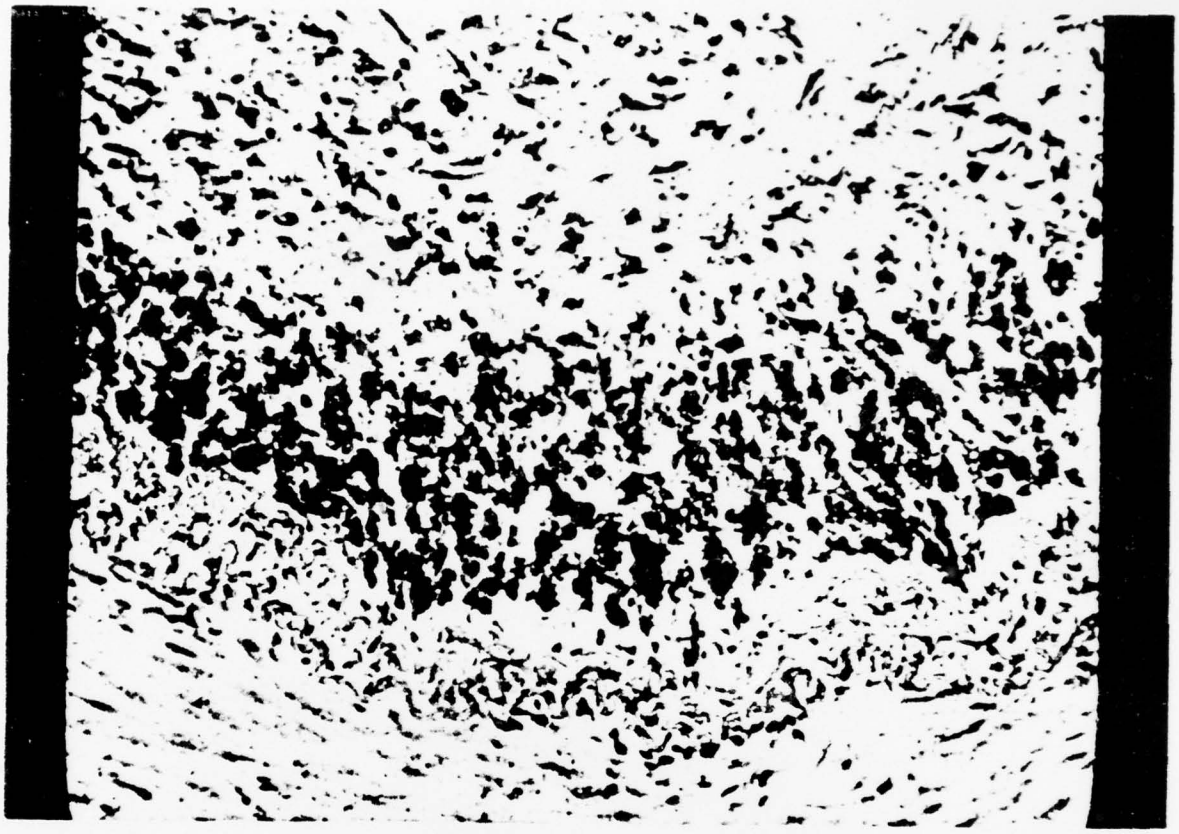

Figure 7

Photomicrograph (stained with Sudan IV and aniline blue) of hypothermal lesion three weeks old produced in monkey with serum cholesterol of 470 mg%. Note numerous lipid-laden macrophages just inside internal elastic membrane. Some elongated mesenchymal cells in more central (top) portion of intimal proliferation also have numerous fat droplets in their cytoplasm. In hypercholesteremic animals, multipotential mesenchymal cells differentiating into lipophages were seen frequently in younger, healing hypothermal lesions.

D Bruce Taylor's Figure 8

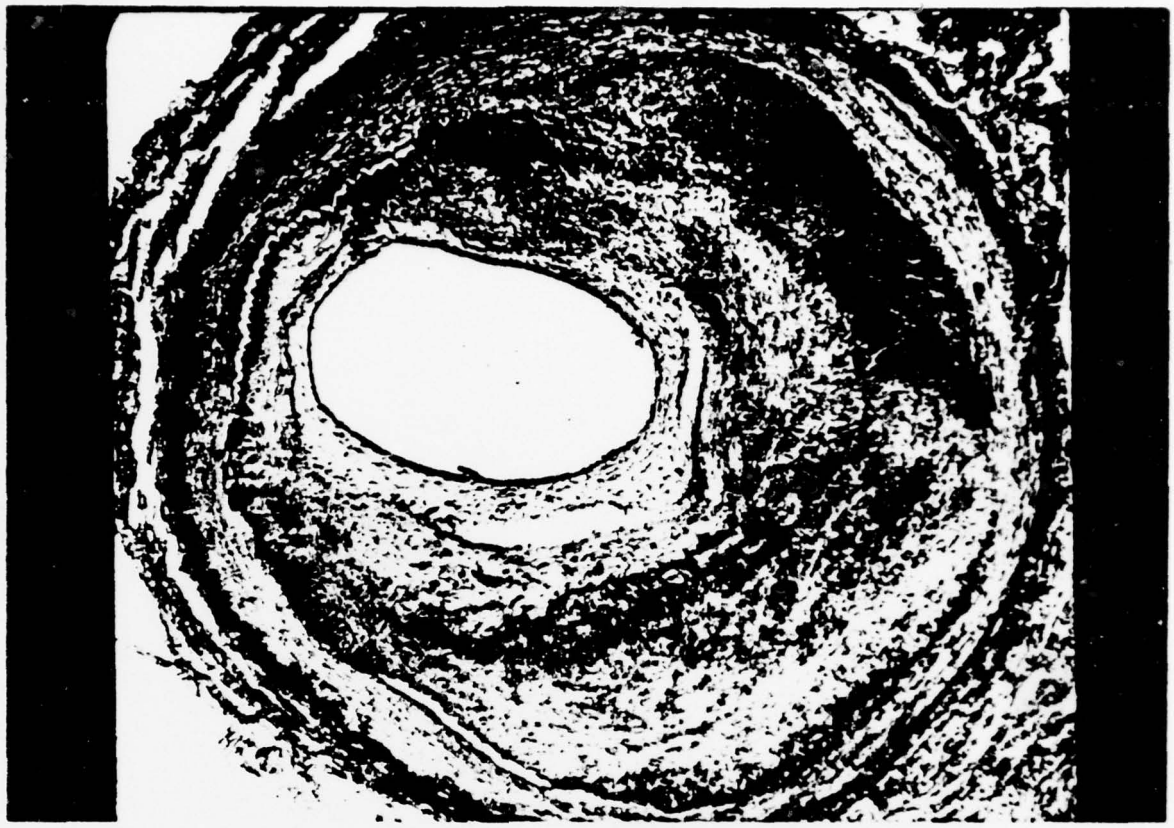

Figure 8

Photomicrograph of Sudan IV and aniline blue stain of ancient healed hypothermal lesion of common iliac artery. This lesion was produced when the animal's serum cholesterol was 419 mg% and healed while animal was maintained hypercholesteremic for period of 136 weeks. It is probably that deeper, red-staining lipid deposits accumulated as hypothermal lesion healed. However, more superficial lipid deposits probably were not related to experimental arterial injury and repair. Lumen of this vessel is reduced by about 80%.

D Taylor's Figure 9

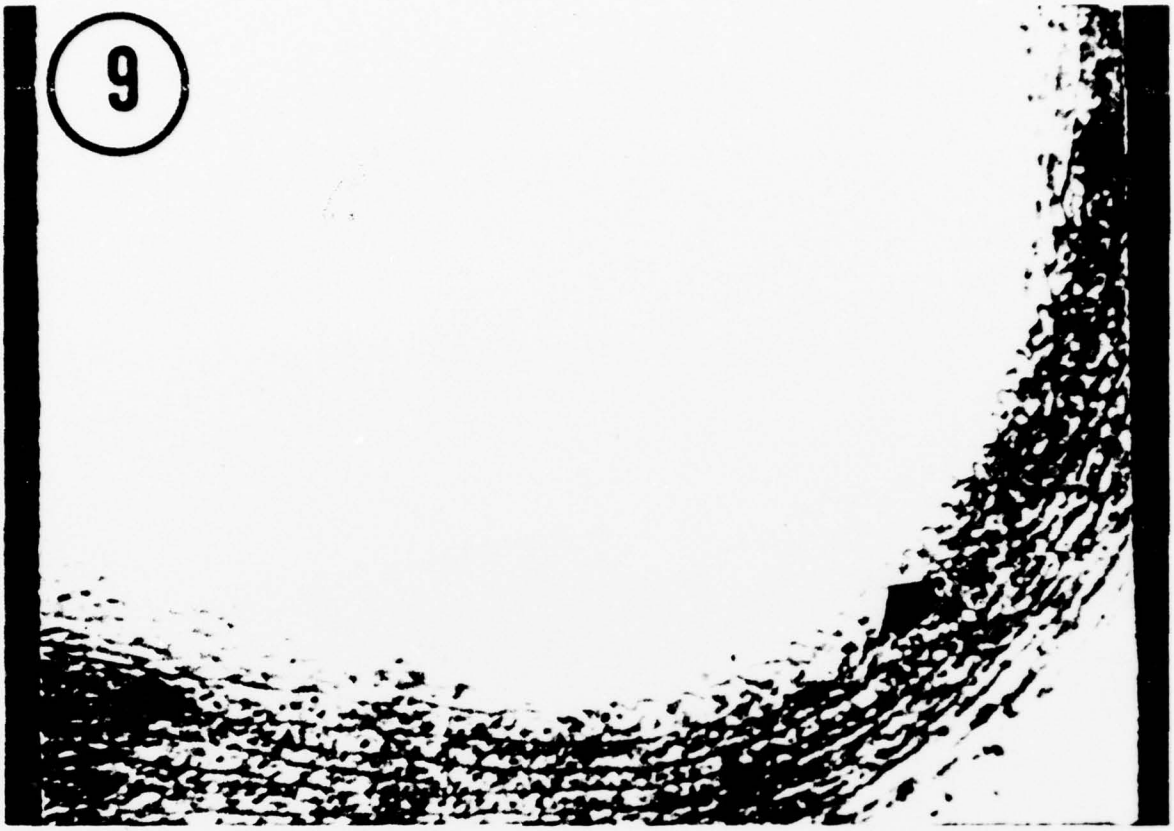

Figure 9

Arrows indicate internal elastica demarcating fibrotic intimal lesion from media of thoracic aorta in rabbit receiving 1,050 mg/kg over seven weeks, of concentrate (modified Altmann-Kull stain, original magnification x 20).

Dr. Taylor's Figure 10

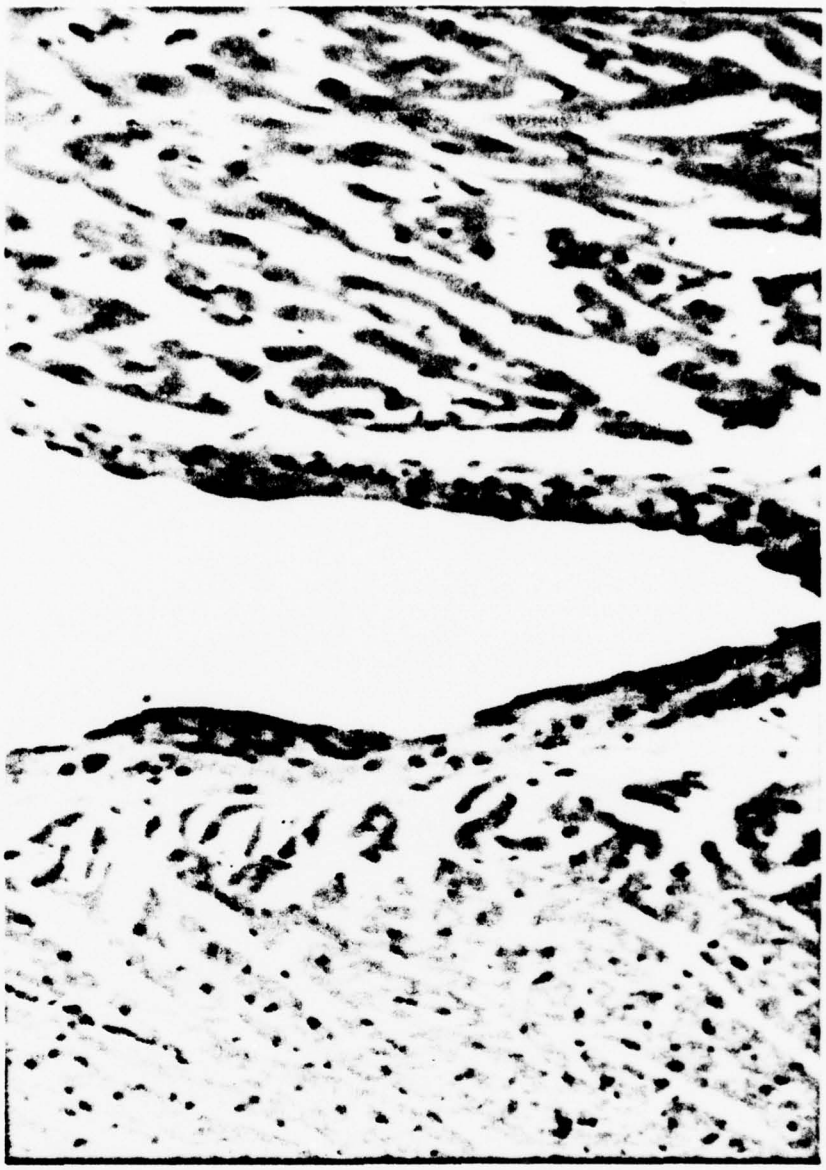

Figure 10

Photomicrograph of a hematoxylin and eosin stain of a rabbit's intrinsic coronary artery showing medial necrosis of smooth muscle cells with an overlying fibro-elastic intimal scar located just to left of center of lower portion of arterial wall. This animal received a total of only 700 mg of auto-oxidation concentrates of U.S.P.-Grade cholesterol over a period of 36 days.

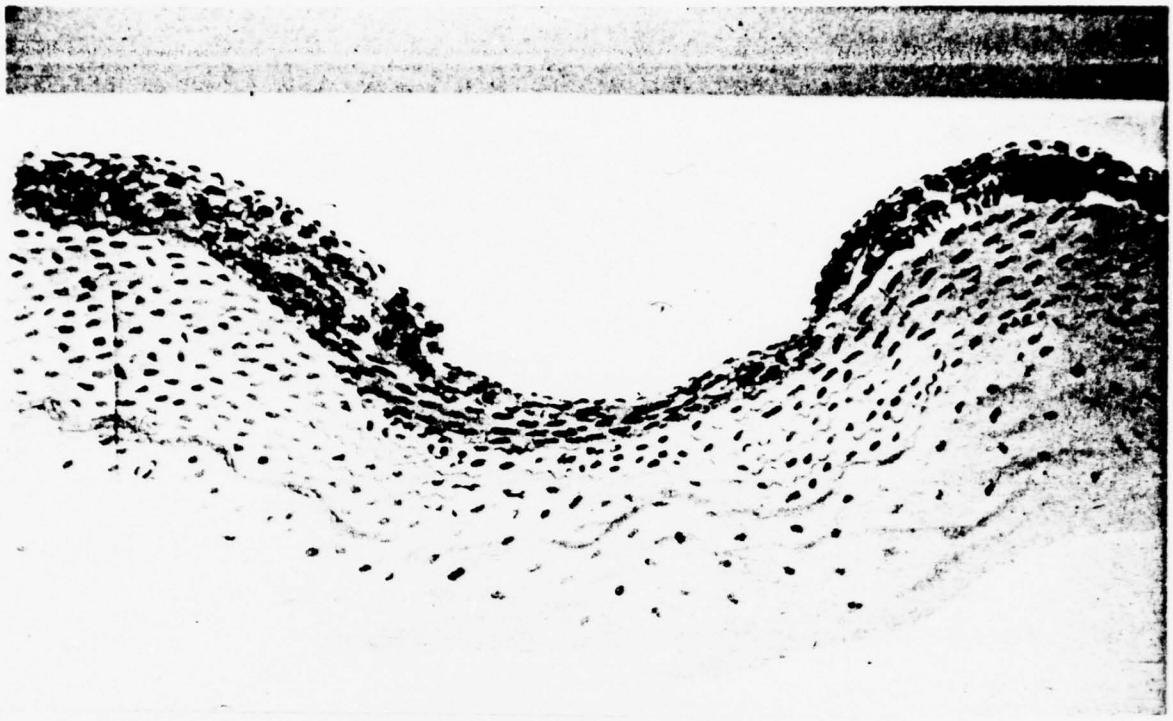

Figure 11

Drawing of rabbit's aorta reproduced from paper by Ssolowjew - 1928. Author produced aortic injury with cautery in rabbit's aortas and allowed 50 to 92 days for an intimal scar to form. After the cauterized lesion had healed, animals were placed on a high-fat, cholesterol-rich diet. Interestingly, the healed scar (center of illustration) produced by cautery was immune to atherosclerosis whereas the adjacent uninjured and unscarred sub-intima developed florid atheromatosis.

Dr Taylor's Figure 12

Figure 12

Photomicrograph of longitudinal section of healed common iliac arterial scar stained with Sudan IV and aniline blue (endothelial surface is towards top of illustration). Artery was locally frozen and healed while monkey had normal serum cholesterol of 157 mg%. Period of 15 weeks was allowed for lesion to heal and for intimal scar to form. Subsequently, a mean hypercholesteremic level of 321 mg% was induced and maintained for 25 weeks. Note complete absence of stainable lipids in intimal scar. Common iliac arterial scars (healed in the presence of normocholesteremia and later exposed to hypercholesteremia) showed even lesser propensity to accumulate lipids whereas adjacent uninjured arterial tissue showed 100% lipid deposition.

Twofold increase in thickness of the iliac arterial wall resulting from the thick intimal scar that has formed over the segment of vascular wall that was injured by freezing is well shown on the right half of illustration. On left margin is thinner, uninjured arterial wall which has no intimal scar and is about one-half the thickness of scarred vascular wall on right. This study was done by Taylor et al. - 1963.

It should also be pointed out that the first 100 pounds of cholesterol used in our studies was composed of about 40 percent autoxidation products of cholsterol. It is of considerable interest to know that newly-purchased (presumably recently isolated) cholesterol is already five percent autoxidized. Apparently the only way one can inhibit continuing autoxidation of cholesterol is to store it under nitrogen in a deep-freeze.

Anitschkow in 1913 first produced atherosclerosis in rabbits by feeding what he presumed to be pure cholesterol. Unfortunately during much of the last 60 years most of us have been using U.S.P. grade cholesterol (stored at room temperature and exposed to air) for feeding experiments for the production of atherosclerosis. In the past several years it has become very apparent that most of the work done over the past 60 years was a study on the effect of cholesterol and its numerous breakdown products on arterial tissue.

About 25 years ago a few sophisticated sterol chemists began to isolate and identify the numerous breakdown products of cholesterol which had been stored in air at room temperature. Finally, during the past six or seven years some individuals who for years had been feeding a mixture of sterols (presuming that they were feeding pure cholesterol) have developed an awareness of the instability of isolated cholesterol and are now doing studies in which pure cholesterol (Imai et al., 1976) has been fed separately from its autoxidation products (Imai et al., 1976). The autoxidation products are apparently very atherogenic, whereas pure cholesterol is not atherogenic (Imai et al., 1976).

DR. FRANK: You mentioned several species of animals. Have you seen any of this in other animal models such as the miniature swine or perhaps in isolated heart preparations?

DR. TAYLOR: I haven't done any work with miniature swine--they do get atherosclerosis. Dr. Imai is a "pig doctor." They do get lesions, very good indeed.

DR. FRANK: I was referring to the challenge with the cholesterol impurities and the rapidity with which the lesion appears.

DR. TAYLOR: First of all the cholesterol impurities. Dr. Imai has handouts there. In the pigs, in the work that was done originally, they used commercially-available cholesterol. When they were fed commercially-available cholesterol, which had breakdown products, they got an increase in mitoses which would imply smooth muscle cell death, thus requiring reproduction.

DR. IMAI: May I make a comment? I have tried this concentrate of impurities from the U.S.P. cholesterol on rabbits, chickens, rats, and hamsters. The rabbit is used because it is a small animal and because the supply of this concentrate is quite limited. What is interesting here is that the rabbit and chicken that are susceptible to cholesterol atherogenesis responded by enchancement of the smooth muscle cell death in the short-term bioassay. On the other hand, the rat and hamster which are resistant to cholesterol-type atherogenesis did not respond by increased frequency of smooth muscle cell death. And more interestingly, in the chicken, the thoracic aorta is elastic and the abdominal aorta is muscular. Smooth muscle cell death was more frequent in the thoracic than in the abdominal aorta. Atherosclerotic lesions induced by cholesterol diets are more severe in the thoracic segment.

DR. WERTHESSEN: Do we have any further questions?

DR. POWELL: Yes, I have one. It's a naive question. Is it true that the atherosclerosis found is more severe in human beings than in animals because we feed ourselves and eat an exotic diet, or are there certain species that develop it more readily than in humans? I'm just curious.

DR. WERTHESSEN: I think that we'll cover that---but you probably won't be here, but some of us will say that humans get it worse than other animals and others of us will say that the animals are as bad as we are.

DR. TAYLOR: It will not occur in rats and dogs unless you reduce their thyroids' function.

DR. POWELL: On what you would call their normal diets? Or on diets that you feed them in the laboratory?

DR. TAYLOR: You have to give them abnormal diets.

DR. ALTSCHULE: What about the trout?

DR. WERTHESSEN: Oh, yes, I forgot to bring that up. Trout get it by themselves.

DR. ALTSCHULE: Going upstream but not down.

DR. WERTHESSEN: They lose it going downstream. Salmon, too. When the salmon come in to spawn, they're loaded with atheromas.

DR. TAYLOR: Do the salmon lose it going upstream or downstream?

DR. WERTHESSEN: They lose everything going upstream....they die. They die, but some of the species that go back, they're clear. Of course they haven't eaten. But the scars remain, as I understand it, don't they?

DR. TAYLOR: Well, they say that the German prisoners lost all their atherosclerosis because they had so little to eat; they had to mobilize the lipids out of their atheromas for nutrition.

DR. WERTHESSEN: Along that one, just to give John another moment to collect himself, it took a year for a friend of mine to get through to the pathologist that ran one of those experiments in the concentration camps. He found that the only way in which a human could lose fat from his atheroma was to die of starvation, and then the scars remained. He did see the data; they have never been published.

DR. WATSON: I've been given the task to orient you to some general aspects of regulation of cholesterol synthesis. Even though we are in the Harvard Club, I don't think this environment would facilitate the direct transfer of the reams of relevant information which is often confusing and conflicting. What I'll try to do is identify salient points of key views on cholesterol synthesis and use my recent studies as paradigms of experimental approaches being used today. Where appropriate, we will present results which relate to the action of cholesterol auto-oxidation products on sterol synthesis and storage. Our main interests in cholesterol auto-oxidation products are derived from the fact that they modulate cholesterol synthesis; that is, they generally inhibit the pathway and we use them as probes to study the regulation of sterol synthesis.

Sterols represent obligatory metabolites which characterize all eukaryotic cells and appear to be required for cellular growth. Sterols or their derivatives are

structural components of membranes. Cholesterol is the primary sterol of mammals and it is a precursor for hormones and bile salts and a major component of plasma lipoproteins. My talk will focus on the regulation of cholesterol metabolism as opposed to sterols in general.

A consistent observation is that the rate of hepatic cholesterol synthesis can be modulated by the level of cholesterol in the diet. This response has been loosely defined as dietary "feedback" control. The first slide shows a dose-response curve for the suppression of ^{14}C-acetate incorporation into 3ß-hydroxy sterols by liver slices from rats fed different amounts of cholesterol for seven days. You can see that suppression is detectable at a dietary cholesterol level of 0.05%. Cholesterol at 5-10% of the diet essentially eliminated the conversion of ^{14}C-acetate to 3ß-hydroxy sterols. This "feedback response" can be detected within six hours.

When mevalonate incorporation into sterols was monitored in livers from animals fed cholesterol, there was no change relative to that for animals fed a control diet. This observation suggested a potential metabolic block in the sterol synthesis pathway between acetate and mevalonate. It was eventually found that acetate was converted to 3-hydroxy, 3-methyl, glutaryl, coenzyme A (HMG CoA--the proximal precursor for mevalonate and ketone bodies). Until recently it was thought that HMG CoA's diversion to sterols or ketone bodies was governed by the relative demand for the substrate by these two major pathways. It appears now that HMG CoA for the two pathways is generated in two different compartments--mitochondria for ketogenesis and cytosol for sterol synthesis. Therefore, no real competition exists between the two pathways for HMG CoA.

The primary source of carbon for sterol synthesis is acetyl CoA. This two-carbon unit is generated intramitochondrially and transported to the cytosol as citrate. Acetyl CoA is regenerated in the cytosol by the action of the citrate cleavage enzyme. This series of reactions is summarized on the next slide.

The overall synthesis of cholesterol involves the polymerization of five carbon (isoprene) units derived from HMG CoA. The enzyme for this process is localized in the cytosol and on the endoplasmic reticulum. On the next slide is a summary of the total pathway for the synthesis of cholesterol from acetyl CoA. Acetyl CoA is converted to acetoacetyl CoA, which in turn is used to form HMG CoA; the latter is reduced to mevalonate, phosphorylated, and, via a series of reactions, polymerized to squalene. From squalene is formed lanosterol (first sterol in pathway). Lanosterol is demethylated, the Δ^{24} bond reduced, and the Δ^{8} bond moved to the Δ^{5} position, and the resultant sterol is cholesterol.

On this slide I have indicated the reaction which has been identified as rate-controlling for the synthesis of cholesterol; i.e., the conversion of HMG CoA to mevalonate. The enzyme that catalyzes this step is called HMG CoA reductase (HMGR). This enzyme is localized on the endoplasmic reticulum.

It should be evident that the synthesis of cholesterol is a complex process and the overall regulation of this pathway probably occurs at multiple steps. However, most investigators have focused on the regulation of HMG CoA reductase activity. Primarily because it is the only enzyme whose activity appears to rapidly change in response to perturbations which modulate the rate of overall sterol synthesis. In addition, measurements of the apparent rate of synthesis of cholesterol from labeled precursors, such as acetate or HMG CoA, or mevalonate, strongly suggest that HMGR catalyzes the rate-limiting enzyme for the pathway.

One of my tasks today will be to convince you that the modulation of HMGR activity is only one of a number of equally sensitive steps of cholesterol synthesis which are regulated in the cellular response to exogenous sterols. In fact, to focus exclusively on the modulation of HMGR activity may be shortsighted.

As mentioned, HMG CoA reductase is bound to the endoplasmic reticulum and it has an approximate molecular weight of about 200,000 daltons with identical sub-units of approximately 50,000 daltons. HMG CoA reductase has been isolated, solubilized, and partially characterized. Although antibodies have been made against it, there is significant debate on the purity of various preparations of the enzymes used as antigens.

The characteristic dietary cholesterol modulation of hepatic sterol synthesis has not been shown for hepatic and non-hepatic tumors. From studies with pre-neoplastic liver which didn't show "feedback" control in response to dietary cholesterol, it's been proposed that the lack of regulation of cholesterol was an integral component of the etiology of the transformation of a normal cell to a malignant one.

The fundamental observation is summarized on the next slide. You can see that the results for normal rat liver shows the characteristic strong suppression of acetate incorporation into sterols. However, a spectrum of minimum deviation of hepatomas shows no response. Some hepatomas showed a significant increase in their rate of cholesterol synthesis. However, my studies and those of others with cultured tumor cells show that they <u>can</u> express the phenotypic "feedback" control response; i.e., rate of sterol synthesis is modulated in response to the concentration of exogenous cholesterol. We don't know whether the mechanism which defines this response is the same as that in the liver. Regardless, a similar regulatory response is observed. We've performed a number of studies to try to understand why malignant cells, in culture, display the "feedback control" phenotypic response but are silent when transplanted in the animal. The next slide summarizes one such set of experiments.

We have shown that tumor 7288C grown in tissue culture (hepatoma tissue culture (HTC)) shows the phenotypic "feedback" response and, when transplanted into Buffalo rats, they do not express any regulation. Upon removal of the cells from infected animals and recultivation <u>in vitro</u>, the "feedback" response was "re-expressed." Thus, if the apparent lack of regulation by hepatoma 7288C was a genetic defect, it was not "lost," but for unexplained reasons not expressed when grown <u>in vivo</u>. It has been argued by Potter et al. and others that it is incumbent upon investigators who don't see a regulatory effect in tumors to demonstrate that it is not due to a lack of knowledge about altered experimental conditions needed to detect the expression of a regulatory phenotype.

We reassessed the lack of feedback control in transplanted tumors by looking at the regulatory effectiveness of cholesterol precursors rather than the sterol itself. Dietary squalene and RS-mevalonolactone were the sterol precursors used; suppression of hepatic HMG CoA reductase activity was used as the index of the "feedback" response. It was reasoned that since cholesterol didn't cause a "feedback" response, maybe we could force the cells to generate an excess of cholesterol <u>in situ</u> and cause a suppression of reductase activity.

A 1.5% squalene-chow diet caused a 40-60% suppression of reductase activity in normal rat liver. Bloch and Lang in the early fifties clearly showed that dietary squalene caused a marked suppression of acetate incorporation into hepatic cholesterol.

Squalene feeding caused no suppression of reductase activity in transplanted, malignant HTC cells. A possible reason for this lack of effect is that squalene is carried by triglyceride-rich lipoproteins which are processed in the vascular space to remnants. The liver removes remnant particles from the circulation, 30-40% per pass with a blood flow rate 10-fold greater than that to the transplanted hepatoma. This efficient sequestation by the liver would strongly reduce the availability of squalene or any other regulatory effector for the tumor mass.

Recently, Edwards et al. have shown that RS-mevalonolactone will cause a marked, rapid suppression of HMG CoA reductase activity (90% of inhibition within 30 minutes; 1 ml intubation of 0.4 M solution). In addition, a single dose will maintain suppression for approximately 30 hours.

We intubated rats bearing hepatoma 7288C with 1 ml (0.4 M RS-mevalonolactone) and measured HMG CoA reductase activity in the tumor, liver, and kidneys at 3, 6, and 24 hours after the doses. Our results are shown on the next slide. HMG CoA reductase activity in normal rat liver was strongly suppressed by RS-mevalonolactone at 3 hours. Hepatic reductase activity remained suppressed for an additional 22 hours. To our delight, reductase activity in the tumor was also suppressed for at least 6 hours. However, the extent of reductase suppression was only 40-50% as compared to 90-95% for the normal rat liver. We monitored the modulation of reductase activity in the rat kidneys to evaluate the effect of RS-mevalonolactone on non-hepatic tissue. Our results show that reductase activity was suppressed (about 50%) and mevalonolactone's effect was sustained for 24 hours in contrast to the recovery seen in the tumor.

We propose that the sustained kidney effect reflects its ability to preferentially metabolize RS-mevalonolactone (Siperstein et al. and Popjak et al.). In addition, the kidney may concentrate RS-mevalonolactone as a result of glomerula filtration and maintain a sufficiently higher perfusate concentration than the interstitial fluid which fed the tumor.

Normal liver was also more sensitive to RS-mevalonolactone than the transplanted tumor and normal kidney. This is consistent with our observation that non-hepatic cells metabolize RS-mevalonolactone 3-10 fold slower than isolated hepatocytes.

Although the transplanted tumor was less responsive than normal liver to suppression by mevalonolactone, significant modulation of reductase activity was observed. Thus, for the first time, the regulation of HMG CoA reductase activity in a transplanted hepatoma has been demonstrated. The use of mevalonolactone as a source of carbon for endogenous sterol synthesis would emphasize that the "loss" of "feedback" control to dietary cholesterol is not related to a deletion in the proximal intracellular regulatory machinery. It would appear that the signal-receptor system for dietary cholesterol is not functional. Published data and our results do not support the thesis that the dietary cholesterol regulatory system is "lost" or "deleted" in malignant liver.

DR. AHRENS: Returning to your squalene work, were these feeding experiments?

DR. WATSON: Yes, they were feeding experiments. At 1.5% of the diet.

Let's move on to discuss how cultured cells have become a superb model to evaluate selected aspects of the regulation of sterol synthesis. The principal cell line used in my laboratory is hepatoma tissue culture (HTC) cells. They were derived from

minimal deviation hepatoma 7288C and retain a limited number of differentiated functions of the liver. The cells are homogeneous and can be grown in suspension or monolayer. HTC cells have been characterized extensively by Gordon Tompkins' research group. In addition to HTC cells, we use primary hepatocyte culture (PHC) cells. These cells' distinct advantage is that they are "normal." Based on a wide spectrum of metabolic criteria, PHC cells appear to function as parenchymal cells in liver. We don't maintain PHC cells for more than 48 hours. However, similar cells have been maintained by others for three to four weeks.

The next slide represents a summary of what I want to cover in this section of my talk. The circle represents a cell. We propose that the rate of cholesterol synthesis is a reflection of the metabolic response to maintain a critical cellular concentration of the sterol. Cellular cholesterol level will be influenced by the steady state between cholesterol input and its output (includes metabolism). To the extent that eukaryotic cells have no sterol input and no cholesterol synthesis, they die. Identifiable potential sources for cholesterol (sterol) input are serum lipoproteins, monomeric and/ or micellular cholesterol, cholesterol analogues, etc. The lipoproteins of primary interest are VLDL, LDL, and HDL_c (unique high density lipoprotein that's found in animals, such as pigs, dogs, rats, and rabbits fed a high cholesterol diet). In addition, partially degraded products of VLDL and chylomicron metabolism (remnants or intermediate density lipoproteins) are included in the list of cholesterol donors. These serum lipoproteins have the capacity to provide cells with "metabolically active" cholesterol; that is, their cholesterol is presented in a form which will lead to a modulation of HMG CoA reductase activity, the overall rate of cholesterol synthesis, and sustain the growth of cholesterol auxotrophs.

There are a number of small molecules which could be viewed as input sterols. This perspective may not be correct, but for simplicity I have put them in this category. Of primary significance are the oxygenated sterols and cholesterol precursors (squalene, mevalonolactone, etc.). Cholesterol associated with albumin or in phospholipid liposomes are also potential sterol donors.

DR. WERTHESSEN: I'd like to cross-check with Dr. Ahrens. Didn't we hear in Philadelphia that HDL was the one that protected you? And now he's saying it's providing cholesterol to the cell.

DR. AHRENS: It is fashionable these days to think that the HDL fraction is protective in respect to atherogenesis. However, what Dr. Watson is referring to is that special lipoprotein rich in cholesterol that Mahley has called HDL_c and that he has shown to be especially effective as a donor of cholesterol in tissue-culture experiments.

DR. WERTHESSEN: Oh, then it's not the HDL that we were just taught.....

DR. AHRENS: HDL itself is not thought to be an effective donor of cholesterol to this cell system.

DR. WERTHESSEN: I was getting confused because within three weeks I was hearing it had different properties.

DR. AHRENS: It's that little "c" in HDL_c that counts.

DR. WERTHESSEN: Oh, okay. Well, thank you.

DR. L. SMITH: In regard to protein binding of oxidized sterols, you are aware that Kandutsch just published a paper on protein binding of 25-hydroxycholesterol (Proc. Natl. Acad. Sci., 74, 2500 (1977)), so there is bound to be some protein binding which needs to be considered in the overall scheme.

DR. WATSON: Andy's work focuses on a potential binding protein that's inside the cell and we can possibly talk about that later. I'm not, quite frankly, convinced his data points to a specific protein for 25-hydroxycholesterol and/or related sterols or reflects non-specific binding to a lipophilic protein. More evidence is needed to put Andy's studies into perspective.

"Output" involves cholesterol acceptors which are present in the cell's environment; i.e., HDL, phospholipid plus protein, or selected lipophilic proteins. In addition to the removal of cholesterol per se, the metabolism of cholesterol to steroid hormones, bile acids, and the serum lipoproteins can be viewed as "output." All of these products are lost from the cell of origin. Finally cholesterol is used to make membranes and cholesterol ester in cells. Both of these usages of cholesterol could contribute to "efflux" or "loss."

Let's consider initially the protein bound sterol effectors--serum lipoproteins. HTC cells were used for our studies. Other investigators have used cells of fibroblast origin. However, recent studies have been described with smooth muscle and aorta endothelium cells. Regardless, the bulk of the published studies report on cells of non-hepatic origin. In general, the basal rate of sterol synthesis in non-parenchymal cells is lower than that for HTC cells. However, one can enhance their rates of sterol synthesis to the same end-point as that found for HTC cells.

The primary experimental protocol used to modulate the rate of cellular sterol synthesis relies on the presence or absence of serum lipoproteins with a density equal to or less than 1.063; i.e., LDL, VLDL, chylomicrons plus any remnants like particles in the growth medium. Sometimes all the lipoproteins with a density < 1.22 g/ml are removed. Sterol synthesis is enhanced 3-30 told by the removal of low density lipoproteins ($d < 1.006$ to 1.063 g/ml). Upon re-addition of specific ($d < 1.006$ to 1.063 g/ml) lipoproteins to the medium, sterol synthesis is suppressed to that found with cells maintained in medium which contained unfractionated serum.

The next slide summarizes our results which show the reversibility of the serum lipoprotein mediated modulation of HMG CoA reductase activity (sterol synthesis) in HTC cells.

It is clear that removal of serum lipoprotein ($1.006 < d < 1.21$ g/ml) causes a marked increase in the level of HMG CoA reductase relative to that measured in cells grown in medium which contained unfractionated serum (DS: dialyzed serum). A new steady state is reached 9-12 hours after removal of DS. The re-addition of lipoproteins from the density range of 1.006 g/ml to 1.063 g/ml led to a rapid suppression of reductase activity to the initial basal level. The rise in reductase activity requires protein and RNA synthesis. Serum lipoprotein mediated suppression does not require continued RNA synthesis.

Reductase suppression by serum lipoproteins was not muted or enhanced by the inhibition of RNA synthesis. This observation suggests a post-transcriptional site of action for the serum lipoprotein. Additional insight into the characteristics of the serum mediated suppression of reductase activity has yet to be obtained.

The next slide emphasizes the serum lipoprotein specificity required for modulation of reductase activity in HTC cells. The steady state activity of HMG CoA reductase and the rate of incorporation of tritiated water into 3β-hydroxy sterols was measured. Removal of lipoproteins ($d < 1.006$ g/ml) had no effect on reductase activity and rate of sterol synthesis. Removal of low density lipoproteins ($1.006 < d < 1.063$ g/ml) caused an enhancement of reductase activity and a marked increase in the rate of tritiated water incorporated into sterols. Removal of the high density lipoprotein fraction ($1.063 < d < 1.22$ g/ml) did not lead to a further increase in reductase activity or the rate of sterol synthesis. Thus, low density lipoproteins are the effective particles which govern the rate of sterol synthesis and the steady state activity of reductase. In addition, the data indicates that high levels of exogenous cholesterol (HDL) per se are insufficient to act as a regulatory signal.

Our results are consistent with the myriad of studies that have been published with a wide variety of cells. Although most investigators have focused on the low density lipoproteins, the literature, however, clearly indicates that VLDL will work with similar efficacy. Although LDL is the major source of cholesterol in human serum, it is important to remind ourselves that the effect of LDL is not exclusive.

From the superb studies of Brown and Goldstein on LDL metabolism by human fibroblasts and supporting experiments of others, a reasonable hypothesis has been developed to explain the modulation of sterol biosynthesis by serum lipoproteins. This hypothesis is summarized on the next slide. Low density lipoproteins are taken into the cell via focal, absorptive endocytosis and bulk phase endocytosis. The former reflects a high affinity process and the latter a non-specific route. The endocytic vesicle, which has the lipoprotein bound (or trapped) on it, fuses with a lysosome to form primary and secondary phagasomes. These vesicles contain enzymes which effectively degrade the lipoprotein to its monomeric components (i.e., amino acids, free fatty acids, cholesterol, etc.) It appears that the hydrolysis of cholesterol ester is required to modulate the regulation of sterol synthesis or reductase activity by serum lipoproteins.

In addition, the lysosomal release of unesterified cholesterol leads to an increase in the rate of cellular cholesterol ester synthesis in the cell. The mechanism(s) by which unesterified cholesterol accomplishes these metabolic changes is (are) unknown. In addition to these rapid (within 1-3 hours) effects of LDL, there is a long-term effect (evident in 24 hours) which governs the number of plasma membrane high affinity receptors to which it binds.

Although the low affinity, non-specific uptake mechanism can account for significant LDL metabolism at high concentrations (> 150 μg LDL protein/ml), it does not lead to the metabolic events described for functional high affinity receptors. Presently, the model proposed by Brown and Goldstein does not provide insight into how the cell can distinguish secondary lysosomes derived from high and low affinity endocytic vesicles.

In general the Brown and Goldstein model has held for non-parenchymal cells. However, it has been proposed that the liver does not readily metabolize LDL. In addition the rate of hepatic sterol synthesis appears to be responsive to another serum lipoprotein--chylomicron remnants and/or intermediate density lipoprotein. There are ample published results which demonstrate that remnant particles are uniquely cleared from the serum by the liver. However, it has yet to be directly demonstrated that clearance of remnants are the regulatory effectors for parenchymal cell sterol synthesis.

Although there has been recent literature which purportedly demonstrates a direct effect of remnant particles on perfused liver and isolated hepatocytes, it must be viewed with caution because the results were based on the suppression of an undefined increase in reductase activity and/or sterol synthesis by the addition of lipoproteins or cholesterol-enriched liposomes. We've taken the position that the cause of this rise is unexplained and its suppression is not necessarily related to the dietary cholesterol mediated regulation of sterol synthesis. In none of the experiments reported have the investigators been able to suppress sterol synthesis below their initial basal rates. This is what occurs with the intact animals given an acute dose of cholesterol. Therefore, until suppresssion of sterol synthesis by cholesterol below a stable baseline is demonstrated, the in vivo response would not have been reproduced.

DR. WERTHESSEN: That rise that you get there--that's over and above the baseline of the tissue in situ?

DR. WATSON: It varies--yes, it is.

DR. WERTHESSEN: In other words, it's induced by the maceration of the tissue?

DR. WATSON: Well, it is observed with isolated cells and perfused liver which are "stressed."

DR. WERTHESSEN: You know what I'm driving at. You disturb the cells and they start synthesizing cholesterol, is that right?

DR. WATSON: If the disturbance could be considered a stressful condition. Sterol synthesis is increased under stress.

We have eliminated the inconvenience of the initial rise in reductase activity with primary hepatocyte culture cells. These cells were obtained from liver perfused with collagenase, dispersed, washed and plated out in a monolayer. We summarize a kinetic study to assess reductase activity with time after plating on the next slide. As you can see there's an initial decrease in reductase activity relative to that measured for intact regenerated liver. With time reductase activity appears to recover and come back to a value similar to that for the intact liver. This restoration occurs 4-6 hours after plating and slowly decreases to a relatively stable level 18-24 hours after plating. Perfused liver and hepatocyte suspension are limited by a period of use (4-8 hours after preparation). Therefore, this cyclic pattern of reductase activity can't be seen with these experimental models. We chose to use our cells 18-36 hours after plating. The cells must be incubated in medium which contain serum in order to obtain metabolically and morphologically normal hepatocytes.

On the next slide we compare reductase activity in cells at two different time periods after plating in the presence and absence of serum lipoproteins in the medium. We chose to evaluate 4- and 24-hour time periods. The key point of these data is: the rise in reductase activity at 4 hours is enhanced by the presence of unfractionated serum in the medium (this has been observed with hepatocyte suspensions and the perfused rat liver). However, this observation is contrary to my earlier remarks about cultured cell lines incubated with unfractionated serum--reductase activity was suppressed under this condition. However, after 24 hours incubation, there was no difference in reductase activity of cells incubated in media which contained unfractionated and lipoprotein-poor sera.

We have attempted to show that liver parenchymal cells respond directly to chylomicron remnants. However, our efforts have been unsuccessful with remnants derived from the lymph of rats intubated with a cholesterol-corn oil suspension. Our results are summarized on the next slide. It is clear that the remanants either had no effect on reductase activity or caused a 1.5-2.5 fold stimulation. We monitored CO_2 production from (^{14}C) triacyl glycerol labeled remants and their effect on (^{3}H) acetate incorporation into saponifiable and non-saponifiable lipids to evaluate the internalization and metabolism of the particles. $^{14}CO_2$ production from the remnants was 7-fold greater than that for their parent chylomicrons and fatty acid synthesis was inhibited 80-90% by remnants. Therefore, this apparent lack of suppression of reductase by remnants was probably not due to their uptake and metabolism.

We have isolated the serum lipoproteins (1.006 g/ml < d < 1.04 g/ml) from cholesterol fed rabbits (source of accumulated chylomicron remants) and used them as a potential regulatory effector. Reductase activity was suppressed (50%) by these lipoproteins. Therefore, reductase activity in PHC cells can be suppressed by serum lipoproteins; however, it is not clear how atypical particles from a cholesterol-fed rabbit relate to the putative normal lipoproteins from a rat given an acute dose of cholesterol. Similar results can be reported with serum lipoproteins from hypothyroid rats fed a high cholesterol diet (abnormal serum lipoproteins are also synthesized in this model).

Our initial studies indicate that there are many unanswered questions with regard to the role of chylomicron remnants as direct effectors of hepatocyte sterol synthesis. This is inspite of the numerous in vivo infusion studies with fractionated and unfractionated lymph particles which show the liver as the primary site for their clearance. We must not forget that liver and parenchymal cells are not the same.

The LDL-receptor hypothesis may adequately describe the "eating and digestion" process for specific lipoproteins, but does not provide insight into the basic questions: "Once cholesterol is in the cell, how does it cause the regulation of sterol synthesis and storage? Is reductase activity the result of an activation or inactivation phenomena or due to an increase in the number of its molecules?"

We've attempted to use antibodies against HMG CoA reductase to gain insight into these questions. The next slide shows an Ouchterlony diffusion plate for different sources of HMG CoA reductase. The antibody was made against "pure" rat liver HMG CoA reductase. It is clear that continuous lines, without spurs, occur between reductase from normal rat liver and HTC cells. Similar results were found for the enzyme solubilized by other procedures. The primary point we want to emphasize is that antibody against normal rat liver cross reacts with the enzyme from minimal deviation hepatoma 7288C (HTC cells).

On the next slide we compare immunotitration curves for reductase solubilized from HTC cells grown in unfractionated and lipoprotein-poor sera. You can see from these results that there was no difference in the antigenicity of the enzyme from cells maintained in lipoprotein poor or unfractionated sera. Therefore, reductase activity must be related to a change in the number of its molecules. The modulation of the number of reductase molecules could be due to a change in its rate of synthesis and/or rate constant for degradation. Indirect measurements with inhibitors of protein synthesis suggest that the rate constant for the rate of reductase synthesis is modulated and not its rate constant for degradation. Therefore, our limited studies are consistent with serum lipoproteins causing the modulation of reductase synthesis.

At intermission we finished our discussion about the role of effectors that put cholesterol into the cells. In addition, I had described studies with HTC and PHC cells which indicated that they do not give the same result with regard to lipoprotein modulation of sterol synthesis or HMG CoA reductase activity. This point may be of interest for future studies directed at the transference of conclusions obtained with growing cells to results obtained with non-growing primary cells.

Let's move from the effect of serum lipoproteins on the modulations of reductase activity and focus on the effect of sterol precursors. Specifically, we asked whether increased carbon flow from RS-mevalonolactone or squalene would modulate reductase activity. I showed you in the first slide that whole animals given an acute dose of RS-mevalonolactone show a marked suppression of HMG CoA reductase activity. This observation can be reproduced with a spectrum of cultured cells. However, there appears to be a differential in the RS-mevalonolactone's effectiveness, which is dependent on the type of cell tested--i.e., normal hepatocytes as opposed to fibroblasts or HTC cells. Normal hepatocytes are more sensitive to inhibition than any other cells we have tested. Suppression can be detected in the presence of unfractionated serum. Thus, in spite of the attainment of the serum suppressible level, the addition of RS-mevalonolactone can result in further decrease in reductase activity. These results suggest that endogenous generated cholesterol or post mevalonate intermediates of sterol synthesis may be important regulatory metabolites. This phase of my talk will describe some of our recent studies to determine whether cholesterol or an intermediate in its synthesis was responsible for the RS-mevalonolactone-mediated suppression of HMG CoA reductase activity.

Our studies suggest that RS-mevalonolactone's effect requires the formation of lanosterol or a related methyl sterol. We used primary hepatocyte cultures for these studies. With 15-30 μM of triparanol (an inhibitor of Δ^{24} reductase), we were able to block carbon flow from RS-mevalonolactone to sterols. The metabolic block occurred at the cyclization to lanosterol with the accumulation of squalene epoxide. Triparanol caused no apparent diminution in carbon flux from RS-mevalonate into the total non-saponifiable lipid fraction.

The next slide summarizes our use of triparanol with high concentrations of RS-mevalonolactone to modulate reductase activity. RS-mevalonolactone caused a marked suppression of reductase (75-80%); triparanol slightly increased (50-100%) reductase activity. However, a mixture of triparanol and RS-mevalonolactone (triparanol was present initially for one hour prior to the addition of RS-mevalonolactone) led to only 25-30% inhibition of reductase activity. Thus, RS-mevalonolactone's effect on reductase appears to require carbon flow to at least lanosterol. Our results also speak against RS-mevalonolactone having a direct effect on HMG CoA reductase. (A similar conclusion has been drawn from cell-free studies with RS-mevalonolactone (2 μM).) We have taken these studies one step further with mutant Chinese hamster ovary (CHO) cells which can't make cholesterol--they're blocked at the demethylation of lanosterol to C_{27}-sterols. When these mutant cells are incubated with RS-mevalonolactone, reductase activity is suppressed. Since these cells can't make C_{27}-sterols, it would appear that reactions distal to block in methyl sterol metabolism are not required for the regulation of reductase activity. This is a very conservative interpretation with no attempt to specify a particular methyl sterol. I will show you later additional other data which are consistent with this conclusion.

The other cholesterol precursor which we have investigated is squalene. I showed earlier (Slide 1) that dietary squalene will cause a suppression of reductase activity in rats. This effect can also be demonstrated with HTC cells. We have not

attempted to evaluate the effect in the same detail as presented for RS-mevalonolactone. Regardless, our results indicate that increased flow of carbon to sterols (probably not cholesterol) is a sufficient regulatory signal to suppress reductase activity. In addition, exogenous sterol is not an obligatory element for the regulation of cholesterol synthesis.

DR. BLOCH: You are measuring the effects on enzyme levels or on activity?

DR. WATSON: Activity.

DR. BLOCH: Activity--these studies ran for how long?

DR. WATSON: These were for a total of four hours.

DR. BLOCH: Four hours, hmmm. But you could very well be affecting. Could you?

DR. WATSON: Yes. We have not measured, with triparanol, processes other than fatty acid synthesis, and it is not affected.

DR. BLOCH: No, I mean the reductase. What you are measuring is a reduction of reductase activity or a reduction in the amount of reductase?

DR. WATSON: Reduction in activity. I have not run any immunotitration curves to determine whether loss in activity was equivalent to a decrease in the number of enzyme molecules.

Now that we have rapidly established a background on the effect of natural effectors which could generate more intracellular cholesterol, let's turn our attention to compounds which represent the theme of this meeting--auto-oxidation products of cholesterol or oxygenated sterols. The next slide compares the inhibitory capacity of a variety of oxygenated sterols on the suppression of reductase activity in HTC cells. The point we want to emphasize is that an increase in the number of oxygen functional groups tends to increase the capacity (relative to cholesterol) of these various sterols to suppress reductase activity. In addition, 3-keto derivatives are much more effective than their 3-hydroxy counterparts; 7-α-hydroxy, 7-keto, and 25-hydroxycholesterol cause approximately 90% suppression of reductase in four hours (5-20 μM concentration). Suppression by cholesterol is variable (30% inhibition at 30-50 μM concentration). Dr. Kandutsch has made similar observations. However, Brown and Goldstein consistently get inhibition of reductase activity in fibroblasts with cholesterol. We can't offer any viable explanation for the differences. I have recently generated a dose-response curve for 3β, 5α, 6β-cholestan-triol; the results were similar to that found for 3-keto, Δ^4-cholestenone. Thus it would appear that increased polarity, per se, is not sufficient to explain the enhanced effectiveness of oxygenated sterols to suppress reductase activity relative to "pure" cholesterol.

The oxygenated sterols will suppress reductase activity in cells maintained in either unfractionated or lipoprotein poor sera. This observation is similar to that made with RS-mevalonolactone and clearly indicates that the serum does not lead to the bottom level of reductase suppression.

In an attempt to evaluate how selected oxygenated sterols cause the rapid and extensive suppression of reductase activity, we measured their apparent rates of uptake and the effect of metabolic inhibitors and divalent cation chelators on their actions. None of these experimental perturbations modified the effect of the oxygenated sterols. In addition, the sterols' action was readily reversible.

Next we determined whether the effect of oxygenated sterols could be modulated by inhibition of RNA and protein synthesis. Initially we ran a series of experiments to determine the effect of 25-hydroxycholesterol concentration on the apparent half-life of reductase in the presence and absence of cycloheximide. In the next slide we show the kinetics of loss of reductase activity at two concentrations of 25-hydroxy-cholesterol in the presence and absence of cycloheximide. The loss of reductase activity in the absence of protein synthesis and 25-hydroxycholesterol gave a $t_{1/2}$ of three hours. The half-life for reductase in the presence of 0.3 µg/ml and 3 µg/ml of 25-hydroxycholesterol was 5.0 and 1.0 hours respectively. However, in the presence of cycloheximide they were reduced to 2.0 and 0.24 hours respectively! Thus, in the absence of protein synthesis, 25-hydroxycholesterol causes a rapid loss of activity which may be due to inactivation and/or enhanced degradation. Our analysis does not allow us to distinguish the possibility. Additional confirmation of a dominant effect of 25-hydroxycholesterol and other oxygenated sterols on the inactivation and/or rate constant for degradation of reductase was obtained from "steady state transition kinetics" studies.

Our results are summarized on the next slide. The data show that the effect of oxygenated sterols on the apparent (because only enzyme activity was measured) rate of reductase synthesis was invariant at all concentrations tested. However, the apparent rate constant for degradation was proportional to the concentration of oxygenated sterol up to a limiting value. As a control to show that the oxygenated sterols did not have a generalized effect on protein turnover, we assessed their effect on the release of TCA soluble radioactivity by pre-labeled (^{3}H-leucine) cells. There was a slight enhancement (10%) in turnover, but this was insignificant in comparison to the four- or five-fold increased loss of reductase activity.

With antibodies against reductase, we asked whether 25-hydroxycholesterol caused a modification of its antigenicity. The next slide summarizes a study which compared the titration curve for reductase from cells exposed/not exposed to 25-hydroxycholesterol for 90 minutes. Microsomal reductase was titrated with antibody. You can see that the slopes of the titration curves for the control and for 25-hydroxycholesterol are similar but their apparent equivalence points are shifted. The enzyme from cells exposed to 25-hydroxycholesterol is less antigenic than the unsuppressed molecule. We have not attempted to determine whether phosphorylation, dephosphorylation, or some other kind of biological modification is responsible for the apparent change in reductases' antigenicity.

DR. AHRENS: John, didn't you say this morning that your reductase preparations were not considered to be pure and, therefore, is it valid to make an antibody preparation to an impure preparation of a protein?

DR. WATSON: The antibody that I'm using was prepared by Dr. Renn Heller. At the time she made the antibody, she thought that she had pure enzyme and monospecific antibodies. However, recent work suggests that her antibodies are not monospecific. Thus radio-immunochemical studies can't be done until a monospecific antibody is made. However, a less pure preparation can be used for enzyme inactivation titration curves. I do not measure the amount of protein precipitated by the antibody--only the reductase activity. Thus the full power of the antibody technique can't be used in our analysis of reductase.

At this stage our results with oxygenated sterols, specifically 25-hydroxy-cholesterol suggest that it can alter the antigenicity of reductase plus cause an increased inactivation and/or an increased rate constant for degradation. 25-hydroxy-

cholesterol is much more effective than serum lipoproteins or cholesterol, and its strong suppression of reductase activity (sterol synthesis) could help explain its potency as a toxin. In the absence of a source of cholesterol, 25-hydroxycholesterol will kill cells.

Let me move into an arena which has captured our current research interest. This is the regulation of cholesterol synthesis by reactions distal to mevalonate production. We have evidence which suggests that regulation in this part of the sterol biosynthetic pathway may be as significant as a narrow focus on reductase activity. We have already suggested that the strong inhibitory capacity of RS-mevalonolactone may be related to modulations in methyl sterol metabolism. 25-hydroxycholesterol and 3β, 5α, 6β-cholestan-triol were also tested for their capacity to alter methyl sterol metabolism.

With the help of Dr. Scallen we have analyzed (high pressure liquid chromtography) the non-saponifiable lipid fraction from PHC cells exposed to oxygenated sterols and pulsed with radiolabeled acetate or mevalonate. Dr. Scallen's analysis confirmed our earlier limited TLC results which showed that 25-hydroxycholesterol and cholestan-triol caused a shift in the distribution of newly-synthesized sterols from C_{27} to 4,4-dimethyl sterols. The results are summarized on the next slide.

25-hydroxycholesterol caused a decrease (48% to 17%) in the relative amount of radioactivity in cholesterol. The change was offset by a concomittant increase in radioactive methyl sterols. In addition, the C_{27} sterols were not exclusively cholesterol--the percent of radioactive demosterol (Δ^{24}-cholesterol) was increased from 2% to 16% in the extract from cells incubated with 25-hydroxycholesterol. Recent experiments indicate that the 4,4 dimethyl sterols are also Δ^{24} derivatives. Thus 25-hydroxycholesterol appeared to inhibit Δ^{24} reductase. This observation, coupled with its effect on mevalonate formation and sterol demethylation, suggests a limited focus on its suppression of reductase activity may be too narrow a window to attempt to understand the mechanism of action of this oxygenated sterol. Since at least three reactions of cholesterol synthesis were modulated by 25-hydroxycholesterol, we wanted to determine whether they were independent or linked events. And, if linked, what is their sequence? To date our kinetic studies which attempted to correlate reductase suppression with altered denovo synthesis of C_{27} sterols from methyl sterols have allowed us to distinguish a difference in the time of onset for the two events. The next slide summarizes such a study.

PHC cells were incubated in the presence and absence of 25-hydroxycholesterol. Thirty-minute (^3H) acetate pulses were used to assess denovo non-saponifiable lipid synthesis; the distribution pattern of the non-saponifiable lipids was determined by TLC and correlated with reductase activity at different times after the addition of 25-hydroxycholesterol.

25-hydroxycholesterol caused a 30% suppression of reductase activity within 60 minutes and 70% after three hours. Total (^3H) acetate incorporation in the non-saponifiable lipid fraction was another measure of the dimunition in reductase activity. However, the ratio of the radioactive C_{27} sterols:methyl sterols showed a marked increase relative to the control cells within the first 30 minutes. With time, this shift stabilizes to a constant value.

We've now done very detailed kinetics of suppression studies (15-minute (^3H) acetate pulses instead of 30-minute) with PHC cells incubated in the presence and absence of 25-hydroxycholesterol. Our results show that within the first 15-minute

pulse, one can detect a diminution in radioactivity in the non-saponifiable lipid fraction and a shift in the C_{27} sterol: methyl sterol ratio; with the 15-30-minute pulse, 80% of the maximal shift in the ratio had occurred.

DR. WERTHESSEN: As I understand this now, as you've put in these different oxidation products, you change the distribution of the sterols. Could one assume from that that the characteristics of the membranes of these cells would be altered because of the difference in sterol type from normal?

DR. WATSON: You could.

DR. WERTHESSEN: Or is that being too rash at this stage?

DR. WATSON: What we lack in our study is a measure of mass. I measured radioactivity and that's the next big hurdle--development, where I'll really be measuring the mass. The real import of these studies is that within a short time frame (0-30 minutes), 25-hydroxycholesterol caused a significant modification in the pattern of newly-synthesized sterols. The rapidity of the 25-hydroxycholesterol effect on metabolite distribution would tend to make us cautious about a limited investigation of the regulation of reductase activity in the absence of an evaluation of post-squalene reactions. In other words, if we had been working exclusively on the regulation of sterol demethylation, our results with 25-hydroxycholesterol would be considered fascinating and pursued without a priority interest in its possible modulation of reductase activity. Therefore, we would argue that to really begin to understand the regulation of cholesterol synthesis by 25-hydroxycholesterol, one would have to consider its import on reductase and sterol dimethylation. Both of the reactions would appear to play important roles in the supply of carbon to make cholesterol. It is possible that reductase plays a secondary role in the overall scheme.

We hope to repeat these studies with lipoprotein effectors to evaluate the constancy of our observations. If similar results are found, then our view of the regulation of sterol synthesis and the putative primal role of reductase will have to be re-evaluated.

DR. LEQUESNE: John, could you tell us whether you've looked into the combination of the 25-hydroxy and the triol and, by combining them in the experiments on the last slide, have restored the ratio of C_{27} to C_{30} to something like you had on the controls?

DR. WATSON: I've done it but not with that intention. I think I just did it as a whim, and obtained the mixed result. You get strong suppression of reductase and change the ratio. However, I could not develop any meaningful interpretation of the results. Both sterols are inhibitors and their effects were seen.

DR. LEQUESNE: No, I wondered if you couldn't restore the ratios, then you might have some evidence for interlocking mechanisms or something like that?

DR. WATSON: No, I suppose if there are no burning questions, I'll turn the floor over to Dr. Subramanyam who synthesized analogs which might generate 25-hydroxycholesterol *in situ*.

DR. BLOCH: Do you have enough kinetic data to indicate that the effect on the reductase and on the sterol pattern are parallel or is one preceding the other?

DR. WATSON: No, the shortest I've done a pulse is between zero and 15 minutes. Within that time frame, I see no dissociation of the events.

DR. BLOCH: Have you considered the possibility that 25-hydroxycholesterol and cholestan-triol might have their effect on the reductase by way of the changed ratio of methylated to non-methylated sterols? One experiment which suggests itself is to try to assess what these methyl precursors of cholesterol do to the system. Has this been done?

DR. WATSON: Yes. We've tried lanosterol and got the same results that you did in earlier animal-feeding experiments--no effect on the suppression of sterol synthesis.

DR. BLOCH: No effect on the reductase?

DR. WATSON: No.

DR. BLOCH: And you have not tried 4,4 dimethyl cholesterol?

DR. WATSON: No.

DR. SHEPPARD: What is your evidence that you feel that the cholestan-triol was a terminal oxidation product in the biological system?

DR. WATSON: I didn't say "in the biological system"--I was referring to the view of chemists on the auto-oxidation products of cholesterol. They state that one of the terminal products of the non-biological catalyzed auto-oxidation is cholestan-triol.

DR. SHEPPARD: Our experience has been it's one of the cumulatives, but other things occur too.

DR. WATSON: I agree.

DR. SHEPPARD: So I'm not sure it's necessarily a terminal.

DR. SUBRAMANYAM: As Dr. Werthessen mentioned this morning, we joined as the last group in the whole team. Initially we were asked only to purify cholesterol--25-hydroxy cholesterol. When the price of the 25-hydroxycholesterol was going to be about $500 a gram, they asked us to synthesize it, and that's how we got into this. The interesting aspect that we came across was that the 25-hydroxycholesterol seems to play a very important role in a number of synthetic products. Dr. Imai's work has showed that it is a potential angiotoxic compound. Brown & Goldstein and Dr. Watson's work reveal that it seems to play a role in controlling the cholesterol synthesis. But at this point if you ask us a question whether that is just a term to probe the synthesis of cholesterol or does it occur in nature as such or is an artifact or if it's a metabolite coming out of cholesterol, it opens up a number of interesting possibilities. So one possibility we wanted to look at was to see if that can occur in the normal process. Dr. Leland Smith has done a lot of work in the metabolism of cholesterol itself and he has identified a very large number of degradation products. But we were looking at it from the point of view of a natural formation and in order to understand our approach, I would like to recall very briefly the mechanism of conversion of cholesterol and squalene to cholesterol. I feel a little odd, like bringing coals to Newcastle when Dr. Bloch is sitting here, but I'll be very brief on this.

The work presented in this slide is the work of Professor Bloch. Then by Corey and Van Tomlin. Just to go over it very briefly: Squalene reacts with molecular oxygen in the presence of the monoxygenase enzyme to form the 2,3 epoxide which then is converted to lanosterol by a cyclase enzyme which then by several steps leads to the formation of cholesterol. We were interested in looking at the possibility of a mechanism by which any of the side chain oxidation products of cholesterol can be formed. And if you go into the literature and read on the mechanism of this formation, Corey has done a lot of work on this. To find out the mechanistic requirements for the conversion of lanosterol and for the conversion of squalene epoxide to lanosterol there during the probe, he has identified that if you substitute the oxygen by a nitrogen that makes 2,3 iminosqualene, nitrogen being a more powerful nucleophile is able to tie up the cyclase enzyme and as a result the squalene epoxide is not able to be converted to lanosterol and it keeps accumulating. One other observation he made during this study was that since the cyclase enzyme is effectively tied up, there is an accumulation of the mono-epoxide and more and more of it accumulates; then the other terminal double-bond also gets epoxidized and he has found out that in a half hour he is able to convert squalene to about 40 or 50% mono-epoxide and about 5 to 10% bis epoxide. I would like to recall that this is a symmetric molecule; for the sake of convenience it is written like that. This bis epoxide then can be allowed to react with the cyclase enzyme and he has shown that the bis epoxide can be converted to 24, 25 epoxy lanosterol.

So this seemed to be an attractive possibility for accounting for the formation of side chain oxidized product, particulary for 25-hydroxycholesterol because that seems to be an attractive compound for many of the biochemists. Here we have the bis epoxide--the 2,3,22,23 dioxide of squalene and, as you can see, it is effectively converted to the 24,25 epoxide of lanosterol which can then go to desmosterol 24,25 oxide and then on to either 24 or 25-hydroxycholesterol by a reduction or ring opening of the epoxide. So this is the early stages and we have made this bis epoxide and we thought we would first try it on Dr. Watson's experiment and see if it has any effect at all. At the same time we also supplied this compound to Dr. Imai to find out if it does have any effect that he has observed with 25-hydroxycholesterol. Depending on the results, we would try to proceed further in a more elaborate way, but at this stage, besides the synthetic work we are doing, this is one aspect of it we are interested in probing further. Of course the added reason for this is because Dr. Smith has identified that in severe atherosclerosis he is able to detect the presence of cholesterol esters and one of them happened to be 24-hydroxycholesterol in esterified form. That again could account for its formation here and again we are still speculating on this stage, but its in the very early stages of the experimental scheme. That will be all. The synthetic work that we have done will be presented by Dr. LeQuesne sometime tomorrow.

DR. L. SMITH: I want to raise one point. I think the hydration of an epoxide is a biological process, but I cannot recall a case of biological reduction.

DR. SUBRAMANYAM: I am not aware of it.

DR. WATSON: I think it's been over a year now since we had the bis epoxide of squalene. When Dr. Subramanyam sent it to me, I tested it right off and checked it with the HTC cell system and I got an effect; that is, it caused a suppression of reductase, and it became evident to me that possibly we should try squalene to see if it had an effect. Obviously if this bis epoxide worked, maybe it wasn't unique to bis epoxide. But since squalene is a precursor, we thought maybe that would work.

Now this is an experiment in which cells which were grown in unfractionated serum. HTC cells were exposed for five hours to varying concentrations of squalene or squalene epoxide. These were added in a vehicle using ethanol; I used serum containing medium because I found that it was best for minimizing, having a nice oil there. In our cultures I use suspension cells and then put on roller drums and there is a constant mixing. Under these conditions we could demonstrate additional suppression above and beyond that caused by the serum. Now there is this weird shaped curve (we consistently get this kind of a shape) and I don't know whether this represents the point when we get the oil separating out of the two phases or what, but the fact of the matter is that both of these two compounds gave an effect with HTC cells. If you consider the micromolar concentration added, it appears to be rather significant. In addition the squalene epoxide based on that kind of data is more effective. If the low concentration is squalene, bis epoxide rather. Recently, like last week, we received the lanosterol epoxide, and I just did a quick test in the system to find out if we get any effect using the lanosterol epoxide. Again I used cells that were grown in serum and so we were looking at an effect above and beyond what serum does and this was done under conditions where I tested two concentrations--1.2 micromole and 12 micromole. Lanosterol epoxide, again it was added through the ethanol vehicle, so obviously I could have had hydration. I can't say what I added after putting it in ethanol and putting it in the medium. But be that as it may, we monitored acetate into 3ß-hydroxy sterols and the summary of these results is as follows:

This is percent of control, the percent of activity remaining. We tried two concentrations, 1.2 micromolar and 12 micromolar and the 25-hydroxycholesterol in 45 and 35 for the "bis", squalene, 50, 45. Lanosterol epoxide we had 50, 45. This is a seven hour incubation and it is already apparent since the additional suppression caused a 10-fold increase in concentration is not that great suggests that we had already begun to saturate the system somewhere in here. I obviously have to do a much more detailed curve, but basically you got inhibition. The 25-hydroxy looked like it was better, but that's the result. So you can, using those particular compounds.

DR. WERTHESSEN: Do you think it would worthwhile having Dr. Imai give a brief resume of what he found with these?

DR. WATSON: I suppose, if he can do it in a very short period of time.

DR. WERTHESSEN: Well, I mean just for the sake of the discussion.

DR. IMAI: What is peculiar or characteristic of the in vivo effects of squalene bis epoxide is that in my short-term bioassay, using the pulmonary artery, the response by this particular agent, squalene bis epoxide, is not as fast as 25-hydroxy or cholestane triol. In a three-day period there was very little response, but there was a lot more response, including the fibromuscular thickening, up to 18 days. This is after three daily injections. And lanosterol epoxide has much less effect than the 25-hydroxy or cholestane triol. In terms of potency, I put this into the third potent category--or weak. Will that be enough?

DR. WERTHESSEN: Yes. Do we have any questions?

DR. AHRENS: In the oxidation of cholesterol, however that occurs, is there peroxide formation preliminary to the formation of the various hydroxy compounds being considered here today?

DR. IMAI: Dr. Dunphy did TLC separation of the concentrate of impurities and he gave me three bands. Band one and band three are potent--angiotoxic; band two was not effective, and I was told that this band two included hydroperoxides. He had also made a group of hydroperoxides from purified cholesterol with U.V radiation and this again was inactive in my hands.

DR. L. SMITH: I will respond to that issue. Certainly hydroperoxide formation is the primary pathway of cholesterol autoxidation. Most of the products come from hydroperoxides being formed first which then decompose into a variety of other products.

DR. AHRENS: The reason I asked the question is because years ago we tried very hard, but without success, to produce deleterious effects in rats by feeding peroxides of fatty acids, not of sterols. We were unable to demonstrate any morphologic changes or accumulation of peroxidized fats in their tissues. Thus, we came to the conclusion that the peroxidized fats had not been absorbed at all, that they hadn't permeated the intestinal wall. I wonder now, in view of the morphologic effects that Dr. Imai has described in feeding oxidized sterols, what is known about the adsorption of these materials?

DR. IMAI: I really have no data on the absorption of these sterols. One of the reasons why I used the intravenous injection is that I wanted to avoid this particular problem.

DR. AHRENS: Well, the question was put to us originally about the practical implications of the possible presence of oxidized sterols in egg products being fed to soldiers. Obviously, the absorption of oxidized sterols is a point of some importance and I wondered what is known about that.

DR. TAYLOR: This is certainly on the drawing boards, it hasn't been done yet, as far as I know. We think it's absorbed, but we're going to do it and try and follow it and see whether it's absorbed as it is, as a peroxide or a hydroxide, and see if it comes out in the bile and how it's distributed in the tissues. Those are some of our plans, which haven't been started yet.

DR. WERTHESSEN: I'd like to add to that one. The original experiments with the concentrate of the contaminants. Those compounds--the mixture of oxidation products--were given by mouth. And the effect was observed within 24 hours. So unless these compounds are not absorbed in the gut when they're all alone, the data indicates that they are absorbed. Otherwise Dr. Imai would not have seen his effect within 24 hours after giving it per os.

DR. AHRENS: The point I'm making, Nick, is that you know what you have fed and you know the effect produced. However, there's a missing link, isn't there between the feeding and the damage?

DR. WERTHESSEN: Agreed, so here you get into this nasty problem, if you want to identify compounds as regards their specific effects, their molecular structure, how are you going to administer them? If you put them in through the gut, particularly these things, you can expect change. For example, a 5,6 epoxide, the chemists tell me, in the acid medium of the gut--the stomach--it's liable to become a trihydroxy. But if you give it intravenously and the blood is neutral or slightly basic, you would expect it to remain as is. This is an old problem.

DR. TAYLOR: I think it's important to do the intestinal study because that's the way humans get all of their exogenous cholesterol--and its oxidation products. Man doesn't get exogenous cholesterol parenterally.

DR. WERTHESSEN: From the epidemiological point, I couldn't agree more. But it depends on the question you're setting up in your experiment.

DR. L. SMITH: I have a comment about that. There are only two papers on pertinent studies that I recall. I do not remember the results in detail, but in one cholesterol 5α,6α-epoxide was administered by intubation to rats (Fioriti, et al., Lipids 5, 71 (1970)) and 5α-cholestane-3β,5,6β-triol was found as a metabolite in the gastrointestinal tract. In the other case, 5α-cholestane-3β,5,6β-triol was administered orally to rats and fecal metabolites examined (Roscoe and Fahrenbach, J. Lipid Res., 12, 17 (1971). Both sterols were metabolized, but there is virtually no evidence to show that either was absorbed. Some absorption may occur, but these studies examined gastrointestinal and fecal metabolites where one cannot be sure of material balance or whether material not recovered represents absorption. In regard to your prior remark about the peroxides, one must question the integrity of the peroxide throughout the whole metabolic system. In the presence of a reducing environment, of peroxidases, etc., peroxides will be destroyed, with the corresponding alcohol derivatives probably formed. One may also expect that the acid of the stomach may modify sterol hydroperoxides. Bacterial metabolism within the gut would also be very likely. However, we know nothing about these matters from experimentation.

DR. WERTHESSEN: On the other hand, Pete, we have some very preliminary experiments underway now, in which feeding one percent 5,6-epoxide has drastic effects on the growth rate of mice. So I think it's reasonable to assume that these compounds are absorbed from the gut, but I'm not going to make any statement as to what they circulate as.

DR. WATSON: I think I second the fact that a number of them of them are absorbed. Gordon Gould has shown recently with 7-ketocholesterol fed to animals that you can get a range from killing the animal to getting some effect in terms of suppression of hepatic sterol synthesis and you don't know exactly what is coming to the liver, but there is some metabolite that's causing an effect. The same has been seen by Andy Kandutsch with feeding experiments with 25-hydroxy. The problem is that a lot of these things will wipe out the animals. If it doesn't kill the animals within a few days, or a week or so, they readjust and so they're now able to restore their cholesterol synthesis, so it seems they began to be treated like drugs.

DR. KUMMEROW: We found that cerebral disorders developed in chicks 1-5 hours after intravenous injection of 10 mg of the hydroperoxide of methyl linoleate or of linoleic acid emulsified in 1 ml of serum. No cerebellar disorders were noted, after injection, in birds kept on an identical diet but supplemented with 8 mg% α-tocopherol. Injection of reduced hydroperoxide, methyl 12-oxo-cis-9-octadecenoate or fresh methyl linoleate caused no cerebellar disorders indicating only lipohydroperoxide initiated symptoms. The severity of cerebellar disorders induced by injection of lipohydroperoxide was greatly influenced by dietary pretreatment. The cerebellum of chicks which had been kept on high protein, high fat, Vitamin E deficient diets was damaged more extensively by injection of lipohydroperoxide than the cerebellum of chicks kept on adequate diets. (Proc. Soc. Exp. Biol. Med. 105, 308-312, 1960.)

DR. SCALLEN: Dr. Subramanyam, do you have any evidence so far that the bis epoxide of squalene can actually be formed enzymatically?

DR. SUBRAMANYAM: Enzymatically there is no evidence so. Under normal circumstances--for example, Corey has done work in which he has tried to reduce some of the six double bonds in squalene, he has produced one of the middle double bonds, the 10-13 double bond, in which case the cyclizing enzyme is not able to act on the epoxide form. In such a case, he had fed squalene subsequently and that was converted to the bis epoxide.

DR. SCALLEN: Do you mean the squalene that was fed was converted to the bis epoxide?

DR. SUBRAMANYAM: Yes. In other words if you are able to tie up the cyclizing enzyme by an iminosqualene, you can convert enzymatically squalene into the bis epoxide.

DR. SCALLEN: Okay, and the product was squalene bis epoxide and not the 10,11 dihydro?

DR. SUBRAMANYAM: I'm sorry, I confused two experiments. In one case he used the 10, 11 saturated compound, in which case the oxidizing enzyme doesn't act at all. In the second case he used the iminosqualene in which case the cyclizing enzyme was completely tied up and the externally added squalene would now be converted to the bis epoxide enzymatically.

DR. SCALLEN: Was this in rat liver or what kind of a system was this in?

DR. SUBRAMANYAM: It was an *in vitro* experiment with liver enzymes.

DR. L. SMITH: There is one case of a plant system where a 24,25 epoxide has been isolated from a Spanish moss and then a few years ago the cyclization by the enzyme system from this same plant was shown to cyclize squalene bis epoxide to the 24,25 epoxide of cycloartenol* so there is a plant system that's similar to the one that you suggested here for the liver. Although there are arguments about whether that epoxide that's isolated from Spanish moss could possibly be an enzyme product or whether it could be some other process where it's being formed.

DR. LEQUESNE: I recall a paper by Derek Barton in which he showed that some of the triterpenes which have no hydroxyl at C-3 can be formed *in vivo* by cyclization not involving the initial epoxide. Is this parallel to the work which you are referring to?

DR. L. SMITH: What he did, if I recall, is that he showed that yeast systems would hydroxylate a 3 deoxy lanosterol to give lanosterol, but I don't recall the cyclization without free oxygen.

DR. TAYLOR: Dr. Peng and I plan to use animals smaller than the rabbit because it takes less of these precious breakdown products. We're using ground squirrels, prairie dogs, and squirrel monkeys which all weigh a good deal less than rabbits. We won't need to use so much of each specific breakdown product when smaller animals are used.

DR. WERTHESSEN: Is there any further discussion on this point? What I'd like to hear from some of the people that have been playing around with this cholesterol regulation phenomena is how they would like to fit in this idea of the bis epoxide being formed and the 25-hydroxy being a natural end result. Now that this has been thrown at you, Pete, have you got any comments to make on it?

*Name supplied by Dr. LeQuesne.

DR. AHRENS: I pass.

DR. WERTHESSEN: With my training as an endocrinologist, I love it.

DR. AHRENS: Has anyone studied the metabolites from the bis epoxide, using labelled epoxide in normal liver systems? Do we know that the epoxide goes to 25-hydroxy-cholesterol? Has that been established?

DR. WERTHESSEN: As far as I know, it has not, and if you want to get in on it, go ahead. That would be my reaction.

DR. SUBRAMANYAM: The bis epoxide was subjected to enzymatic cyclization and it was found that it stops at the 24, 25 epoxide after about an hour of reaction in the in vitro system.

DR. AHRENS: Is that the 24,25 epoxide of lanosterol or cholesterol?

DR. SUBRAMANYAM: Of lanosterol.

DR. AHRENS: It stops at that point?

DR. SUBRAMANYAM: The experiment was done only for a short time. At that point they could not identify any further conversion. At this stage the terminal product was the 24,25 epoxy of lanosterol.

DR. AHRENS: Were you using a system known to be competent at synthesizing cholesterol from normal precursors?

DR. SUBRAMANYAM: Yes.

DR. SHEPPARD: In his handout here, he shows a 5,6 epoxide of cholesterol and when Dawes and Stein in 1907 demonstrated that there could be a 5,7 epoxide. Leland, in your experience as an organic chemist, do you think this is possible with molecular oxygen?

DR. L. SMITH: A 5,7-epoxide might be possible. There would be a four-membered ring involved, but we have not encountered such structures yet.

DR. LEQUESNE: Could I respond to that briefly by saying that they have been generated further chemically in some reactions and by free radical syntheses, but they have not, to my knowledge, been detected as natural products.

DR. L. SMITH: Maybe the reason they had a 5,7-epoxide in 1907 is because the numbering system used then was different from the present one. One needs to translate all numbers in pre-1932 literature to the presently used system.

DR. WERTHESSEN: I've got the impression sitting here that some of our people would like to do a bit of digestion of these data before we go forward. If I'm not mistaken, there's coffee just outside the door.

COFFEE BREAK

DR. WERTHESSEN: When we broke for coffee, Dr. Bloch was talking over here about the possibility of the bis epoxide being present and he rashly agreed to talk about it for the record.

DR. BLOCH: This is really hardly worth talking about and even less worth taking down on paper. In routine assays in which you study squalene conversion in isolated systems and chromatograph the products by a conveyor, you invariably find a fraction which has the correct polarity for squalene bis epoxide. It is more polar than the well characterized mono-epoxide. However, these are in a sense artifacts of artificial conditions. We always add a cyclase inhibitor which is known as "ammo" which is some aromatic compound which was first shown to accumulate a squalene epoxide in plants. And this is how we do our squalene epoxidase assays. And under these conditions, there is as I said a material with the polarity suggesting that it might be a squalene bis epoxide and perhaps stimulated by this meeting, we will try to find out.

DR. WERTHESSEN: Is there any further discussion on the material which has been presented so far?

DR. FRANK: I'd like to just bring up a question about intestinal absorption of these compounds. Is it my understanding from what was said this morning and this afternoon that there is no definitive information as to whether or not the compounds that we were discussing, the epoxides and the 25-hydroxy are actively absorbed--not actively but by a mechanism with transport across the GI tract membrane? Is there any documentation you people are aware of?

DR. WERTHESSEN: The only documentation that I know of is Dr. Imai's original work where the--I beg your pardon, we now have two--the original work on the concentrate of the oxidation products. I'll have Dr. Imai describe that to you in a moment--the precise technique. Some very preliminary experiments which we've started, using a supermarket-available egg custard powder and, more to the point that you're asking, one percent 5,6 epoxide in the diet--a synthetic diet--there are unequivocal effects on the growth of the animal and its ability to become pregnant. Of course, I don't want to go ahead of the story, but I decided to use females because of my background formerly as a reproductive physiologist and I thought it would be highly probable that these agents could upset the standard steroid synthesis.

DR. WATSON: I don't know how specific Dr. Frank wishes the answer. Obviously there have been at least 7 keto and I know 25-hydroxy has been fed to the animals and they looked for a suppression of sterol synthesis in the liver and in the intestine and people have been able to observe. The assumption is that the material got across the intestinal wall and appeared at the active site to cause suppression. But there was a curious experiment of Alan Cooper's in a recent paper where they were trying to definitively explain how 7 keto in fact caused suppression of cholesterol synthesis. It was that when they used radioactive 7 keto in their feeding experiments, they could never find counts in the liver, but they automatically assumed that the counts would be in the liver and there's a quantum jump in the paper until we went to perfused liver, so we can get counts in the liver. But they never did ask where the counts went. So they did perfused experiments and were able to show that the bulk of the material appeared in the bile.

DR. FRANK: This is essentially what I was driving at--the compartmental analysis for example--to find out to what compartment within the body the material is going to be found. It's very interesting what you just commented on. I guess what I was driving at is that in ordinary drug studies we can determine, quantify, the amount that crosses the gastro-intestinal membrane. There are a number of techniques that we routinely use. These are assaying after the drug is administered or simply the

unligated GI tract, or you can ligate it and simply separate it off into segments and then instill the material into those segments and catch it as it comes out through the venous system. There are any number of techniques that can be used. So it seems to me that we can quantify that. Then the next step would be to quantify this distribution and perhaps use regular labelled material of some what--perhaps do a whole body scan or do a slice down the middle, separate it out, and put it on a beta camera or something like that and take a look at it. And the other, of course, is to look for metabolites. There could be a good documentation if it's necessary perhaps to determine the fate of these materials. Perhaps this would supplement the subjective analyses essentially that are done by looking for changes--well any number of things such as an arterial injury or so which is to some extent a subjective analysis rather than objective data. I just wanted to bring this up. This can be done and we have the methodology, I'm sure, for doing these kinds of studies.

DR. L. SMITH: I would reiterate my remark of awhile ago, that other than a paper or so on cholesterol epoxide and cholestanetriol metabolism done some years ago, I do not recall other similar work with any of the available cholesterol oxidation products of the sort you mention. Such work is long overdue. I do not believe we know what the absorption from the gut would be in any case.

DR. IMAI: In that handout that I just passed out, there are references concerning the effects of cholestane triol and absorption and papers by Cook & McDougall indicate that the cholestane triol at 80 mg/kg for 5 days is quite toxic to rabbits. So it undoubtedly does go through the intestine and does something to the animal, and this is no subjective matter. This is a life and death matter. On the other hand, Ito et al. reported a study on the absorption of the cholestane triol and found this chemical is retained to a large extent in the intestinal mucosa. They concluded that this mucosal retention may explain inhibition of cholesterol through the intestinal lymphatics, and subsequently, cholestanetriol's hypocholesterolemic effect.

DR. TAYLOR: A thoracic duct fistula would be useful in this study too.

DR. FRANK: Just reflecting on what Dr. Imai said, one of the things that we would observe or look for is perhaps injury to the GI membrane and that gross injury might lead to permeability increases which are not really an absorption phenomena but simply a partitioning across into the lymph or into the serum. So that as part of an examination of this, we would certainly do a histological examination of the integrity of the GI membrane. If it's toxic to other tissues, one would expect it is to that also--at least in the high doses given. Perhaps on chronic administration of very low dose, there is no appreciable injury effect and maybe it's just carried across by the normal methods of lipid absorption via the bile salts or such.

DR. WERTHESSEN: I think myself that it would probably be requisite in a study of that sort to see what effect cholesterol itself had on the absorption of these things. It may be that it accentuates it.

DR. FRANK: Well, that's a very good observation, and certainly dietary lecithin and other materials would be essential in this case. It could be that by taking all this lecithin as we're doing, we're just screwing up matters by adding additional solubilizing agent, but it could materially increase the amount of cholesterol inhibitors that are absorbed.

DR. AHRENS: Could I ask the chemists whether they can postulate what tissues would do to these hydroxylated materials? Would they leave them alone or would they convert them to other products?

DR. L. SMITH: I have a brief answer. We have some evidence from the literature that esterification of hydroxyl groups of cholesterol autoxidation products may occur. In special tissues, other metabolic alterations may occur; thus, 25-hydroxycholesterol might be converted to bile acids in liver, and 20α-hydroxycholesterol may be transformed into pregenolone in the adrenal cortex. Some A-ring dehydrogenation to steroid 4-ene-3-ketones may also occur, but generally, esterification is the only metabolic event for which there is present experimental support from the literature. These esters have been detected in tissues under conditions where air oxidations were recognized and attempts made to limit artificial oxidations.

DR. FRANK: The comment was made earlier about the fact that it was found in the bile. Was my assumption correct that these derivatives were excreted in the bile? If indeed they are, that indicates that there could be very little conversion and simply a recycling of material which leads to the fact that we could end up with a cyclical pattern where once you get it in your body it's hard to get out because it keeps coming back at you everytime you excrete bile and then it's picked up like bile salts are, simply recycled from the lower parts of the ileum or some other part of the intestinal tract.

DR. L. SMITH: I do not recall studies of bile where cholesterol autoxidation products were sought. Most interest in bile has been in the natural intermediates implicated in bile acid biosynthesis.

DR. WATSON: I was under the impression that in those studies with cholestane triol that one of the things that had been shown was that the 6-hydroxy could be oxidized to the ketone, metabolically.

DR. L. SMITH: The oxidation of cholestanetriol to the corresponding 3β,5-dihydroxy-5α-cholestan-6-one also is readily accomplished by air oxidation and by mild chemical oxidations.

DR. WATSON: The other thing is that I think that in one of the cholesterol ester storage disease, Wolmans, I think it is, says that you can find esters of the 7α-hydroxy or 7β-hydroxycholesterol.

DR. L. SMITH: If you refer to the report of Assmann, et al., J. Lipid Res., 16, 28 (1975), the tissues studied were stored for years at -20° before analysis.

DR. WATSON: That's data that's been published in reputable journals.

DR. L. SMITH: I have discussed this paper with several of the authors. The tissues were stored for years. I do not believe you can take these results for anything other than that if one keeps tissues for years, cholesterol autoxidation products may be detected.

DR. FRANK: A short time ago I think Dr. Imai mentioned that there was storage in the intestinal mucosa. The question is, "Why?" I mean is it an inhibition--does the material just simply infiltrate into the intestinal wall and then have difficulty passing through into the plasma or lymph or what is the blockage mechanism? Or is it injuring the tissue sufficiently so that it's making its own blockage or barrier?

DR. L. SMITH: I am not following what you mean. I do not recognize the data or the comment on Dr. Imai's work.

DR. FRANK: All right, let me just clarify. Did you mention, Dr. Imai, that there was a Japanese group who found the storage of this cholestanetriol?

DR. IMAI: That is correct.

DR. FRANK: In the intestinal mucosa. Now if this occurred, the question would be, "Why would this be site specific in this particular case?"

DR. L. SMITH: I do not know of these data. I would be pleased to hear more about the matter.

DR. WERTHESSEN: That has also been done by an American group at a site meeting where these data were presented in a very early stage, one of the visitors on the committee had been investigating the use of the trihydroxycholesterol as a means of preventing the absorption of cholesterol. And one of the site visitors was quite shocked (he made the inference himself) that apparently they were using an atherogenic agent to inhibit the absorption of cholesterol. I spoke to him and said, "I didn't say it, you did." But apparently it will go into the mucosa, stay there, and interfere with the absorption of cholesterol That is not to say, of course, that it doesn't get out eventually.

DR. FRANK: That's a rather unusual circumstance. I'm not aware of too many drug molecules that will do that sort of thing. They're usually metabolized at the GI membrane, but a storage within the mucosa is a reasonably unusual phenomenon. It leads me to think that there might be some cellular involvement in there that's trapping the material or something of that sort.

DR. WERTHESSEN: Well these workers were quite delighted at their finding. Do you remember who the investigator was, Dr. Imai?

DR. IMAI: Who did the work on the mucosa?

DR. WERTHESSEN: I mean at the site visit in Albany.

DR. IMAI: Oh, that was Thomas.

DR. WERTHESSEN: Have you got the name there, Pete?

DR. AHRENS: William E. Connor and associates, working then in Iowa.

DR. WERTHESSEN: So this is quite a remarkable property of that compound.

DR. FRANK: This is just on that last comment, but it's not uncommon, of course, for drug molecules to saturate membrane transport, in fact one of the rate-limiting steps in absorption is the membrane's ability to pass it. What we're actually talking about is storage in this particular compartment. I think this is a very rare event and certainly is something that should well be looked into and we find storage compartments throughout the body, of course, for many drug molecules. They seek out a specific site or else they just end up being stored there for some reason and then limiting it. But one doesn't usually think of the mucosa of the intestine as site

for this action. This may offer cellular chemists some interesting aspects to look at what's really going on in the cell membrane in structure of the mucosa.

DR. WERTHESSEN: It would be fascinating.

DR. AHRENS: Does anyone know whether the hydroxylating enzymes of the liver could be responsible for producing any of the hydroxylated materials you've been talking about?

DR. L. SMITH: The question has not been asked as such often, but there are allusions in the literature regarding 25-hydroxylation of cholesterol. The Karolinska Institute group has suggested several times that they have encountered a liver cholesterol 25-hydroxylase, but unfortunately they have not published data to substantiate their claims. Without very careful examination of data and conditions, whether autoxidations have been limited, etc., it is very idfficult to determine whether 25-hydroxycholesterol formation is enzymic or not. I am anxiously awaiting publication of data. The case of 7α-hydroxycholesterol which is a liver metabolite of cholesterol implicated in bile acid biosynthesis is, of course, an established metabolism origin for this sterol. Metabolism origins for other cholesterol autoxidation products do not appear to have been described.

DR. WATSON: I think in addition to the Swedish group, there is a group who had a paper in JBC a few years back on omega oxidation of cholesterol in the mitochondria--the rat liver mitochondria, and their consistent finding was that you get maybe 88-90% 26 hydroxycholesterol and it varied from 2-8% or something like that of 25-hydroxy. They put significance in that and tried to argue that it was not due to experimental error. But there's no other data that I'm aware of.

DR. L. SMITH: This would be one of the papers I mentioned, but I do not know whether one can rely on these items. In order to show an enzymic event by means other than the difference between results with enzyme and results without enzyme, I would want some more information. The artificial generation of 25-hydroxycholesterol in liver incubations dates back to Fredrickson's early work, but controversy and possible misidentification of the 25- and 26-hydroxycholesterols clouds some later work. One needs to be quite critical of data purporting to support 25-hydroxycholesterol formation by an enzyme. What controls are had? What are the control levels? One might want to show a dependency upon NADPH or upon a cytochrome P-450 system. Recovery of a cholesterol 25-hydroxylase free from 26-hydroxylase activity would be additional support.

DR. WERTHESSEN: Now that you've put yourself nicely on a limb, Leland, one of the articles of faith I had, although he warned me not to have it, was provided by Dr. Brooks from Scotland. This is a very personal communication. It occurred in the Natick Laboratories, so I can't cite you the references. But they had chased the 25 to verify your findings, and the way Brooks put it, he has the suspiciousness that you have about the reality of the material because it might have happened through the isolation process. But they repeated it several times--not just once--and they had done everything humanly possible to prevent spontaneous auto-oxidation. When you set up conditions such as you have, unless you identify an enzyme that can make it, I have to agree with you that it could still be a fortuitous finding. On the other hand, if I understand your work properly, the 24 and the 26 do not fall into that class. Am I correct on that?

DR. L. SMITH: I think we can accept that the yields obtained in 26-hydroxylation of cholesterol and the stereospecific nature of the indicated 26-hydroxylation by mouse liver mitochondria overwhelmingly support enzymic 26-hydroxylation. We can also accept our own work on cholesterol 24-hydroxylation by brain microsomal enzymes because a stereospecific product is obtained (cerebrosterol, (24S)-24-hydroxycholesterol). Only the (24S)-24-hydroxycholesterol epimer is obtained, not the other (24R)-epimer. With this kind of stereospecificity, one more or less has to accept the intervention of some asymmetric agent, and we do not expect this result from air oxidations. I regard the 24- and 26-hydroxycholesterols as a separate system from 25-hydroxycholesterol, but the matter is not definitively settled by any means. We do not have an easy means to use the principle of asymmetry to settle this point. Furthermore, in that enzymic hydroxylation cannot progress without molecular oxygen, one must aerate enzyme incubations to get products. In so doing, by aerating a dispersed system containing cholesterol, one also has autoxidation occurring, and 25-hydroxycholesterol is among the products. I have thought about this problem for some time, but I do not have a satisfactory approach which we can use that would settle the matter. Unless the 25-hydroxylase can be isolated and purified and its properties examined, with specificities, cytochrome P-450 involvement, kinetics, etc. provided, this matter may remain unproven. I would be very pleased to see someone establish his point, but I do not believe the matter has been adequately addressed yet.

DR. WERTHESSEN: Well, one of my regrets, as I said this morning, was that George Popjak couldn't come. When Dr. Watson and I saw him this summer at the Gordon Research Conference--I can't give you all the chemical details--but he worked up a beautiful way of proving it, by putting C^{13} into one specific carbon of mevalonic acid. But now to get back to the point, has anyone tested the 24 or the 26 as regards their ability to inhibit cholesterol biosynthesis? We've been trying to, but making the 26 apparently is an unholy job. What about the 24?

DR. L. SMITH: Kandutsch has tested a (24RS)-24-hydroxycholesterol mixture of epimers and found it to inhibit HMG Co A reductase, and we have sent him pure (24R)- and (24S)-epimers, both of which are also active. I do not recall the numbers, but the inhibitions were of the same order of magnitude as for the other active sterols. Both 24-hydroxycholesterol epimers were active, the (24S)-epimer cerebrosterol found in mammalian brain and the (24R)-epimer made only by chemical methods and not found in tissues to our knowledge. I do not believe anyone has tested 26-hydroxycholesterol. I have samples which I would be happy to have tested. Our 26-hydroxycholesterol is the (25R)-26-hydroxycholesterol isomer derived from kryptogenin. We have evidence that human aortal 26-hydroxycholesterol may not be a pure isomer but may have small amounts (ca.5%) of the (25S)-26-hydroxycholesterol present as well as the major component (25R)-26-hydroxycholesterol. This is a disturbing matter at present which will have to be clarified.

DR. WERTHESSEN: I think this would be one of the more critical next experiments. Because going back to the steroids, we have three different estrogenic steroids, so I wouldn't be necessarily surprised if we had one or more, i.e., 24,25,26-hydroxyls that would have the same pharmacological or regulatory effect. It wouldn't bother me.

DR. L. SMITH: I will be provocative here and predict that 26-hydroxycholesterol will be active because all the other side-chain derivatives are active.

DR. WERTHESSEN: As far as I'm concerned as a biologist, the evidence is there that these might be the natural regulating systems. Does that bother you?

DR. BLOCH: At the coffee break, someone mentioned the possibility, I think it was you, Dr. Smith, that we may not be dealing on, let's say, negative feedback effects. Because if we were, I think one would want much better structural specificity, and as far as I know, and I may be inadequately informed, but the 7 keto compound, the various side chain hydroxylated materials all have the same effects to a smaller or greater degree. But the possibility which you have just mentioned is that all these compounds do something to the membrane or the membrane system which harbors all these membrane-associated enzymes we are talking about. And in order to create local disturbances, you don't need an awful lot. I mean everyone working with artificial membranes always talks about membranes containing sterol phospholipid in the ratio of one to one which is a very exceptional situation and probably occurs only in two situations in myelin and the red cell, but in other organelles there is much less cholesterol and yet there is reason to expect that cholesterol has profound effects in very small concentrations in the membrane on membrane properties. So, since all the oxygenated steroids are freely soluble in the membrane, usually without requiring any specific uptake process, I could well imagine that one simply wrecks membrane structures which are essential for the functioning of all these enzymes beginning with HMG CoA reductase. An interesting test might be the effects of these sterols on the enzyme preceeding HMG CoA reductase, the HMG CoA synthetase. Now there is some work, I believe, I don't know the literature very well, is it Goldstein & Brown or Lane which shows coordinated effects on the synthetase as well as on the reductase?

DR. WATSON: Primarily the adrenal work of Brown & Goldstein show coordinated.....

DR. BLOCH: Not the liver enzyme?

DR. WATSON: Lane's work shows some coordination, but it's not as strong as ....

DR. BLOCH: And this is all with cholesterol or lipoprotein, right?

DR. WATSON: Right.

DR. BLOCH: Well, what I'm suggesting, it might be worthwhile looking at the effects of these oxygenated sterols on the HMG CoA synthetase which I believe--is that a soluble enzyme?

DR. WATSON: Yes, soluble.

DR. BLOCH: That's a soluble enzyme and not membrane-associated.

DR. L. SMITH: If we examine this thesis, to which I also subscribe, I agree one must look at membrane-bound enzymes in general. I believe this is what information on enzymic reductase and demethylation systems suggests, that these are all membrane-bound systems.

DR. BLOCH: The curious thing is the gut. You see the HMG CoA reductase is membrane-associated and all the phosphorylating enzymes up to farnesol phenyl phosphate are not. They're cytosolic. And then you start again with squalene synthetase and from there on everything happens in the ER system. So it is very tempting to believe that all these effects on regulatory effects are on membrane-associated enzymes primarily and the membrane structure as well.

DR. WATSON: I think that we've begun to recognize that this possibility is a frightening possibility because it terminates your research. It's very difficult to get

into the diffuseness of microviscosity in membranes, but I think with our liver cell system since there are a number of liver-specific membrane enzymes that you can measure and since we have normal liver now, we're going to check, just randomly, a number of ER-associated activities which can be done with fibroblast readily and see if it's broad enough.

DR. WERTHESSEN: I'm dredging up some information from the past, and you probably know this better than I do, Konrad, but isn't there a huge species difference? You worked on it harder than I did. Isn't there a big species difference between the various sterols that you find? For example, Schwenck told me that he used to get cholesterol, lanosterol, and some other form from sheep, and if you extract the liver of the sheep.....

DR. BLOCH: I don't know of any systematic investigation of hepatic sterol. Comparison of species of let's say lanosterol content. I think we think of lanosterol because it's primarily found in wool. I don't think that's reflected in the lanosterol levels in internal organs.

DR. WERTHESSEN: Well, let's assume that it was the same; there could still be a difference in the kind of sterols in different parts of the same animal's body.

DR. BLOCH: Oh, yes, the question is that in the body is its secretion. Now I think one would have to look at the sebaceous gland, for example. Is it--with lanosterol that there's an interference with demethylation in sheep that you do not find, let's say, in non-fur-bearing animals? I have no idea.

DR. WERTHESSEN: Yes, but what I'm driving at, of course, is that there's a distinct possibility here that each organ could make its own group of sterols as regards to the specific requirements of its structure. And this, to my knowledge, is only crudely available data from people like Schwenck who had to use the material by the ton. And they went to the best source. In his case it was sheep's wool. Have you got data on it, Leland?

DR. L. SMITH: Sheep wool fat is very subject to autoxidation and is generally full of cholesterol and lanosterol autoxidation products. A recent systematic study of wool fat sterols has not been undertaken, and work with lanosterol derivatives is not easy. Work with 24,25-dihydrolanosterol 3β-acetate has been attempted, and analyses of raw wool fat shows it to contain peroxidic material and many secondary autoxidation products. It is unlikely that any of these oxidized sterols is specifically made by the sheep, but sound studies have not been made.

DR. WERTHESSEN: What I was driving at more, were the compounds that we expect to see, say in the liver, like lanosterol, desmosterol, etc. These compounds are present in different concentrations, if my memory is correct, in the various organs from various animals. There's not a uniform distribution. I guess I'd better go back to the ancient literature and verify it.

DR. SCALLEN: In rats you see quite an abundance of methylated sterols in the skin, but again in the liver they're only present in trace quantities, so I think it is the same thing that Dr.Bloch was mentioning in the sheep. There may be organ differences, but I'm not sure that these are going to be something to be capitalized on as far as being uniform within the animal.

DR. WERTHESSEN: Well I'm just being a wild-eyed biologist here. The cellular chemists say that the sterols are important in the structure of the cell and skin has a different function from liver.

DR. AHRENS: I haven't seen two fingers raised here today. Let me be the first. May I change the conversation for a minute to ask whether the group is aware of how much squalene we human beings have in us? People have very large pools of squalene, especially in skin and adipose tissue, and these pools seem to be quite separate from those metabolically active pools involved in cholesterol synthesis. Even overlooking the very large amount of squalene that each of us excretes daily in our surface lipids, it appears that less than 10% of our squalene stores are present in metabolically active pools. I'm not at all sure why it's there. There's an enormous amount of it in adipose tissue; that's the biggest store we have. But it's present in practically every human tissue we've examined except the red cell. Those inert pools appear to be mobilizable: when people reduce their body weight and lose triglyceride from their adipose sites, they mobilize squalene as well as cholesterol. I don't know what becomes of it, and I don't know whether it has anything to do with what you've been talking about all day, but perhaps this is a substrate for formation of your hydroxylated sterols.

DR. BLOCH: Did you remember Nancy Bucher's paper on squalene pools many years ago? I forgot who the co-authors were, but this was, I think, in the late 1940's and she clearly demonstrated two pools in liver, but as I recall it, the pools were exchangeable. It was not an inert pool, I mean you could demonstrate different turnover times for the two pools. But the inert squalene was, as you said, convertible to metabolically active squalene.

DR. WERTHESSEN: Would you say there was enough squalene present to interfere with synthesis as Watson showed it?

DR. AHRENS: I can't make that transposition, I'm afraid. I'd have to get together with John and figure out the quantities. I just don't know.

DR. WERTHESSEN: And this in the liver and all our organs?

DR. AHRENS: Every tissue we've looked at except the human red cells.

DR. WERTHESSEN: Well, in old fashioned terms what's the milligram percent, say of the liver? Is it the same order of magnitude as cholesterol?

DR. AHRENS: Oh no. The ratio of cholesterol to squalene in human liver is about 150 to 1 and 200 to 1 in the small intestine, but 10 to 1 in adipose tissue and 20 to 1 in skeletal muscle. In the body as a whole there's something like 3 grams of squalene in each of us, of which it would appear that only 300 or 400 milligrams is in the metabolically active pools.

DR. BLOCH: I don't think it's anything to worry about, do you?

DR. AHRENS: The main worry is the analysts', since it's a major contaminant in all our laboratories, where it presumably arises from the squalene in our fingerprints.

DR. BLOCH: You know we all produce about 3 grams of ethanol a day, not coming from any yeast but coming from some unknown enzyme systems.

DR. AHRENS: That's the cheeriest news I've heard today.

DR. WERTHESSEN: That's why martinis are so good--we make about three of them a day according to one calculation. Have we got any more burning questions? If not I think we can enjoy a well-earned rest. Tomorrow morning we'll put Dr. Imai on the griddle.

SECOND DAY, 13 OCTOBER 1977

DR. WERTHESSEN: Since Dr. Altschule was once a pathologist and now we're going into pathology, he's going to do the "Chairing."

DR. ALTSCHULE: A small part of the general nutritional problem is the phenomenon that we're discussing. We must remember that atherosclerosis is a very spotty disease. It does not occur as a general phenomenon in the body; it occurs in certain areas--bifurcations, branches, curves, etc. That doesn't necessarily rule out a toxic process, because when we produce vascular disease with high doses of Vitamin D, that too is spotty; it's not uniform although if continued long enough, it may become so. Hence the idea of spottiness does not necessarily rule out a toxic factor. It merely points to the fact that we're dealing with something much more complicated. Although we should bear that in mind, it's wise to stick to the very limited aspect of the problem that we have faced in the last couple of days. Dr. Imai, as usual, is loaded with cheery news--like how bad things are.

DR. IMAI: Thank you Dr. Altschule. I'm sure glad you said we are going to talk about one aspect because that's what I'm going to do. One aspect, that is, tissue injury, is my major concern in this collaborative study in sterol chemistry and pathology, mostly vascular pathology. The objective is a better understanding of the mechanisms involved in the initiation of atherosclerotic lesions. Among the components of the arterial wall, my particular attention is directed toward the smooth muscle cells because it is the major cell type and also mainly responsible for the architectural distortion. Among these manifestations, my attention is focused on tissue injury. Necrosis is one of the hallmarks of severe atheromatous lesions. In the course of a study on necrosis, we found that dead and dying smooth muscle cells were demonstrable in atheroma and also in grossly normal arteries after only three days of cholesterol feeding. Concurrent to this enhancement of smooth muscle cell death, comparable increase in DNA synthesis and mitotic index of the medial smooth muscle cells have been demonstrated by my departmental chairman, Dr. Wilbur Thomas, and others in Albany.

These are our immediate objectives in our study in progress. Slide 1. First we wish to do short-term bioassays using the rabbit in order to determine the degree of arterial tissue injury by individual or combined oxidation products of cholesterol. At present we are more interested in finding the combination with potentiating effects rather than to work on an antogonist. On a hypothetical basis, manipulation of the molecular structure of some of these compounds are expected to produce antagonistic effects. Secondly, when we have an effective angiotoxic individual or combined sterols, we plan to do long-term experiments in order to induce gradable arteriosclerotic lesions in the rabbit and swine.

Before going into the data from our current experiments in progress, I shall describe the background and tell you how my particular interests on smooth muscle cell death evolved. Necrosis and lipid-rich debris are the two major hallmarks of

atheroma. Atheromatous lesions are clinically significant in that these are the ones to develop complications such as thrombosis, ulceration, and hemorrhage in the plaque. Yet this necrosis is only vaguely defined. In an attempt to understand this tissue necrosis better, we examined moderately severe atherosclerotic lesions in the cerebral artery of swine induced by high-fat and high-cholesterol diets for one year. By electron microscopy we found the products of cell death. What we mean by the products of cell death are (1) collections of cellular debris, (2) the remains of cells that have severely changed but retain recognizable structural features to make the distinction possible of the nucleus and cytoplasm. Both of these are often surrounded or sequestered by the smooth muscle cell. This grey band is the internal elastica and the thickened fibromuscular intima is here and the endothelial cells are way out of the field. There is a collection of vesicular and dense particles that are no longer recognizable as a cell but only the remains of what used to be cells. The surrounding cells or sequestering cells have the basement membrane and thus these are characterized as smooth muscle cells. Again a product of cell death sequestered by smooth muscle cells. In this portion the distinction of the nucleus and cytoplasm is possible. On a closer inspection in this area, you will see some particles that can be recognized as the mitochondria by the characteristic double membrane structures and other vesicles such as these, the sarcoplasmic reticulum and so on. Some of these dead and sequestered cells have some features suggesting smooth muscle cell origin. In order to test this possibility of smooth muscle cell origin of the product of cell death, 10-kg weaning piglets were fed 800 mg/kg per day of USP cholesterol suspended in rehydrated whole milk powder for three days. Controls were given the vehicle only. Sections of the terminal abdominal aorta were taken on the fourth day for electron microscopy. Conventional criteria for light microscopy for cell death by pyknosis and cytolosis were modified to suit our purposes. Only the cells with the nucleus in the plane of section were counted. The frequency of dead and dying smooth muscle cells was expressed per hundred cells. The cholesterol-fed piglets had a significantly higher frequency of dead and dying smooth muscle cells. We also know from other experiments that this mixture of cholesterol and milk can induce atherosclerotic lesions in four months. The vehicle, or milk, controls had infrequent but definite dead and dying smooth muscle cells.

We have tested this working hypothesis in other situations. Based on consistent results, we postulated that if a regimen could induce a significant rise in the frequency of dead and dying smooth muscle cells on a short-term bioassay, the same regimen could induce atherosclerotic lesions in long-term experiments. One crucial point is the degree of enhancement of cell death. Our current threshhold value is two per hundred cells if the regimen is to be continued for several months for the induction of lesions.

An example of cell death by pyknosis. The ones on the right are normal and usual and, thus, viable smooth muscle cells. The ones on the left are markedly condensed and shrunken but retain enough features for identification as the smooth muscle cell, such as the basement membrane, streaky pattern of the myofilaments and fusiform densities. At the same magnification as in the last micrograph, this cell is markedly swollen and the nucleus rarefied. Thus, this is an example of cell death by cytolosis. For further test of this possible correlation of short-time angiotoxicity and long-time atherogenicity, a system of short-time bioassay was devised. Since our initial interest was in the dietary induction of lesions, the preferred mode of administration was by gastric tubing. Later we added intravenous injection of certain agents. Nembutal anesthesia and fixation of the heart and blood vessels by pressure perfusion fixation are particularly important because our principal indicator is the dead and

dying cells. We like to preserve the tissues under defined conditions without undesirable artifacts. For systematic quantitative comparisons, specimens are routinely coded with random numbers in order to enhance objectivity in the observation. And these are some agents that we have used in the short-time bioassay.

In this experiment the effects of 5% cholesterol diet, which is a conventional cholesterol diet, was compared with that of a control diet. In the past we have used this cholesterol diet in the rabbit for four months and induced moderately severe artherosclerotic lesions in the aorta. One hundred grams of this atherogenic diet was divided into three portions, made syruppy by adding water and fed to the rabbit by gastric tubing. Twenty-four hours after the first feeding, the aorta was perfusion fixed under Nembutal anesthesia. Controls were given the stock diet in the same manner. As shown here the frequency of dead and dying smooth muscle cells are significantly higher in atherogenic diet-fed rabbits than in controls at 24 hours. In controls the products of cell death are present at a much lower frequency but are not absent. Thus we used the word enhancement to describe this higher value in the diet-fed rabbit.

DR. WATSON: The numbers say total count. Does that mean that you only counted five degenerated smooth muscle cells? You have 1.9 and 1.0. You counted 1.9 cells--I don't understand what those numbers mean.

DR. IMAI: These are frequency of cell deaths. To begin with, these are debris. They are no longer recognizable as cells but are only the remains of what used to be cells. These are the degenerated smooth muscle cells. They are markedly changed and thus are considered dead, but they are still recognizable as cells.

DR. WATSON: So that's 1.9% of the 630?

DR. IMAI: Yes, these are the total cells counted and the total cells counted are the ones with a nucleus in the plane of the section. The cytoplasmic portions were disregarded.

DR. WERTHESSEN: But the question, Hide, is, "What is the 1.9?" Is that 1.9 per hundred?

DR. IMAI: 1.9 per hundred, yes.

DR. WERTHESSEN: So in other words, John, in the control there he saw six cells in the 600. Is that right?

DR. IMAI: Right. The bioassay system of the late Dr. Shimamoto which Dr. Werthessen mentioned yesterday deals with an edematous reaction of the arterial wall and changes of the endothelial cells as seen by scanning electron microscopy. I recognize these changes as those mainly associated with transport of blood components. Besides, I find it difficult to translate these changes into numbers for quantitative comparison. The next step I wished to take was to identify the active agents for the enchancement of smooth muscle cell death on short-term bioassay of atherogenic diet. At about the same time Dr. Werthessen had an extract obtained from cholesterol that had been aged by Dr. Taylor. This extract was brought to my attention through our mutual friend, Dr. K. T. Lee. This extract had a characteristic rancid odor and yellow color. Dr. Werthessen also gave me a small sample of cholesterol purified by dibromination. This concentrate of impurities was suspended in aqueous gelatin and a single dose of 250 mg/kg was given by the gastric tubing to the rabbit. Electron microscopy cell counts of

the aorta (again expressed in terms of per 100 cells)--these are the total nucleated cells counted and these are the frequencies per hundred cells--indicated statistically significant enhancement of the smooth muscle cells in 24 hours.

Purified cholesterol at the same dose of 250 mg/kg in 24 hours induced only a slight increase in the aggregate of debris but not that of the dead whole cells. The minimum effective dose of this concentrate is 10 mg/kg/24 hours by gastric tube feeding, as shown by these electron microscopy cell counts.

At the conventional dose of 1 g/kg per day, both old and new U.S.P. cholesterol had significantly higher values of both aggregate debris and degenerated whole cells than in the controls. The old cholesterol was more potent than the new.

When 25 to 100 mg/kg per dose of this concentrate was given at a total of 1 g/kg extended over 7 weeks, this concentrate induced fibromuscular thickening in the aorta. The purified cholesterol at the same dosage, 1 g/kg extended over 7 weeks induced no lesion. And as I showed you before, this fibromuscular thickening induced by the concentrated impurities in U.S.P. cholesterol was characterized by the absence of foam cells and stainable extracellular lipids. In contrast, the conventional cholesterol lesion in the rabbit would be characterized by the abundance of foam cells and stainable extracellular lipids. This electron micrograph was taken from the thoracic aorta 24 hours after a gastric tube feeding of 250 mg/kg of the concentrate. The dead and changed cells by pyknosis and a portion of cytoplasm or marked distortion of the plasmalemma are present in the immediate vicinity of this normal and thus presumably viable smooth muscle cell. Another example of cell death due to the concentrate. This is again at 24 hours and 250 mg/kg. The nucleus is pyknotic and shrunken. The cytoplasm is distorted and plasmalemma has extensive diffusiveness and focal exaggeration of the trilaminar structure. The cellular boundary is obscure and the extracellular substance is seen right next to the nucleus. Concurrent to these changes of the plasmalemma, characteristic dense bodies occur in the cytoplasm of dead cells. These dense bodies are mostly single-membrane bound and seen in the cytoplasm or occasionally in the sarcoplasmic reticulum or in changed mitochondria. By combined scanning electron microscopy and X-ray microanalysis, calcium and lesser amounts of chlorine and phosphorus have been demonstrated by Dr. Nakamura at the Tokyo Metropolitan Institute of Gerontology. I have no answer as to the question, "Is this deposit of calcium extrinsic or intrinsic to the smooth muscle cell or to the arterial tissue or is it the cause or a manifestation of cellular injury?" I have not seen these dense bodies with metallic calcific electron density in conventionally cholesterol-fed animals. The occurrence of these dense bodies in the concentrate-administered rabbits may be due to the high concentration of the impurities.

This is an example of intimal fibromuscular thickening induced by a total of 1 g/kg of the concentrate extended over seven weeks. The endothelium is flat. The internal elastica is way out of the field. As you would expect, the thickened intima consists of smooth muscle cells, occasional poorly differentiated cells with no characteristic signs of differentiation and connective tissue. No foam cells or extracellular lipids are present. This absence of stainable lipids and foam cells is one of the differences between the concentrate-induced fibromuscular thickening and the usual foam cell lesion induced by one to two grams per kilogram per day of cholesterol feeding.

DR. TAYLOR: Did you determine the serum cholesterol levels?

DR. IMAI: They are all in the normal range and the average was something like 40 or 45. Next question is, "What is this mysterious and potent MP4--or the concentrate of cholesterol impurities from U.S.P. grade?" I was told that the data from the Army Research Laboratory indicated that half of the mass is cholesterol. On the hypothetical basis, two possibilities can be considered for the remaining half. One is the impurities carried over from the original biological source of the cholesterol. The other is auto-oxidation products of cholesterol. This graph is from the Army's Natick Laboratory. Dr. Merritt would be able to explain this procedure and findings more properly than I. The important point for me here is that all these peaks are sterols and that some of these sterols have been demonstrated by others as auto-oxidation products of cholesterol, such as cholestane-triol and 7-keto-cholesterol. There are two peaks of dihydroxy sterols and I was told one of these may be 25-hydroxy-cholesterol. These oxidation products of cholesterol have profound effects on cholesterol biosynthesis and are cytotoxic to culture cells as discussed by Dr. Watson yesterday. Some of these sterols have been demonstrated in the artery and brain. References for these are cited in the handout in your hand. Thus our current effort is directed toward the evaluation of angiotoxicity of this class of sterol. The epidemiological implication of these sterols in a human situation seems real in view of the data from Dr. Merritt. We also have some data to suggest that excess Vitamin D is injurious to the artery. The references for these are also given in the handout. Dr. Kummerow will be able to elaborate on the degree and extent of possible involvement of hyper-Vitamin D in the American population. Thus our approach to a better understanding of the initiation of arteriosclerosis is through a better understanding of arterial tissue injury. Rationale for this is that, if the tissue is sufficiently damaged, the damaged tissue would be healed by repair whether the insult be physical, chemical, or other, such as immune or infectious. In the case of the arterial tissue this repair occurs in the form of diffuse intimal fibromuscular thickening. Dead cells are clearly not going to divide or produce the connective tissue matrix, so what we are dealing with in long-term experiments are either at least two functional phases or two distinct population of cells. A relatively simple example for this is as Dr. Taylor showed us yesterday, thermal injury followed by arteriosclerosis or a patch on the inner side.

Our data suggest that a more subtle form of tissue injury occurs in conventional cholesterol feeding. We began evaluation of this complex situation of cholesterol feeding. Our particular attention is now directed to the oxidation products of cholesterol rather than cholesterol itself. What we wish to accomplish is to understand the pathogenesis and be able to control the initiation of a basic form of arteriosclerosis. Some individuals, like centenarians I saw in Tokyo could live happily ever after if it were not for the cancer, infection, or massive cerebral hemorrhage. Included in the future projects are modifications of this response and basic form of innocuous type of injury so that the end result would be atheromatous lesions that are clinically significant. The problems that I am directly involved in are (2) and (3). For this part I depend on the sterol chemists and the analytical chemists in Massachusetts. And these two agents were added by the brainstorm of Dr. Subramanyam. Currently we are using female or male New Zealand White rabbits with the body weight of 1.5 to 1.8 kg. I have tried younger and older rabbits, but this body weight range of rabbits seemed to respond the best. Test agents are suspended in physiological saline using a glass tissue-grinder and given to the rabbit by intravenous injection. This intravenous injection was adopted at the beginning of bioassay of discrete oxidation products of cholesterol because of the uncertainty of intestinal absorption and also the high cost of the sterols. This mode of administration is preferred at present because the response of the pulmonary artery to angiotoxic agents can become grossly visible in three days making this system

quite suitable for rapid screening for both injury and response. Traditionally we have used gastric tube feeding of a suspension in 3% aqueous gelatin in order to insure the dosage and also to regulate the timing. Primary fixation of the heart and blood vessels is by pressure perfusion of glutaraldehyde at 25 mm/Hg as I mentioned before. For light microscopy the descending thoracic and abdominal aorta and other systemic arteries are cross-sectioned at regular intervals. Samples of representative viscera are also taken to evaluate the possible systemic effects. The lungs and pulmonary artery are important for short-term bioassay of angiotoxicity by intravenous injections of saline suspended agents. Specimens of the aorta for electron microscopy are taken from the thoracic segment at the level of atrioventricular junction. These are the agents tested so far. I will mention major findings first and illustrate some of these findings later.

Negative results were obtained with a group of hydroperoxides prepared from purified cholesterol using UV radiation by Dr. P. Dunphy. Band 2 by TLC of the concentrated impurities of cholesterol of U.S.P. grade includes these hydroperoxides. A sample of this Band 2 was used on short-term bioassay and the results were likewise negative. Among all these agents the concentrate of the impurities in the U.S.P. grade cholesterol is the most potent. As I mentioned before the concentrate induced transmural necrosis of the aorta three days after intravenous injections of a total of 30 to 120 mg/kg of saline suspension. When the rabbit was left alone on the stock diet for 10 weeks after such a single chemical injury, the damaged aorta was repaired by fibromuscular thickening. The pulmonary artery responds to two of these agents by grossly visible thickening in three days at 5 mg/kg three times. That is cholestane-triol and 25-hydroxycholesterol. These two are the most potent. These two are mutually potentiating in that half a dose of each induces slightly greater thickening of the pulmonary artery than the full dose of 5 mg/kg of either agent as a single agent. This grossly visible thickening of the major pulmonary artery does not occur at either 8-10 mg/kg three times for three days or at 2 mg/kg three times in three days. At higher doses, the rabbits tend to die immediately after the first or the second injection. Such immediate death is preceded by sneezing, characteristic posture of hyper-extended neck, dilated pupils, labored respiration and apnea. Part of the cause of such immediate death is apparently due to sudden infusion of large amounts of particulate matter. Systemic toxicity is also a likely mechanism for this immediate death because 4 mg for 2 ml/kg of cholestane-triol or cholesterol-5,6-epoxide on the second day after the first day's injection of 8-10 mg/kg kills the rabbit. These two, cholestane-triol, and cholesterol-5,6-epoxide, are the most potent systemic poisons in terms of reduction in the body weight, the wasting of the depot fat and musculature and the collection of pleural and peritoneal serous effusion. Cholesterol-5,6-epoxide and 7-ketocholesterol are the second order angiotoxins in terms of the changes of the main pulmonary artery. The main pulmonary artery near the hilum does not become grossly thickened but develops microscopic necrotizing inflammation and focal fibromuscular thickening. Smaller branches of the pulmonary artery become affected by these changes at a moderate to severe degree. The optimal dose for this class of angiotoxin is again 5 mg per ml per kg. At 2 mg per ml per kg, the angiotoxic effects become less and tend to be confined to the smaller branches. In the peripheral arterioles, embolic crystals are seen with associated thrombosis, organization and collections of inflammatory cells in the arterioles, capillaries and in the lung parenchyma.

Cholesterol purified by dibromination is a third angiotoxin in that as long as it is fresh and pure, the changes are confined to the peripheral arterioles. These changes are embolic crystals, thrombosis, organization and collections of inflammatory

cells in arterioles, capillaries, and in the lung parenchyma. The main pulmonary artery and smaller branches are not affected. Lanosterol epoxide and squalene bis epoxide are a class by themselves. Tentatively I call these delayed-action angiotoxins because a total of 15 mg/kg of lanosterol or 80-120 mg/kg of squalene bis epoxide in three days induces no grossly visible change in the pulmonary artery, but only microscopic necrotizing inflammation in the intima of the main pulmonary artery and smaller branches. The adventitia and perivascular connective tissue are heavily infiltrated by chronic inflammatory cells. When thus-treated rabbits were left alone on the stock diet for as long as 18 days, the main pulmonary artery is still grossly normal. Microscopically, however, acute necrotizing inflammation and foci of fibromuscular thickening are seen in the luminal side of the main pulmonary artery and smaller arteries. I am aware of some objections to the use of pulmonary arterial changes for rapid screening of angiotoxicity. I am also aware that the pulmonary arterial changes do not occur when the same angiotoxic agents are given by gastric tubing at 5 to 10 times the dose for intravenous injections. The process for the induction of pulmonary arterial changes could not be totally dependent upon the particulate nature of the agent because (1) repeated intravenous injections of 20- to 50-micron-lucite spheres or carbon particles do not induce necrotizing angitis or fibromuscular thickening. A sample of cholesterol purified by dibromination induced no grossly visible change in the pulmonary artery when it was fresh in Albany in the year 1976. After a half year of improper storage and transport to Tokyo, the same purified cholesterol induced grossly visible thickening in the pulmonary artery. Furthermore, I'd like to remind you of the systemic effects of the intravenous-injected of the concentrate on the aorta in the Japanese White rabbits. We also have a combination of five sterols that can induce renal arterial changes in three days at a total sterol dose of 6 mg/2ml/kg by intravenous injection. This combination is 1 mg/kg of 25-hydroxycholesterol, .5 mg/kg each of cholestane-3 beta, 5 alpha, 6 beta-triol, cholesterol-5,6-epoxide and lanosterol epoxide. Among these 5 sterols, cholestane-3 beta, 5 alpha, 6 beta-triol appears to be the most crucial agent in this combination because this sterol alone at 2 mg/kg three times can induce similar changes in the renal artery to a lesser degree. Cholesterol-5,6-epoxide may also be participating because a total of 20-30 mg/kg induces acute inflammation of the renal arterial intima. These changes in some of the viscera are listed here because I did see them in sections by light microscopy. I feel that more careful examination, especially electron microscopy, is warranted because these changes are only vesiculation and the possibility of osmotic damage at the primary fixation must be considered, especially in the case of renal tubules.

This is from the renal artery of a rabbit given the combination of the five sterols I just mentioned. The internal elastica is at this level so there is an area of diffuse fibromuscular thickening of the intima, and this happened after three days of intravenous injections of the combined sterols at 6 mg/kg. An arcuate artery of the same rabbit. Cytoplasmic vesiculation of some of the medial smooth muscle cells was accompanied by the presence of nuclear debris in the media.

DR. WERTHESSEN: Excuse me, Hide, which artery is that?

DR. IMAI: This is the arcuate artery--one of the branches of the renal artery inside the kidney. An extension of the renal artery. And because of the co-existence of this nuclear debris and vacuolation of the cytoplasm, I feel that this sort of change is real. This is a portion of the renal artery at the branching site. This rabbit was given 3 daily injections of 2 mg/kg cholestane-3 beta, 5 alpha, 6-beta-triol and sam-

pled on the fourth day. This arterial cushion is much bigger than in the controls and there is an increased amount of cells and connective tissue and probably some nuclear debris.

I think this is a good place to have a break and ask for discussions.

DR. FRANK: I'm not denying at all the obvious irritation effects of the oxygenated cholesterols and so on, but I still am troubled about the possibility of or what the effects would be of the suspension of material being injected intravenously. I'm a little unsettled on what effects are being produced simply by particulate matter, particularly as it gets into the narrower branches of the arterial tree as what effects can occur in that respect. You mentioned that certain particulate matter does not elicit the same type of tissue response--well, purified cholesterols for example.

DR. IMAI: Purified cholesterol does affect the small caliber peripheral pulmonary artery, yes.

DR. FRANK: Okay. It's probably because of its--or it may be related to its particulate function. I was wondering if there's some kind of control that can be applied. Just off the top of my head I was thinking of pulverized teflon or something like this. I don't know what could be used, but some inert material which is known not to elicit a very demonstrable tissue response.

DR. IMAI: I have used lucite spheres of 20 to 50 micron in diameter and these can produce pulmonary hypertension of significant degree yet they do not produce any fibromuscular thickening or any inflammation or necrosis in the pulmonary arteries--they just get all plugged up. And the same applies to the carbon particles. But if you inject fragmented fibrin thrombi intravenously, they will get lodged in the pulmonary artery and they can produce severe fibromuscular thickening. The mechanism for this pulmonary-arterial response does not depend entirely on the particulate nature.

DR. FRANK: I guess there are two questions: What is the relative size of the particulate matter that you're injecting compared to this control that you just described essentially, and what is the effect in the renal arteries by the latex particles--or lucite particles that you used? I guess I'm asking, what is the approximate particle size of the oxygenated cholesterol derivatives that you're injecting in suspension?

DR. IMAI: I have not really measured the size of these particles and this varies depending on the kind of oxidized cholesterol. The pure cholesterol itself is the hardest to make a fine suspension of. It tends to become fragmented and it gets all clumped together. The 25-hydroxy is the easiest. It can make a very fine suspension. The best is actually is the mixture, the concentrated impurities from U.S.P. cholesterol, half of which is cholesterol itself. This makes a very fine--almost milk-like--emulsion.

DR. WERTHESSEN: May I speak to that? The point you are raising, Dr. Frank, Imai and I considered rather carefully and this is why he used the purified cholesterol and the way the thing worked out, in my opinion, was rather nice. The oxidation products grind nicely. The cholesterol does not. So actually the cholesterol forms (if you want to put it that way) a larger chunk than any of the oxidation products do. So that if you were looking for an effect due to obstruction or scouring or injury of the endothelium

of the smaller arteries, you would expect more of it with the cholesterol than you would with the oxidation products. It's a very fortunate coincidence.

DR. E. SMITH: Do you get these effects when you feed by mouth? I feel very strongly with Dr. Frank that this is essentially a non-physiological system and I would much rather see these materials go in by the normal route so that they become incorporated into a lipoprotein molecule. It's quite obvious you're dealing with a very cell-toxic material which is obviously going into the cell somehow, but I would certainly feel happier if I thought it was going in in a more physiological particle.

DR. WERTHESSEN: May I speak first, Dr. Frank?

DR. FRANK: Yes.

DR. WERTHESSEN: What you're talking about, Elspeth, has bothered me no end. We have tried half a dozen different ways of getting it solubilized. Dr. Soloway was due to be here and he had a freak accident, and when I checked up on him, he told me about Dr. Frank, to whom I think we will look for getting these damned things into solution. They're atrocious--particularly the oxidation products--and I agree with you totally. But what we had to do here was to get them in somehow, and the cost of these things is absolutely prohibitive. I agree totally that it's an improvised assay and it's at least semi-quantitative because you don't have to worry about what is being absorbed. And last, but not least, I think Subramanyam's big contribution is the fact that it might not necessarily be administered "per os" all the time.

DR. FRANK: Okay. This was the major concern that I had from the very beginning as I expressed to Dr. Werthessen. I think there could be some mechanisms by which we could more physiologically direct these components as they travel through the bloodstream. Without going into a long discussion of this, we'll just keep it very brief, and perhaps I can present something on this later on. I think that one could apply some solubilization techniques and perhaps utilize some of the technology that we have and others have--for example, liposomal work. By constructing a liposome, for example, which is simply a phosphide lipid vesicle and we like to use the "love vesicles" as we call them--the large unimolecular vesicles in which the cholesterol or cholesterol derivatives or what have you, since they are soluble in the lipid phase, would simply act as a component in forming the vesicle. And then this thing traveling through the bloodstream which is of dimensions approximately the size of a red blood cell and relatively fluid should clear the entire vascular tree and be transported throughout setting up the various equilibria that are necessary in the serum to produce concentrations of the material sufficient perhaps to elicit a response. Now the liposome itself is not a typical circulating substance, but it isn't very much different from the lipoproteins that we have which are essentially solubilized systems in the bloodstream. So that this is one approach which I think would be a very promising technique. It's interesting, of course, that the conventional liposomes tend to aggregate in both the liver and spleen and could provide an additional benefit in that respect with regard to looking at hepatotoxicity and splenic toxicity although their duration in the bloodstream is sufficiently long to give us some idea perhaps of arterial disposition. That can be argued as to how long they persist in these tissues; for example, in the bloodstream, but that's something that can be worked out, I believe. I think this is one approach that can be taken. I shudder at the thought of suspensions because of the very randomness of the attempt. I think it's a fine idea in the sense that it's perhaps proven some valid points, but it might be

at this point wise to direct our attention, as Dr. Smith has indicated, towards more physiological presentation of the materials and see what effects come from there.

DR. IMAI: Thank you for the suggestion. I do share your concern about the non-physiological nature of this experiment. However, once these particles--suspended particles--go through the pulmonary capillaries, they are in the order of less than 5-6 microns in diameter--perhaps smaller--and by the time they reach the systemic arteries, the aorta and renal arteries, they must be in a much dispersed state. Before I went into this pulmonary arterial system by intravenous injection, I did feeding--gastric tube feeding--of the 25-hydroxy and cholestane-triol by gastric tubing of the gelatin suspension. At 100 mg/kg, I can show you the same significant enhancement with smooth muscle cell death. This is by electron microscopy cell counts. This is very expensive and time-consuming. In contrast the intravenous injection system gives you an answer that is grossly visible in three days and we are using this not to prove any point but to make differentiation between the grossly angiotoxic substances from non-grossly angiotoxic substances, such as pure cholesterol or carbon particles or lucite spheres. So this is being used as a convenient, rapid screening bioassay.

DR. FRANK: I understand the reasons for it and I commend your types of work that have been done. I'm perhaps simply looking for a refinement of this or perhaps a mechanism by which we could look at a more controlled release of the material or perhaps for a longer period of time or at least to regulate its control over a more steady-state type of release. There is one problem, of course, with very fine particles of solid matter and that is that their charge picture becomes quite crucial. The magnitude of charge which is accumulated by subdividing materials even of organic nature is quite high and there can be a degree of self-aggregation and a higher tendency for them to adhere to other structures, so that it's possible that we might not have a completely dispersed system of solid matter--semi-rigid material floating through the bloodstream--but rather aggregates or material clinging to other substrates.

DR. IMAI: I might mention that the substance we are dealing with is in the same class as cholecalciferol. These are very potent chemicals at small doses. You can give Vitamin D by mouth or by injection and you will induce perivascularitis, vascular damage, and arterial smooth muscle cell death. Regardless of the route, Vitamin D can induce vascularitis that is recognizable by light microscopy and, of course, by electron microscopy.

DR. L. SMITH: There may be some confusion about what a suspension means in this regard. It has been known for a long time that aqueous colloidal dispersions can be made of cholesterol autoxidation products without any dispersing agent or surfactant. These are very fine emulsions with no visible particles present in the system. Of course, albumin, blood serum solids, and other agents possibly present in blood are also known to aid in the dispersion of cholesterol and its oxidation products. I am not aware of how you disperse your preparations, but ordinary cholesterol mother liquor (methanol) solids from cholesterol autoxidations are quite easily dispersible by themselves to give fine emulsions.

DR. ALTSCHULE: I should like to point out that in clinical practice we introduce into the circulation an astonishing variety of materials--that's called treating the patient as a hole (h-o-l-e) into which you pour all these things. Some of the

things we pour in are particulate. You see that a good deal in bloodbanking where, after the blood lies around for a while, aggregates of platelets, fragments of red cells, and various other kinds of debris accumulate--enough to clog a filter, if you happen to have a filter. But if you don't have a filter, you let it run into the patient. Nevertheless, no lesions like this ever occur. However, if too much of that debris runs in, if given unwisely, the patient will develop pulmonary insufficiency and clogging of the very small vessels of lungs with fibrosis. However, nothing like the lesions we see here occurs. Moreover, there are other things that get into the circulation; for example, in fat embolism. This develops after trauma and masses of lipid material big enough to clog capillaries and cause the death of a patient may form, but again, nothing like the lesions we see here occurs. The occlusion of capillaries by particulate matter is a well known phenomenon and it produces a well known pathological and clinical picture--but nothing like this. On the basis of half a century of clinical experience, I cannot accept the concept that these changes shown by Dr. Imai are due to particles. Undoubtedly there are better ways of doing this experiment, but Dr. Imai should have every possible compliment because the amount of this work and the quality of it is phenomenal. Nevertheless I'm sure there are somewhat better ways of doing some of these things and they will be done, but I should like to emphasize that on the basis of clinical experience involving thousands of patients--and a few animals--this lesion cannot be due to particles.

DR. WATSON: I was going to try to get back to the purpose of the angiotoxicity assay in terms of using it as a screen, because obviously every time you test a compound, you kill a rabbit and you have to do all your sectioning. And if your purpose is purely a screen, would not a smooth muscle culture or something of this nature when you're asking the question.

DR. SCALLEN: I wanted to get a clarification on the feeding experiments. You did feeding experiments at 1 g/kg for 7 weeks, is that it?

DR. IMAI: That is the total amount of cholesterol--not per day. This one gram was divided into 25 to 100 mg/kg per dose.

DR. SCALLEN: I see, so this would be a dose that would be totally incapable of producing atherosclerosis in the animals then, I assume. In other words you would not have raised the serum cholesterol enough to .....

DR. IMAI: No. The serum cholesterol level remains normal.

DR. FRANK: I hope it's not an attempt to belabor a point to death or to bore you to death, but I want to clarify a couple of things that you reflected upon which I think might have been a little unfairly directed to what I said. First of all, I'm not questioning Dr. Imai's work. I think it's not only extensive but quite illustrative and as everyone else is appreciative of what has been done, I think that there's a point demonstrated. However, my concern was directed at eliminating one of the potential possibilities in the experimental protocol so that we could eliminate the effect of particulate matter. Reflecting on what you said, it's indeed true. Of course particulate matter functions within the body. If it wasn't for that we wouldn't shut off an inflammatory response. We wouldn't have MI's; we wouldn't have pulmonary emboli and what have you and so forth. Certainly that's true and I don't think the effect of the arterial lesions that are seen here are entirely due to particulate matter. I didn't mean to imply that and I think I

clarified that in an earlier statement. But what is a concern to me is that in the illustrations that I just mentioned and that you mentioned, we are not dealing with solid matter; we're dealing with semi-solid structures which are biodegradable and flexible fluid, not rigid. So that the clot that forms, while it is an occlusive clot of course by virtue of its viscosity and its integrity maintains the occlusion of the artery or the buildup of platelets or fibrin in the closing off of a vessel that's been broken during the inflammatory response is similar to that also. What I'm talking about are actual solid particles traversing the bloodstream. Now whether they're colloidal dimensions or not--colloidal dimensions, of course, range a wide spectrum--nonetheless they are solid in nature and this is what troubles me. Now the FDA would never approve for any clinical administration any solid suspension of material for intravenous use. If you apply a filter to it, of course, you're going to remove this drug. Now infiltrating platelets from whole blood is certainly acceptable because you don't want to introduce a circulating clot. But under the current regulaions of FDA only one emulsion is permitted for introduction into the bloodstream and that's an egg lecithin emulsion which is provided for increasing caloric content, and that went through one heck of a rigorous thing until it was proven that the dimensions were sufficiently small and that the system was sufficiently fluid to be able to pass the bloodstream intact. So that's where my concern lies and I'm simply trying to alleviate this one element of this protocol and to provide a more physiologically acceptable system. No attempt is made to deny what you said or what Dr. Imai has said.

DR. E. SMITH: Dr. Imai, have you looked at the lipoproteins after the administration? Can you show that any has gone into the lipoproteins?

DR. IMAI: No I have not done the study.

DR. E. SMITH: This would be very interesting, I think.

DR. WERTHESSEN: I must say that I've been in part responsible for what my colleague did. Let me go down the path in this fashion. We tried solubilizing it; that is getting these things into solution in the usual methods. We tried dissolving them in olive oil, peanut oil, and such like. Cholesterol goes in beautifully. They do not. I did know that we could get them into serum, but here you face a monstrous task. If you're going to use serum, you have to do it under absolutely aseptic techniques. It's tedious. The procedure is to dissolve it, say in methanol or ether; pump off the solvent so that you have it on the side of your flask and you add your absolutely sterile serum and you rotate the thing, and this is very nice. And it can be done, but the problem was to get the thing done in a hurry. Now let's go back to what he used as a control--that was cholesterol. And I think in any bioassay procedure, if you do your control in the same fashion as the experimental side, the comparisons are valid. I won't argue with you that because the more polar nature of the oxidation products, the charge on the end of the crystal and so on and so forth may be different. But I doubt very much, as a first approximation, that the toxicity that you observed is due to that difference in charge, particularly by the time it got down to the renal artery. So that this is a simple bioassay procedure, the same as that I mentioned in my opening remarks when we were dissolving carcinogens in olive oil, injecting them subcutaneously and examining the response of the mouse's vagina to those same agents. But I think we can get awfully concerned now that we don't deal with bioassay anymore because we don't remember the simple rules of the game: "Set up a good control and make your comparisons with it." And under those conditions, I think these observations are sound as regards the relative potency of the different oxidation products.

DR. WATSON: In one of your ealier slides you had pointed out that in the gavage experiments you got lesions with the cholesterol and what I was wondering was, have you or anyone else gone back and used highly purified cholesterol and re-fed rabbits and asked the question, "Do they develop lesions and at the doses that poeple have used in the past to show they could get lesions in rabbits?"

DR. IMAI: No, I have not, and this is due to the supply of purified cholesterol.

DR. TAYLOR: Although this is not a direct answer to Dr. Watson's question, I think that it should be mentioned that there are three reported studies where endogenous hypercholesteremia of about 600 to 1000 mg/100 ml. were induced. These studies were (1) (Seifter & Baeder, 1956) in which hypercholesterolemia was induced by injection of pituitary extracts into rabbits, (2) (Chaikoff et al., 1948) production of endogenous hypercholesteremia in chickens by administration of diethylstilbesterol, and (3) (Ho et al., 1974) induction of endogenous hypercholesteremia in hens with an hereditary inability to lay eggs by lengthening the photoperiod to 16 hours per day. Even though all of these animals had profound (endogenous) hypercholesteremia, they developed no, or minimal, atherosclerosis, whereas animals with equal levels of diet-induced hypercholesteremia had advanced, severe atherosclerosis.

DR. IMAI: I'd like to add one more strain of rabbit to this category. This rabbit was found by Dr. Kondo at Kobe University in Japan and he called this hyperlipidemic rabbit or HLR. This is a genetic hereditary hypercholesterolemia rabbit and they do develop quite severe atherosclerotic lesions in about two years. I do have some specimens but I have not really studied these specimens yet. The levels of serum cholesterol are high, but I don't know how much of it is pure cholesterol and how much may be oxygenated products.

DR. WATSON: Well, I think one of the things you have to concern yourself with, in view of the emerging information that's coming out on lipoprotein metabolism, is that hypercholesteremia *per se* may not be the signal you have to concern yourself with. It would be more in terms of, if the large amounts of cholesterol are being carried on, the very low density lipoproteins or the HDL's or the LDL's etc.

DR. TAYLOR: I personally think that one of the very urgent new things that should be done is to feed absolutely pure cholesterol to a number of animals (rabbits, chickens, and pigs). This is an urgent study that should be done.

DR. L. SMITH: Perhaps your request that pure U.S.P. cholesterol be used has already been attempted. However, it does no good to start with pure cholesterol because, unless it is kept pure, you cannot be sure of what is transpiring between adding pure cholesterol to rabbit chow and getting the chow into the rabbit. A typical protocol for such studies involves dissolving cholesterol in ether, adding the ether solution to chow, allowing the ether to evaporate in air, etc. These conditions are very likely to provoke cholesterol autoxidations. The prepared chow is effective in inducing aortal lesions in rabbits, but this protocol is not one to be followed if an effect of pure cholesterol is being examined. Cholesterol in ether exposed to air is an acknowledged means of oxidizing cholesterol. One would have to design a protocol which would use pure cholesterol but would keep the cholesterol free from air and other oxidizing conditions over the course of preparation of the food, storage, and feeding.

DR. WATSON: What I was going to respond to that in terms of a protocol: Dr. Scallen and I were talking about this and sometime ago decided that the only way you could do this is tube-feed those animals every day.

DR. WERTHESSEN: Schwenck actually tried to do this. Now he was an extraordinarily persnickety person and they fed, I think, about 5 rabbits a day if I remember the thing--well let's just use 5 rabbits as an example. That meant that he had to go through that dibromination and produce 5 grams per day with a 50% yield and I don't think if we publish I'll let this get into the published record, but the reason I assumed that the cholesterol when it got into the rabbit was not pure was because I knew the technicians in the animal room, and they were going to mix it with food and I'm sure that they did what Leland was talking about. So, in spite of all his work and the daily purification, by the time the animal got it inside its gut, it had had a chance to oxidize, and I never could tell the man that because he'd have been furious, but that was my hunch--the one I'd mentioned earlier.

DR. LEQUESNE: I think that we should also consider that once the cholesterol is introduced, even if it's purified as Dr. Werthessen says, once it's introduced into a chow, the chow is not a chemically inert substance and the catalysis of decomposition, peroxide formation, and further decomposition is probably greatly accelerated by the materials in the chow. What pH is chow, for example? We're going to see the cholesterol dispersed on the surface of solid particles of food. There's no telling what's going on here and in how short a time.

DR. SCALLEN: Well perhaps that problem could be taken care of by dissolving it in a rather hydrophobic substance where it would be stable such as an appropriate oil and storing the oil under conditions anaerobically perhaps at very low temperatures or else perhaps just simply making it up fresh from material that is known to be pure and has been kept under nitrogen in the deepfreeze. So I think that those methodological problems could be overcome and one could treat the animals as Dr. Watson has suggested and feed the cholesterol dissolved in the oil and presented in that manner.

DR. IMAI: I'd like to say one thing about the use of oil. One time Dr. Werthessen gave me a pair of unknown samples, one was dark-colored olive oil; the other was light-colored olive oil, and I didn't know which was what. I did intraperitoneal injection of these two oil specimens. Both were negative in that the enhancement of cell death was below 2 per 100 cells. Nonetheless, between these two, the light-colored one had slightly lower value than the dark-colored one. Later I was told by Dr. Werthessen that this light-colored one was the mixture of one of his agents and upon mixing with olive oil, the color of the oil changed and became lighter, suggesting that there was some interaction.

DR. WERTHESSEN: It wasn't just the reaction in the olive oil that we had purified and the collection of oxidation products.

DR. ALTSCHULE: Dr. Frank hasn't taken his hand down yet.

DR. FRANK: I had my hand down for quite a long time. It's pink. I might just want to add about the oil problem. We worked quite extensively with vegetable oils and it's almost impossible to get pure vegetable oils. We have some procedures for extracting soybean oil, for example, that require you to use it almost immediately

after it's been prepared or else, if we make up quantities of it, it's reasonably free of oxidation products and all sorts of other things. It's stirred under nitrogen, kept in the dark, and put in the refrigerator and we pray. That's about the size of it and that's a pretty tough deal and if you're going to put something else into it, as we do, the other stuff gets all messed up because it's invariable that there's going to be some reaction--or more likely that there's going to be some oxidation system going on.

DR. IMAI: Along the same lines, I'd like to mention that I tried intravenous injections of "Intralipid." This is FDA-approved oil emulsion and when it is fresh, it's okay. There's no visible response by gross or light microscopic examination, but one time I used an old preparation that had been opened and kept in the refrigerator for about three months and this had tremendous effects on the pulmonary artery--very bad.

DR. FRANK: The reason for that, of course, is that "Intralipid" is stabilized with the purified egg lecithin which is a zone-refined material and you've got a very good model obviously now to consider because there you're dealing with the potential oxygenated compounds and I think that's an interesting experiment. We'll look into that a little bit more. Intralipid, of course, was the only product that was approved for intravenous administration--it's an emulsion that has a particulate matter. Everything else has to be a solution.

DR. SHEPPARD: I'd like to point out to you that oils, even though they're pure, supposedly pure oils, in the cracking towers and the processing of making oils, there are catalysts introduced, there are traces of these and no one knows what they would do to these oxidation products. As far as getting pure cholesterol, we scaled our system up to where we can process 150 grams of cholesterol at a crack, so as far as keeping up with somebody with rabbits, that's no real problem, but how to keep the stuff in some kind of shape until you introduce it to the rabbit. Now one way of doing it would be to mix your diet in a nitrogen box and spoonfeed that immediately to the rabbit right there. That would be one way. And if you're that interested in doing the business, then you're going to have to be meticulous, or else by gavage. Well, it's true that it's going to be a very expensive process, but if you're that devoted to the experiment, then that is the price you have to pay.

DR. WERTHESSEN: Dr. Merritt got here with some very nice slides of what is in U.S.P. (high-grade U.S.P.) cholesterol and it was thought that if he showed those, we'd have a little bit better ground for comparison of cholesterol feeding and what purified cholesterol is. Do you want to go ahead, Charlie.

DR. MERRITT: I'm not sure exactly what you want me to do.

DR. WERTHESSEN: Well, just show us what you found.

DR. ALTSCHULE: You're expected to lay it on the line.

DR. MERRITT: I'll show you what I've got here anyway. First of all I understand that Dr. Imai has already shown the slide, but I don't know to what extent he commented on it.

DR. WERTHESSEN: He's a pathologist.

DR. MERRITT: There are one or two things that perhaps ought to be said. First of all is the occurrence of this methyl progesterone which seems a little bit unusual. I'm not sure that we have any explanation as to how it may arise except that it's in there as a natural constituent. I am aware that Dr. Smith says that 16 methylation is not possible and so on enzymatically, but.....

DR. L. SMITH: Is that 16-methyl that you mentioned? 16-Methylprogesterone?

DR. MERRITT: Now the only thing that can be said about this is that it matches exactly the reference compound in the mass spectrometry file.

DR. ALTSCHULE: Dr. LeQuesne, do you have some comment?

DR. LEQUESNE: 16-Methyl. I'm very surprised that that would occur in such a mixture as that. In fact I know of no naturally occurring 16-Methyl steroids.

DR. ALTSCHULE: Well, you all came here to learn and now you're learning.

DR. L. SMITH: Such a compound in nature is absolutely unprecedented! I would seriously caution you to prove the matter beyond further doubt.

DR. MERRITT: I agree. This is not stated as being unequivocally identified, but I would like to say--I recognize there's doubt about this compound; that's why I brought it out. But on the other hand, and of course matching that spectra is not entirely unequivocal either, especially when you have possibilities for isomers and so on, but the mass spectrum of this peak does match that compound in the reference library. That's all I can tell you about it at this point. The other thing which I think is important to notice is the presence of the cholesterol acetate. This is one of the major components. And it appears in almost every sample of U.S.P. cholesterol that we have analyzed, and we have at least triplicate analyses; that is, three separate sample sources of U.S.P. cholesterol which have the acetate, and it's one of the most abundant compounds.

DR. WATSON: Did you saponify your mixture and then show that peak disappear?

DR. MERRITT: No, no. But we did some reading on the preparation, the isolation of cholesterol from lamb's wool, which we presume is the source of this cholesterol; and acetic acid is used somewhere in the cleanup procedure, so it is not unreasonable to find a compound like this cholesterol acetate. Anyway, one of the reasons I mention it is because in the subsequent analytical work that I'm about to describe, we can eliminate it from the analysis of the other compounds which might be present at that same region in a gas chromatogram, and that's important, because if you notice these two little shoulders here, the first one corresponds to the retention time of the epoxide. If that is present in a very small quantity, you won't want to have it obscured by this large acetate peak. The other peak down here is a keto compound. We're not sure what it is. It could be 3-keto with the hydroxide or 7-hydroxy or something like that. It has the retention time for such a compound, but there's not enough of it there and it's obscured in the mass spectrum by the acetate. I guess the other compounds are straightforward. The thing to notice is that this first one--this dihydro sterol, this is the right retention time for the 25-hydroxy and this one over here for the 24-hydroxy. But we can't tell from the mass spectra which is which, so we just call them a dihydro sterol. The triol stands out by itself way out here.

You might be interested in the procedure. These analyses are all done by taking the cholesterol up in methanol and then crystallizing and removing the mother liquor and then drying down the mother liquor and taking it up in the appropriate solvent, which is usually chloroform for the GLC analysis.

Well anyway, we became interested in separating the bulk of the cholesterol from the oxidation products by some prior step. This becomes very important in the analysis of the food products because whenever we're dealing with eggs on the GC--I'll kill two birds with one stone, I'll put another chromatogram here. This is exactly the same as the one that was shown previously except that it's a different lot and you see you have essentially the same patterns or it looks very much, except for the relative amounts of these components--like the one which you were shown previously. But the point that I wanted to make was, if we have a non-saponifiable fraction, which we take directly for GC from egg yolk or dried egg products and then--especially the food products to which corn oil has been added, we have a number of minor sterols which are present such as stigmosterol and desmosterol and so on which elute in this region and obscure this spectra for any of the oxidation products.

So it's useful to use some sort of separation which will separate the sterols from their more polar oxides before going to gas chromatography, as a class. And we find that this can be done very well on silica gel and by liquid chromatography. Here is a liquid chromatogram and this is the solvent front. The sterols elute here and, as a matter of fact, this large peak here includes the acetate--and the sterols and the more polar triols and so on, or rather diols and triols are out here. The elution is actually carried out with solvent programming from about half a percent isopropanol in hexane up to 7% isoproponal in hexane over a period of 30 minutes up to about this point. From here on it is continued with 7% isopropanol and hexane. This was a small particulate silica gel column about 25 micron particle size and was a narrow bore column about a foot long. Also I want to point out we ran this at two wavelengths simultaneously. The 254, of course, is the fixed wavelength ultraviolet detector usually found, but we also have a variable wavelength detector and this upper trace is at 210 nanometers where, of course, absorption is much greater. So you can see the presence of compounds in this region here at the lower wavelength that are not visible and not detected by the detector having the higher wavelength. Depending on where you cut--for example, if we take the cut from here to here, let's say, then a gas chromatogram after the lipid chromatography you see that the acetate in this region has been removed. And now we clearly see a peak here --this peak here which could be the epoxide. Also of course, it depends on where we take the cut. If we take it before we get way out to the end, then we would remove the triol. Actually I'd like to show you the difference between what's taken between this point and this point, which is before the cholesterol, and then one at the region where the sterols are eluted and finally where the oxides elute. This is a GC after the L.C. cuts. The acetate now appears with the sterols like cholesterol, dehydrocholesterol, and so on. Thus in the last fraction, we can enhance the intensities of these oxide products in the cholesterol. For example, here we have the 25-hydroxy and this is probably the 24. If we carry the elution out far enough to make sure that we bring in the triol, that will also appear in the subsequent gas chromatogram.

Finally a chromatogram which is essentially the same as that I showed previously but a slightly larger amount. Again we see the enhancement of the oxidation products in this fashion which have been recrystallized. The mother liquor is then put through an LC separation then that polar fraction has been taken after the elution of the sterols on the silica gel column was taken and run as a GC chromatogram and that is

what we're looking at here. The enhancement of the 25-hydroxy or 24 peak and that of the triol. So that's the story as far as the U.S.P. cholesterol is concerned, and I guess if you want me to talk about it, I'll do that later.

DR. WERTHESSEN: May I ask a question? I'm going to direct it to Leland. I assume that each one of those bumps in there is another compound. Is that equal to, or more than, the number that you've listed as potential oxidation products?

DR. L. SMITH: Our publication on this matter from 1968 did not use the pre-fractionation by liquid column chromatography, but I recall we just got tired of numbering all the resolved components. There are 25-50 components which can be resolved with ordinary care, and we can account for at least 50 distinct compounds produced by known mechanisms of cholesterol autoxidation. I believe you can expect to encounter up to 50 components in such preparations. What is shown here is typical of autoxidation mixtures which have been enriched, that is, much of the cholesterol removed by recrystallization.

DR. MERRITT: That was a procedure to be able to get at the oxidation products which are in very low concentration. No particular effort was made to oxidize this cholesterol. This is cholesterol from the bottle. I think one of the considerations though that we ought to make here is: this procedure is a good way and a quick way actually of telling whether you've done a good job of clearing things out. If you were to take the other part of it, mainly the cholesterol itself, which has been recrystallized, that should be free of some of these oxidation products. If you go through dibromination or some of those other steps, it would be even better. But if you take your mother liquors after successive crystallizations and apply liquid and gas chromatography, then you should be able to establish the purity of what you're working with with some degree of assurance.

DR. WERTHESSEN: You didn't happen to bring in the analysis of the dibromo purified stuff, did you? Where Walter found an extra peak?

DR. MERRITT: I don't think so--well, possibly, I have some other things here. You might recognize it, Nick.

DR. WERTHESSEN: This was a thing that was a disappointment to me. That is that freshly dibromo purified showed another minor peak.

DR. L. SMITH: This was work I did some time ago. One must not speak of dibromination and debromination as if that were the end of it. Such preparations must be recrystallized repeatedly to remove products formed during bromination and debromination. The bromination procedure removes oxidizable and unsaturated congeners of cholesterol, but bromine and air also oxidize some of the cholesterol.

DR. WERTHESSEN: Of course this purification was with repeated crystallization.

DR. MERRITT: This is the first one we did that you brought over with this OXP 3.

DR. WERTHESSEN: Yes, that was from U.S.P. cholesterol.

DR. MERRITT: And this is a chromatogram before we did anything to it.

DR. L. SMITH: Are those gas chromatograms or high-pressure liquid chromatograms?

DR. MERRITT: Gas chromatograms.

DR. L. SMITH: Why is the background rising? This is disturbing.

DR. MERRITT: Probably it is column bleed-off.

DR. L. SMITH: One cannot tell what is under the rising background. How many components might be missed?

DR. ALTSCHULE: Dr. Watson, you've been leaning forward so tensely that I think we'd better call on you or you'll fall on your face.

DR. WATSON: I'm struck by the high proportion of cholesterol acetate that was in the U.S.P. cholesterol. What fraction of the total cholesterol was represented by cholesterol acetate?

DR. MERRITT: Well, we haven't actually measured that quantitatively. I would say it is somewhere in the order of perhaps 80% of the amount of cholesterol which remains in solution in the mother liquor based upon the peak areas of the acetate versus the cholesterol in the GC, but, after all, most of the cholesterol has been recrystallized so the amount of it relative to the cholesterol is very small.

DR. WERTHESSEN: Walter and I estimated that it is less than one-half a percent.

DR. ALTSCHULE: Were you just scratching your head or do you want to be heard? Would you advance to a microphone, please?

DR. LISANTI: All I'd like to ask is a naive question, and that is, when does a compound or a preparation stop being U.S.P.?

DR. ALTSCHULE: There's a stunned silence. We can now pass on to Dr. LeQuesne.

DR. LEQUESNE: Could I comment? First of all purity, like some other things, is a negative quality and to the extent that anything is pure, it is only until it's proven to be impure. And cholesterol is a substance which as we know oxidizes very readily and even if you purified it by the most rigorous means that you had, you could undoubtedly establish that even 10 seconds after exposure to air, if you had a sufficiently refined technique, you could see some impurities in it. And so the classical organic chemist's view of purity is that a substance is pure where it is shown to be homogeneous by all the methods at his disposal, but each pure substance awaits attack to show that it is in fact impure.

DR. L. SMITH: The organic chemistry view may not be adequate. One must apply every strength of the analytical chemist as well. Generally the organic or biochemical approach to purity might not take into account moisture, solvent residues, ash, bromide, zinc, etc. As these extraneous items, particularly halogen and metals from purifications procedures, may exert influences on the stability of cholesterol, these matters need attention too.

DR. LEQUESNE: Sure, you and I are saying the same thing.

DR. L. SMITH: Yes, with different words.

DR. LEQUESNE: The real question I wanted to ask is, does the acetate arise as an artifact of processing cholesterol, because we know that the alcohol at C3 in cholesterol can be acetylated if you just dissolve the cholesterol in acetic acid? I want to ask as a point of information, is acetic acid used in the purification of cholesterol from brain, for example?

DR. MERRITT: Yes.

DR. LEQUESNE: Well, that must be how it comes then.

DR. TAYLOR: I think an interesting thing is the sources of cholesterol over the years. I got my first batch of cholesterol for nothing from Armour; they extracted it from freshly-killed spinal cords of cattle. Under these circumstances, probably the oxidation opportunities during extraction were minimal. Apparently the only current source of cholesterol is that extracted from wool in Holland. The lanolin and cholesterol are attached to the sheeps' wool for about a year and probably get a pretty good chance to oxidize. They extract these two compounds (lanolin and cholesterol) and sell the cholesterol for $500 per 100 lbs. The new cholesterol we're getting now is already 5% degraded. So there's been a change in the commercial sources of cholesterol over the years.

DR. SHEPPARD: I think that when we talk about U.S.P., you have to look at the criterion on which their specifications are based, and their specifications are based on old-type classical techniques. They were fine at the time of the state of the art when they made the specifications--well, it was the state of the art that they had available to them, Leland. Ignorance was bliss at that time in many respects. For instance in Vitamin K, when I have taken modern procedures and looked at the compound, in no way did even the infrared and UV spectras meet their old criteria. And so we're able today with techniques of high pressure and good GC to see characteristics beyond the older techniques. For instance the cholesterol specification "U.S.P." doesn't say a thing in the world about GC--those specifications were written long before the advent of GLC. And so they've continued with no revisions and U.S.P. has a very special reverent reputation. It's like this hall of Harvard University; it's a hallowed piece of ground, and you don't attack it.

DR. L. SMITH: I agree that the term U.S.P. cholesterol is like saying CP cholesterol or some such. The U.S. Pharmacopoeia does not have any criteria for cholesterol purity which is adequate to the problems we are addressing.

DR. SHEPPARD: I agree, the specifications are archaic.

DR. ALTSCHULE: If Dr. LeQuesne has nothing to say, we can proceed; otherwise I was going to ask Dr. Imai to sit down again.

DR. LEQUESNE: I was just going to paraphrase Oscar Wilde and say that purity is like ignorance: it's a delicate and exotic fruit; touch it and the bloom is gone.

DR. ALTSCHULE: Well, that was great, but I didn't like your gestures.

DR. MERRITT: I'll make one more comment and that is from my experience in analytical chemistry. Anyone who is performing experiments in a laboratory does not regard the material in the bottle as having any necessity in conforming at all to what it says on

the label, so you have to approach the use of any reagent with the thought that it may have changed and the first thing you do in any experiment is to verify the quality of the reagent you use and this has to be done in biochemical and physiological experiments just the same as you would in an analytical laboratory.

DR. IMAI: The rest of my presentation will be on the pulmonary arterial changes induced by the concentrate of impurities and two of the oxidation products of cholesterol; namely, 25 hydroxycholesterol and cholestane triol. The reason for further pursuing these pulmonary arterial changes is that I think it is about time for someone to study what happens on the cellular level in this complex situation of cholesterol atherogenesis. In 1913 Anitschkow presented his data on the induction of atherosclerotic lesions in the rabbit by very artificial means and 10 years ealier than that the instability of cholesterol samples had been pointed out. This is a portion of the lung from a rabbit that was given 5 mg/kg of cholesterol purified by dibromination, suspended in saline, daily for three days and killed on the fourth day. The pulmonary artery near the hilum is unchanged. So are the bronchus and pulmonary vein. Thus such a picture is similar to that of the saline-injected or untreated controls.

Changes due to intravenous injected angiotoxic agents are qualitatively similar, that is among these three agents. The difference is in the caliber of the affected artery and the degree and extent and timing of changes. The vein and bronchus are again unchanged. The pulmonary artery becomes swollen, necrotic, and inflamed. And the lumen remains narrow despite pressure perfusion fixation. In three days some segments of the pulmonary artery become not only inflamed but also thickened. This thickening is by an increase in the fibromuscular tissue as shown here and also at the site of branching. The media is edematous. A slight degree of inflammatory change and edema are seen in the adventitia. In this segment, again from the 3-day experiment, the adventitia is almost normal except for slight collections of inflammatory cells, but the media and the intima are both thickened by the fibromuscular tissue. There is a slight degree of hypercellularity in the lumenal side, suggesting some inflammatory change. As a partial explanation for this increase in thickness of the media and the intima, there are mitotic figures as shown here in random sections of the pulmonary arteries.

This electron micrograph and the following ones are from the rabbits given 5 mg/kg of 25-hydroxycholesterol daily for three days and killed on the fourth. The ultrastructural changes due to oxidation products of cholesterol are qualitatively similar and the differences are in the degree and timing. The endothelial cells here are changed but not absent. Many of the medial smooth muscle cells are vacuolated and rarefied. Inflammatory cells in the wall are identified by these specific granules as true eosinophiles. Some examples of cell death by pyknosis with condensed cytoplasm except for the distended vesicles. This smooth muscle cell appears less changed but has vesiculation of organelles. This vesiculation of the organelles is in part due to enlargement of the sarcoplasmic reticulum and, in part, due to swelling of the mitochondria. The endothelial cells are continuous. This is not an endothelial cell, but this cell is underlying the endothelial cell. If you note that this layer here is the endothelial cell which is about normal except for slight vesiculation of the cytoplasm. This cell adjacent to the endothelial cell has no characteristic features of differentiation and thus belongs to the category of poorly differentiated or so-called mesenchymal, primitive, or Langhans' cells. The cells underneath can be identified as smooth muscle cells by the basement membrane, fusiform densities, and streaky cytoplasm suggesting myofilaments. Again a portion of fibromuscular thickening of the intima, the internal elastica being far out of the field. There are some cytoplasmic

and nuclear fragments. This cell has no differentiated features and belongs to the category of poorly differentiated cells. Now I would like to finish this slide show with just two illustrating the 24-hour effect of intravenous-injected impurities concentrated from cholesterol of U.S.P. grade. This particular epoxy section was taken from one of the segments of the pulmonary artery. These segments were selected on the basis of thickening that was descernible under a dissecting microscope at 6 to 10 times magnification. This area will be shown in the next and last slide. These wavy lines are the duplicated internal elastic membrane. There is this much of an englargement of the intima that probably occurred in 24 hours after the intravenous injection of a single 5 mg/kg of the concentrate of impurities of U.S.P. cholesterol. Some of the intimal cells have light stained cytoplasm suggesting that some of these are the primitive or poorly differentiated cells. The underlying media has an abnormal arrangement of smooth muscle cells. The exact nature of such intimal collections of cells is yet to be determined. Possibilities include mitosis of either smooth muscle cells or poorly differentiated cells. And migration of the medial smooth muscle cells into the intima must also be considered.

To summarize, we have presented some data to support the notion that extensive medial and intimal necrosis or statistically significant enhancement of arterial smooth muscle cell death beyond a certain degree is followed by healings by repair which takes the form of a basic type of arteriosclerosis or fibromuscular thickening. In the case of the pulmonary artery, such injury and repair can occur in three days, after three daily intravenous injections of 5 mg/kg of individual, combined, or naturally occurring mixture of oxidation products of cholesterol (or MP4). The same naturally occurring mixture after total doses of 30 to 120 mg/kg in 3 days induced transmural necrosis in Japanese White-Nipponese Institute of Biological Sciences, or JW-NIBS, in addition to the pulmonary arterial changes. When thus treated rabbits were left alone on the stock diet, fibromuscular thickening of the aorta occurred in 10 weeks. In New Zealand White rabbits, enhancement of arterial smooth muscle cell death was demonstrable by electron microscopy cell counts after 10 to 250 mg/kg of MP4, 1 g/kg of cholesterol of U.S.P. grade, or 100 grams of 5% cholesterol diet in 24 hours. In long-term experiments aortic fibromuscular thickening occurred in 7 weeks after a total dose of 1 g/kg of MP4. Cholesterol purified by dibromination given by the gastric tubing of the gelatin suspension at the same small total dose of 1 g/kg extended over 7 weeks did not induce demonstrable lesions. We have bioassayed these oxidation products and two others as listed here for pulmonary arterial changes after intravenous injections of saline suspensions. That is with the exception of squalene bis epoxide which was dissolved in olive oil. These agents are angiotoxic in this bioassay system, individual agent's potency being in the listed order. Combinations of these agents, each at less than the minimum effective dose, which is 2 mg/kg, were effectively angiotoxic, suggesting potentiating effects. When 1 mg/kg of 25-hydroxycholesterol and .5 mg each of the cholestane-triol, cholesterol 5,6-epoxide, and lanosterol epoxide, and 7-ketocholesterol, necrotizing angitis and fibromuscular thickening occurred in the renal artery in three days after three daily intravenous injections. This renal arterial response after intravenous injections seems to be triggered by cholestane-3β,5α,6β-triol because 2 mg/kg each of cholestane-triol and 25-hydroxycholesterol or 2 mg/kg of cholestane-triol alone induced similar changes of a lesser degree. None of the other agents induced renal arterial changes when used individually except that cholesterol 5,6-epoxide after a total dose of 20 to 30 mg/kg induced acute inflammation in the intimal layer in the renal artery. Thank you.

DR. E. SMITH: This picture you showed of the reduplication of the elastic lamina and the intimal thickening after 24 hours, what sort of extent of intimal thickening did you find? I mean, what area of the vessel wall was involved?

DR. IMAI: That I cannot really give you in quantitative terms because this was done with two or three rabbits, and these thickened segments were selected using the dissecting microscope. So to begin with there is a selection at the microscopic level. Random blocks of these selected areas were then made and these thickened areas were found.

DR. E. SMITH: You don't find thickened areas in an untreated rabbit?

DR. IMAI: No. These are young rabbits, no more than 2 kg. And in these untreated control rabbits, there is no change in the pulmonary artery or in the thoracic aorta.

DR. TAYLOR: It's my experience that an artery first begins to repair itself about two weeks after you've killed the whole artery by freezing and you actually don't get a respectable intimal proliferate for three weeks.

DR. IMAI: And that is in the aorta. The ones I showed you here are from the pulmonary artery. The pulmonary arterial response is quite a bit different from the aortic response. But I agree with you in that the aortic response takes at least 7 weeks--maybe 10 weeks--but the pulmonary artery response is quite rapid.

DR. LISANTI: What do these doses of the oxidized materials represent in terms of a percentage or a qualitative relationship to the contamination in whatever we want to call that U.S.P. cholesterol? In other words, how many times the expected contaminant do these doses represent in the experimental procedure?

DR. IMAI: To that I refer to Dr. Merritt.

DR. MERRITT: What's the question please?

DR. LISANTI: The question is since these oxides or compounds that he's been using in the experimental procedure are considered to be contaminants of cholesterol, what do the doses that he used represent in terms of the contamination of the original compound--the original mixture?

DR. TAYLOR: Dr. Peng in his tissue culture study has got some percentages of how much of the various oxidation compounds there are.

DR. MERRITT: I'll try to answer the question--actually by not answering it. The only thing I can say is that we haven't made the calculations and so at this time I can't answer the question, but I think the data is available by which we could make this comparison and perhaps we'll try to work that out.

DR. PENG: Dr. Taylor just mentioned that percentage is a percentage of a concentrate so that Dr. Werthessen mentioned that 5 kg--how many mg come out of 5 kg of old cholesterol, so that will be some kind of a guess of one percent or two percent.

DR. WERTHESSEN: Walter and I did do a rough comparison, particularly in those analyses of what I would call the third mother liquor, and it looks as though, at best, all of the oxidation products would add up to about a half a percent. Now obviously when he gives 5 mg/kg, he's giving an extraordinarily high concentration of cholesterol equivalent. But what you must recognize in this comparison is that in order to get the effects which have heretofore been ascribed to cholesterol in dietary

stress experiments, it takes a minimum, if I'm right, Dr. Lee, of about six weeks of feeding according to Dave Kritschevsky, one gram of cholesterol U.S.P. per day. Now that gets hellishly confused because you don't know what has happened to the cholesterol that's in the diet. And there are some data that I gathered on Lee's experiments where inside of a week two-thirds of the cholesterol was gone. So we are really up a tea-total creek in trying to make the comparison you've asked for. There's one man--Hugh Lofland, with whom I've discussed this problem. He was very, very careful. He ran a gas chromatogram of the cholesterol that he bought and was happy to obtain just one peak, but he put the cholesterol alone on the column and in the comparisons made in Dr. Merritt's lab, in order to see any contaminant, the column had to be woefully overloaded. And, furthermore, the retention time of the oxidation products on a chromatograph is such that, unless Lofland had run it for half an hour afterwards, he wouldn't have seen anything. So fundamentally, pragmatically your question is totally unanswerable, because it depends on how the man handles his cholesterol, what kind he bought, how he mixed it in, etc.

DR. L. SMITH: I will add to that in agreement that U.S.P. cholesterol may have a variable amount of other materials present, depending upon the immediate past history of the sample. If U.S.P. cholesterol sits in its bottle on the shelf for very long, an increase in autoxidation products surely results. Specific samples analyzed by adequate chromatographic methods at different times depending on conditions are necessary to proper control of the purity of cholesterol to be used.

DR. WATSON: Dr. Imai, I'd like to follow up on Dr. E. Smith's question. You did selection of the thickened portion of the pulmonary artery--your slices. I'd like to know, although you didn't see at the light microscope level, thickening in the control, were sections made of the control to go beyond your eyes?

DR. IMAI: I have thousands of sections of the lungs and I have not seen any thickening of this kind in controls.

DR. WERTHESSEN: John, you also have to recognize what's extremely difficult in the atherosclerosis problem and that is that these lesions are not all over the place. They're discrete.

DR. WATSON: Yes, I just think of the classical problem that you always have to remind a student that his eyes are not a spectrophotometer and eyes aren't electron-microscopes.

DR. TAYLOR: Dr. Lisanti asked about Dr. Imai's studies on the coronaries in the rabbits, and since I am his co-worker, I happened to have a slide in my box upstairs. This particular rabbit had only 700 mg of cholesterol over a period of 36 days, and he has a respectable amount of coronary arteriosclerosis. This is what you get when you kill the media which is followed by intimal proliferation. Three areas of intimal damage with overlaying intimal scars are present in this section. So you do get coronary disease. I might point out that just intrinsic coronary disease of rabbits get arteriosclerosis. Their epicardial coronary arteries don't develop dietary-induced atherosclerosis. They get all their disease in the arteries within the myocardium.

DR. LISANTI: Did you say 100 milligrams total?

DR. TAYLOR: A total of 700 mg, over a period of 36 days, and that's not very much

cholesterol. I had the good fortune to spend a couple of months in Africa and study the Masai and I think we might take some lessons from their dietary habits. They don't have any refrigeration, so their food preservation consists of a herd of cattle which they move about with them, and milk them. They keep the milk for up to 24 hours and they store it in a gourd. It ferments and probably undergoes very little oxidation. The ladies have the short haircuts and the men have the long haircuts. This is one of their homes. They make their huts of manure and tree branches. At night they keep their cattle in this central area surrounded by their huts. The manure gets several feet deep in this central area. All the kids have tetanus. They all have their lower two teeth knocked out because tetanus is so common. They have to feed them milk with their jaws locked tight. They milk their cows directly into this large gourd which they dig out with a knife and then burn it; they keep a tight cork on it and they milk the cows twice a day. They rinse their gourds out with cow's urine--water's scarce over there. They have to save it all for their cows to drink. As I mentioned their food doesn't sit around very long. During the dry season, they have African borsch, which is half milk. They half fill their gourds then tie up a cow and stick her jugular vein and mix the milk with blood, then drink it. Now all this food contains fresh, endogenously synthesized cholesterol. According to Leland Smith (Smith et al. 1967) if it's all made inside the body of a mammal, it's protected by anti-oxidants. This is the type of serum cholesterol level they maintain (Fig. 14). It runs around 135. At 65 years it is still only 135. We call ourselves a developed country--I'm not so sure but what we're undeveloped when it comes to improper nutrition. This is an aorta from a 67-year old male (Fig. 13) Masai. This is better looking than that of a 3- or 4-year old aorta in this country. By contrast, this is an aorta from a 42-year old north suburban Chicago person (Fig. 15). There's a little bit of normal area here--and a little bit here. All the rest of it is an atheroma. So there's a big difference. We had the privilege of autopsying 10 Masai. Their aortic disease up to 70 is less than a 5- or 6-year old U.S. child. They also have excellent lipoproteins. They have almost no low density lipoproteins. They carry all their fats around as high density lipoproteins. This is a cross-section of an aorta from a 67-year old male Masai (Fig. 16). They do have some fibrous intimal thickening. This is a fat stain. There's very little fat in there. If you do a polarized light study, they have a few cholesterol crystals; they have a little disease, but it sure is minimal compared to what we have. This is a coronary artery from a 67-year old man (Fig. 17). They do repair their arteries by putting new elastic and muscular tissue in a thickening intima, but there is no fat in this thickened intima. So they do a little repair of their arteries with new intimal tissue, but they don't have a whole bunch of lipids plugging up their arteries like we do. Right now the eggs you buy at the chain grocery stores are probably six months old and they call them fresh eggs; they probably have significant amounts of auto-oxidation products of cholesterol. Arterial disease is definitely on the increase. I think our food preservation, shelf-life, transportation, etc. are playing a big role in our high incidence of atherosclerosis, which is 54%.

DR. LEQUESNE: Could I ask you what the current view of the epidemiological aspects of this is? Do you think the Masai owe their freedom from arteriosclerosis to genetic causes? The Finns, for example, have the highest coronary disease rate in the world, don't they?

DR. TAYLOR: I have a reference to that (Ho et al. 1971). I suggest you look at this paper. We felt that the Masai tribe at the time we did this study probably represented genetic phenomena. They have twice as strong a negative feedback of cholesterol synthesis as a person in this country. We challenged them with dietary choles-

Dr. Taylor's Figure 13

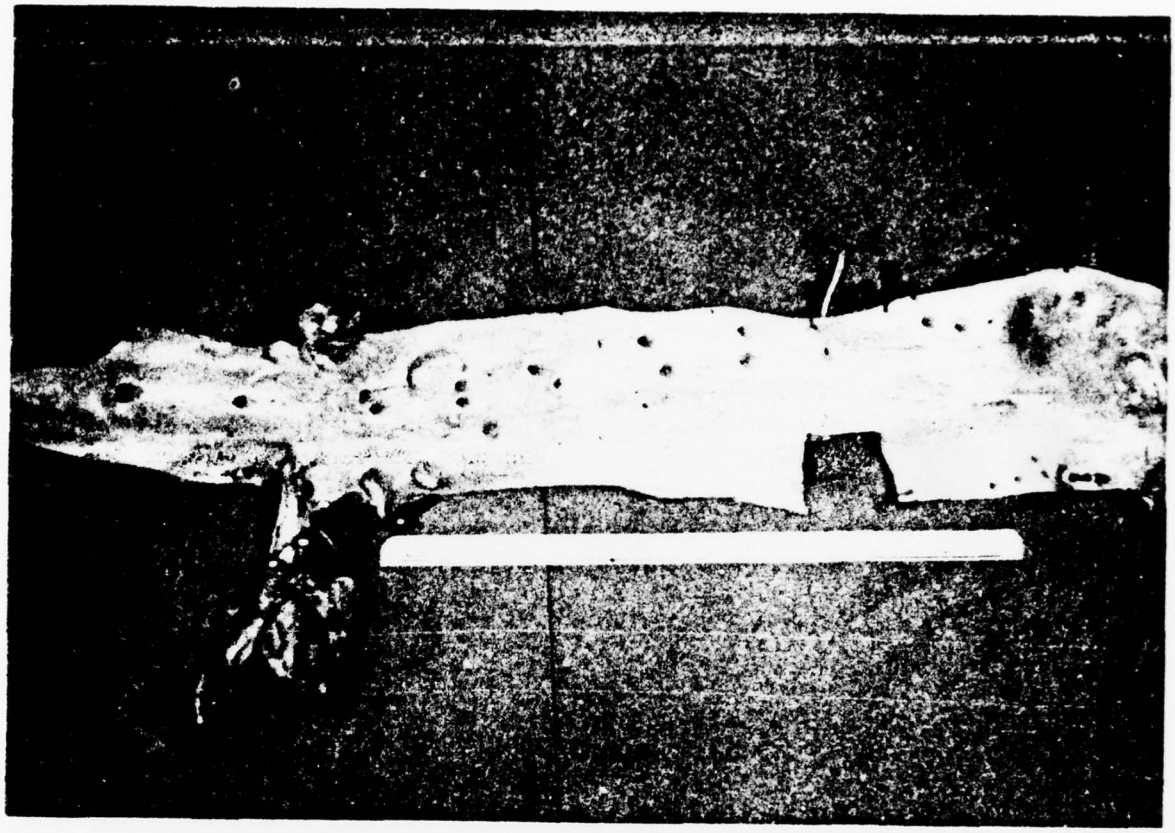

Figure 13

Gross photograph of aorta from a 67-year old East African Masai male. This aorta has less atherosclerosis than one generally sees in five- to 6-year old children in this country. (Ho et al. - 1971.)

D. Taylor's Figure 14

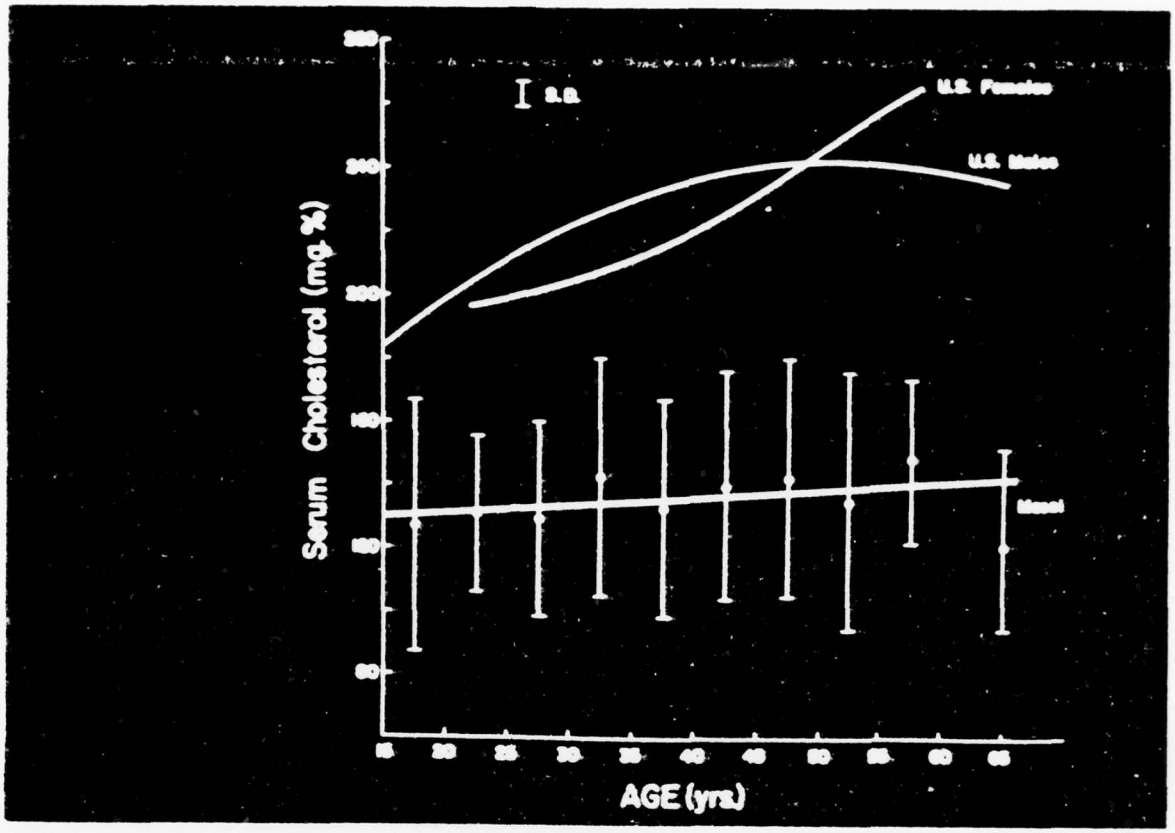

Figure 14

A plot of serum cholesterol levels of 254 Masai ranging from 15 to 65 years of age. An unusually low average value of 135.4 ± 33.5 mg/100 ml (mean ± standard deviation) was observed (Ho et al. - 1971). Average serum levels of U.S. males and females--based on a large sampling of U.S. subjects of various ages (Lewis et al. - 1957)--is given in the two upper curves.

D. Taylor's Figure 15

Figure 15

Gross photograph of a severely atherosclerotic aorta from a 42-year old U.S. Caucasian male. Disease as severe as this is not infrequent in U.S. males dying during the fifth decade.

Dr. Bruce Taylor's Figure 16

Figure 16

Fat stain of abdominal aorta from a 67-year old male Masai. Photograph taken under partial polarized light; a modest number of birefringent cholesterol crystals can be seen. The darker staining patches at the left of the illustration represent cholesterol esters and neutral fat. Although the Masai had minimal atherosclerosis, their arteries (coronaries and aorta) did reveal mild atherosclerosis (Ho et al. - 1971).

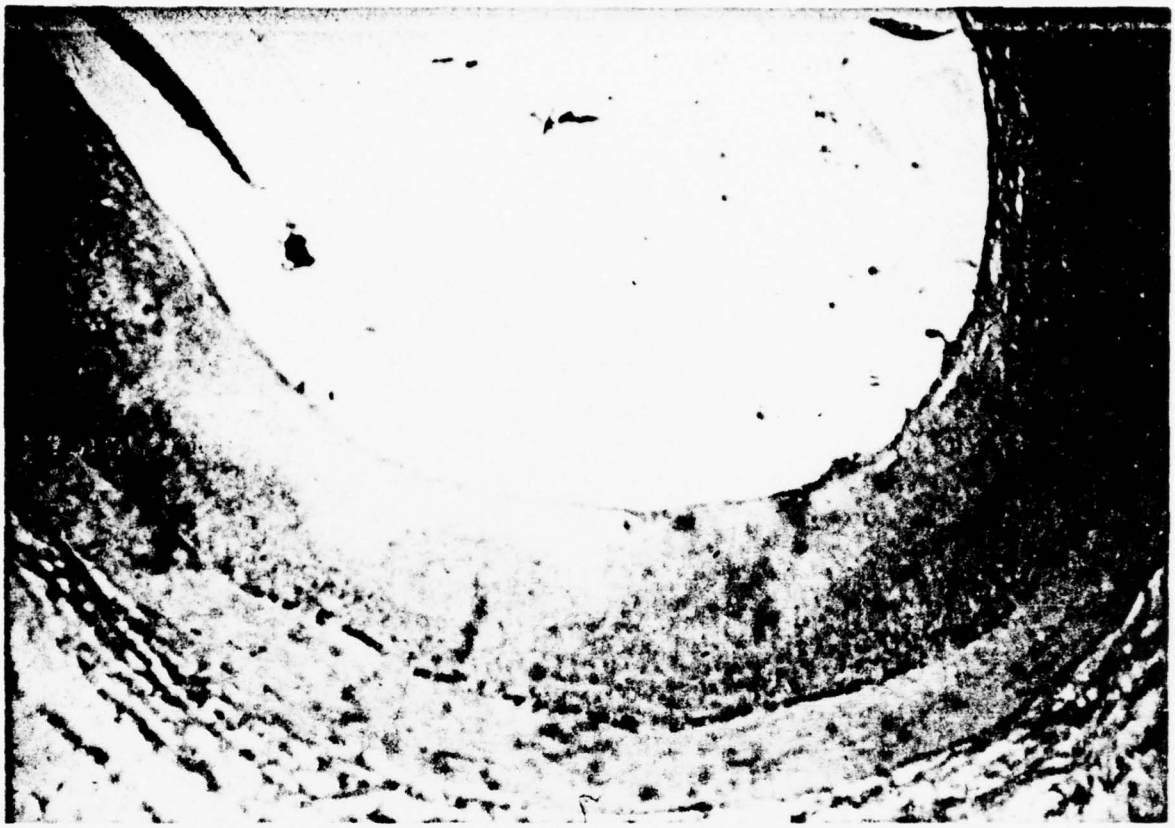

Figure 17

Section of coronary artery of a 67-year old male Masai. At this age there is a thick fibrous intimal scar which is somewhat thicker than the original media. There is, however, essentially no atherosclerosis. Note that the internal elastic membrane is markedly disrupted in this 67-year old coronary artery. (Ho et al. - 1971.)

terol, and they could reduce their cholesterol synthesis by 600 mgs per 24 hours, whereas the average person in this country can only reduce his cholesterol synthesis by about 300 mgs per 24 hours. Food preservation is a new phenomenon and these people eat their food just about as soon as it comes out of the cow's vein or the cow's teat, so it's very fresh food. I think this may be another very important factor.

DR. WATSON: Do you have any comments on the suggestion that the curdled milk has in it a magic factor like HMG?

DR. TAYLOR: I suspect that, since the Masai usually consume their milk and blood within 12 hours, there is not nearly enough time for toxic oxidation products of cholesterol to form; so they probably ingest quite pure unoxidized cholesterol.

DR. WERTHESSEN: There's one queston that I'd like to ask, Bruce. Did you do any postmortems on women?

DR. TAYLOR: Yes, one person was a 47-year old female. Her disease was also extremely minimal.

DR. WERTHESSEN: Did they have lesions?

DR. TAYLOR: Yes, a minimal of early disease. One of the more striking cases was that of a 27-year old male who died after being hit by a truck. His disease was principally the juvenile fatty streak lesions involving the thoracic aorta.

DR. WERTHESSEN: You mean the same as Rusty found in New Orleans?

DR. TAYLOR: Yes. And he had none in his abdominal aorta. The older the Masai got, the less atherosclerosis they had. The best arteries we had were in the seven people who were over 60.

DR. WERTHESSEN: The reason I raise this point is that I know a little bit about the way the Masai live. It's a real male chauvinist world. The men do nothing but stand around all day. The women do all the work and Stewart Wolf, who isn't here, raised the question about the hypertension in the next to youngest wife. I think if you'll think about it for a moment, you'll understand the point. They do all the work and that's why I raised the question. I have a hunch they don't live as long as the men do.

DR. WATSON: But don't the men in herding the cattle walk an average of about 12 miles a day?

DR. TAYLOR: No. The children do all the work over there--the children and the women. The men just lean on their spear and think great thoughts and take care of their six wives. I don't know how hard that is, but they don't do any work at all during the day. The cattle herding is all done by the children.

DR. WERTHESSEN: You can spot a Masai five miles off in the distance because what you see is a thing standing absolutely motionless. As far as I can make out the one traumatic period in their lives is when they are forced to prove their manhood. Maybe this is why you saw the lesions in the young one. You know the technique, Bruce. The warriers find a pride of lions. The boy who is to prove his manhood is placed

in a particular position. The other men prod the group of lions into action and the big male lion is supposed to jump at this youngster. His job is to stand there and hold the spear and kill the lion as he jumps at him. If he fails, he's usually not inducted into manhood--even if he survives.

DR. TAYLOR: We had one female autopsy in our group and this person had very minimal atherosclerosis.

DR. WERTHESSEN: How old was she?

DR. TAYLOR: Forty-seven.

DR. WERTHESSEN: But she had a worse looking aorta than your old man of 60.

DR. TAYLOR: No, she didn't. Yes, there's one 60-year old male that had the same atherosclerotic index as we found in her aorta; there was another one of 67 who had a little more disease; there was another of 63 who had less. But they all had minimal disease.

DR. WERTHESSEN: So now, Dr. Peng, do you want to be quantitative?

DR. PENG: My presentation today is an "in vitro" study of the cytotoxicity of the barrel of old U.S.P. cholesterol we have stored for five years in which Dr. Taylor and Dr. Imai felt strongly that there was a substance (or substances) in there that was probably angiotoxic or atherogenic or both. My opinion is that a simple and better way to screen that very complicated mixture of compounds is to use cell culture and test them individually in the cell culture. In this case, we used the aortic smooth muscle cells. The reason is simple. Since we deal with atherogenesis, the aortic smooth muscle cell is most suitable for testing these compounds. In addition, Drs. Taylor and Imai have shown that the most important component in the arterial wall is the aortic smooth muscle cell and also that the most important initial manifestation of atherogenesis is the smooth muscle cell death. Therefore, a compound (or compounds) which has the capability of inducing smooth muscle cell death was considered to be potentially atherogenic.

Let's go back to that barrel of cholesterol which we had stored for five years. Figure 1 shows the thin-layer chromatogram of this old cholesterol, irrigated with the solvent system containing ethyl acetate:heptane, one to one by volume. Then we sprayed it with 50% sulphuric acid. This old cholesterol was no longer pure and contained lots of other compounds which Dr. Werthessen had analyzed 40% or more as auto-oxidized cholesterol. Now I have to correct my statement in this morning's session which was misunderstood: new U.S.P. cholesterol is about one or two percent auto-oxidized. But this is not so in the barrel of old cholesterol.

There are two ways to approach the problem to find out which one is cytotoxic in our mixture of those compounds. One way to do it is simply to test them individually in the cell culture; the other way is to test pure compounds comercially available and prove to get the comparable result. We have done it both ways. Before presenting the result, I would like to summarize the literature regarding the pathway of auto-oxidation.

I have a couple of schemes for it (Figs. 2 and 3). The most active site for cholesterol auto-oxidation is in the A-B ring you can see, either forming hydroperoxide as an intermediate or forming the 5,6-epoxide, and then further oxidation from hydroperoxide forms the hydroxy compound or the keto compound or deyhdrated at the third position to form another keto compound and hydration of 5,6-epoxide forms triol on the other hand.

DR. L. SMITH: Your compound No. 1 is not an autoxidation product of cholesterol but a product of the attack of electronically-excited signlet molecular oxygen on cholesterol. The isomerization of this 5α-hydroperoxide (compound No. 1) to cholesterol 7α-hydroperoxide does occur as indicated.

DR. PENG: Thank you for the comment, but we didn't have a chance to test this one. This is a compound that is difficult to get a sample.

DR. LEQUESNE: I think the point that Dr. Smith is making is that that compound No. 1 in the top of the slide has a different mode of origin from all the other oxidation products that you see there. It's a different mechanistic pathway to get to that and so, while it may be present there in the mixture, it should be considered slightly apart from the other compounds in view of its origin. That's a different reaction pathway.

DR. L. SMITH: It is very unlikely that one would encounter the 5α-hydroperoxide in autoxidized cholesterol preparations.

DR. LEQUESNE: It could occur in nature, but it just has a different origin.

DR. WERTHESSEN: Unfortunately he wasn't able to secure the compounds, so we won't know about it

DR. PENG: Of course there's a further oxidation that occurs after this, and as in 6 position triol can further oxidize to form ketone compounds, which I consider the minors. We are in progress to test those minor compounds, but my presentation now is limited in the major compounds we found which are 7α and β-hydroxycholesterol and 7-ketocholesterol and then triol and the epoxides and so forth.

DR. L. SMITH: It must be pointed out that the 5α,6α-epoxide of cholesterol is the minor cholesterol autoxidation product and that the isomeric cholesterol 5β,6β-epoxide is present in several-fold excess. The 5β,6β-epoxide has been generally disregarded by investigators, but this should not be the case.

DR. PENG: Yes, I really feel that way that the beta is a major one, but always together in there, I have to check it both. In the TLC it always comes so close, so I feel strongly that the beta is a major component in the mixture.

Fieser had suggested that the A-B ring auto-oxidation mostly occurs in the dispersed state which is randomly attacked by the oxygen but in the case of crystalline cholesterol like ours, stored for five years, in which molecules arrange as bilayer with the hydroxy group in the juxtaposition, making it difficult for oxygen to attack on the A-B ring, in turn, they attack the side chain like the tertial position over 25 or over 20 more easily. Therefore 25-hydroxy or 20α-hydroxycholesterol appears to be more abundant in the crystalline state. We also test these two compounds. We

had separated that barrel of five-year old U.S.P. grade cholesterol with thin layer chromatography and divided it into six different fractions. Hopefully each fraction will contain very few major components. Table 1 shows the fractions we got, and we checked them with references. Here is the fraction one containing triol and other compounds. Fraction two consists mainly of 7α and β-hydroxycholesterol. This olive color is 25-hydroxy we got in quite a large amount in fraction three. Then this is fraction four, which is another blue band over here. I assume this is the hydroperoxide because the peroxidase stain positive which we used N,N-Dimethyl-D-phenylene-diamine-dihydrochloride and starch:KI. In the band three, you notice that 7-keto has no color by spraying with the 50% sulphuric acid, but did have a fluorescence show up when we treated with 2'7-dichlorofluorescene or Rhodamine B, so I think that in addition to 25-hydroxy in the band three, we do have a 7-keto right behind it, although you cannot see the color right in there. In fraction five we have pure cholesterol, and in fraction six we have some minor compounds like cholestadiene etc.

I like to go back to the aortic smooth muscle cell culture preparation. We used New Zealand White rabbits, about 2-3 kg in weight, and we sacrificed them aseptically and removed a segment of aorta and cut it into about one-millimeter pieces. They grew in the tissue culture medium containing 10% of fetal calf's serum and antibiotics. Up to three weeks the cell had grown to confluence and then they were trypsinized subsequently. The cells were transferred into new Falcon flasks and a confluent monolayer had developed again in another two weeks or so.

Figure 4 shows the monolayer which we fixed with glutaraldehyde, and we also studied this with the electron microscope. The electron microscopic finding (Fig. 5) confirmed that the cultured cell is indeed a smooth muscle cell which is characterized by the presence of the myofilament with fusiform density and pinocytotic vesicles. The majority of them show various stages of maturation, but some of them are rather immature and have abundant mitochondria and E.R.

Next we treated cell monolayers with all different kinds of sterols, for which we used ethanol to dissolve those sterols, including the purified cholesterol. The ethanol in the tissue culture medium is less than 0.8% by volume. We shook them for about an hour in the incubator before we replaced the culture media we had for the monolayer. The control containing vehicle of 0.8% ethanol only showed no toxicity and no visible change. Cultures to which test sterols were added to culture medium showed varying degress of cytotoxicity which was measured as percentage of dying and dead cells.

My criteria are something like this: the cells which have shrunken cytoplasm but still maintain spindle-shape or polyhedral shape are classified as degenerating cells. The dead cells were small, round, and stained darkly with no internal cellular details nor nuclear configuration as shown in Fig. 6. Ultrastructurally they have lost the continuity of the membranous structure which we call "myelin" figure materials, and aggregate of granularity represent a dispersion of chromatin material. However, we still had a chance to demonstrate some myofilaments on the periphery and pinocytotic vesicles which confirmed that these cells originally were smooth muscle cells. We first tried, of course, the old barrel of the cholesterol in the mixture and.....

DR. WATSON: On that dead cell, do you interpret those results to suggest that the cell started eating itself?

DR. PENG: Eating itself?

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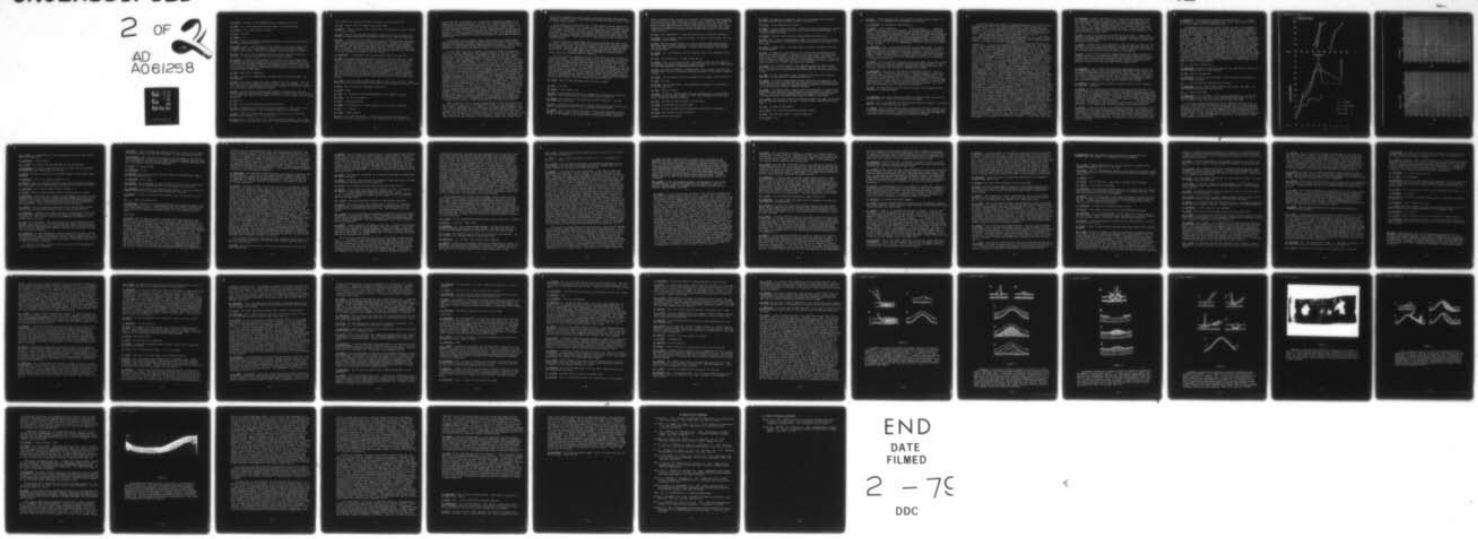

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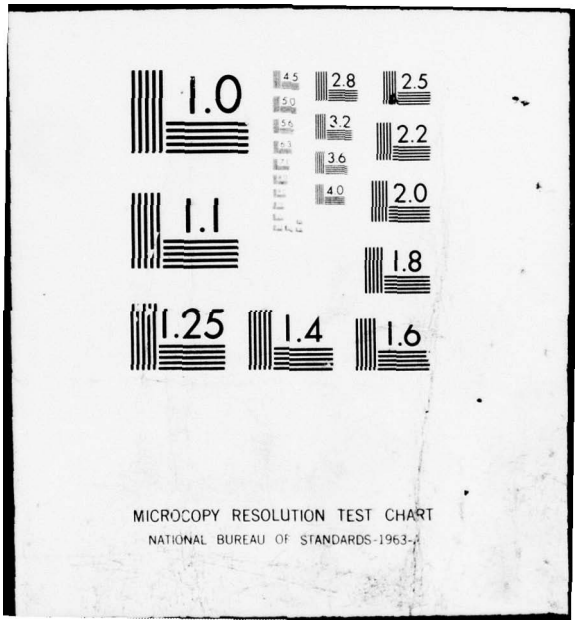
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DR. WATSON: Yes, with all the membranes inside a lysosomal-type vesicle?

DR. PENG: No. I don't know what the exact content is inside the vesicle.

DR. WATSON: No, no, where you see the whirls.

DR. PENG: This one?

DR. WATSON: Yes.

DR. PENG: Those are degenerating membranous materials--so called myelin figure materials under electron microscopy.

DR. WATSON: Right. I got the impression that that forms in digestive vacuoles like lysosomes, and to form, it would suggest to me that the cell is eating itself in response to these toxins. Why would it accumulate there rather than just disrupt?

DR. PENG: Well, that's up to speculation how that--either autolysis or eating up themselves, or just disintegrating there and then sticking together.

DR. SCALLEN: You can see similar pictures in a variety of cells from animals that are treated with inhibitors of cholesterol synthesis and these cause a proliferation of lysosomes from the smooth endoplasmic reticulum and you see structures that either look like that or are even more organized and membrane limited, so it is possible that these particular compounds may indeed cause a proliferation of lysosomes in an attempt to sequester them in some manner.

DR. PENG: Thank you for commenting.

DR. IMAI: Am I right to understand that you think there is extensive damage to the cell membranes?

DR. PENG: Yes, there's a defect in the cell membrane, so-called "leaking." But some of them are totally disrupted and you don't see them. They just disappear in the medium. The cells you still have here are rather well preserved. Those held up together for a while before becoming completely disrupted.

DR. IMAI: You also said that there are many pinocytolic vesicles which seem to be rather incongruous with the extensive membrane damage. The other thing I am interested in: did you do any studies on the remains of the nuclei, such as Feulgen did on the same preparation?

DR. PENG: No.

DR. IMAI: You see, these dark particles may be the nuclei...

DR. PENG: The purpose of this study is the relative potency. I don't want to speculate on the mechanism behind it, but the relative potency will be presented.

DR. IMAI: What is at stake is that if the cell is dead or still alive and doing something--maybe abnormal, but it may not be dead.

DR. PENG: Well, I would say here I don't think those cells are normal. This is only an individual cell, one particular cell which is studied under the electron micrograph.

Now in general you can see the severity of toxicity in this monolayer cell.

DR. IMAI: Agreed. They are changed, but are they dead?

DR. PENG: I think so--some of them, not every one--you can see here, but some of them I would say are dead.

DR. IMAI: Have you done the phase microscopy on these to see if you can see, for instance, the Brownian movement of the particles within the cell that you assume dead?

DR. PENG: I took a look at every flask every two to four hours under the phase microscope, and the changing is just continuously progressive. I would like to remind you that the degree of the progression of cell toxicity can be different and depends on the compound. Some were quicker than others, but they progressed slower afterwards. What I am saying is that this is a generalized finding. I don't speculate what mechanism is behind it, but you obviously can see some compounds are more toxic than others if you have comparison of response in the cell culture.

DR. WERTHESSEN: I would suggest that we go on because I know you've got some....

DR. PENG: We counted dying and dead cells that way as I have just mentioned. Adding 1 mg of the concentrate of that old barrel of cholesterol in the 5 ml of the tissue culture medium means 200 micrograms per ml concentration in which we had 35% dying cells and 38% dead cells. Now we had tested all the compounds of auto-oxidation derivatives of cholesterol which are commercially available. The result was shown in Table 2. Cholestan-3β,5α,6β-triol is most toxic and 25-hydroxycholesterol is the next; 20α-hydroxycholesterol follows; 7-keto and 5,6-epoxide and 7β- and 7α-hydroxycholesterol are relatively untoxic up to 100 micrograms per ml. Purified cholesterol shows no cytotoxicity and the monolayer remains the same as the control except that there are some small particles which are not completely resolved in the higher concentration.

DR. WATSON: It doesn't seem as though you're going to get very far, Dr. Peng. Where here did you begin to see floculation of the sterol--at what concentration in the cultures?

DR. PENG: Most of this had no floculation except that....

DR. WATSON: None?

DR. PENG: No. The majority of oxidation products are very soluble in ethanol.

DR. WATSON: Yes but in the medium.....

DR. PENG: In the medium we put....

DR. WATSON: My experience with 7α and 7β usually at 5 or 6 mg/ml I could tell...

DR. PENG: Is it turbid?

DR. WATSON: Slightly turbid.

DR. PENG: Well, maybe very minimal. I did look under the phase microscope and I didn't see any good-size particles at all. But cholesterol, yes, up to about 100 mg

or more than that; it was quite a bit. Another way to approach the problem is to separate and test them individually. Now we go back to check each fraction of old cholesterol for response of the monolayer cell. The result is shown in Table 3. In the relatively low concentrations, only fraction three showed striking changes. At a higher concentration fraction three as well as fraction one start to show some toxicity and, of course, at very high concentrations, fraction four is showing similar changes too. But my explanation of fraction four may be due to the contamination of fractions three and fraction five which is pure cholesterol, which shows no toxicity at any level.

In conclusion only two compounds, mainly cholestan-triol and 25-hydroxycholesterol have the most potent biological activity which is consistent with the result obtained from bioassay of the pure, commercially-available compound. Because hydroxycholesterol is more abundant than the cholestan-triol in the mixture, that may be the reason for 25 hydroxycholesterol showing an effect at the lower concentration.

The next study I would like to present is the activity of HMG CoA reductase activity in the aortic smooth muscle cell in vitro stimulated by the literature in recent years. But they're working on a different cell line than ours and I wanted to see the effect of cholesterol biosynthesis by these sterols in the aortic smooth muscle cells. Now the preparation we have started with is the same material which is a monolayer of aortic smooth muscle cell. It contains about 9.3×10^5 cells per flask. We scrubbed it with rubber policeman. The reason we use the cell homogenate is because we fractionate it into microsomal and mitochondrial fractions; then all of those show only one-quarter portion of the total activity of HMG CoA reductase. Since the smooth muscle cells contain less amounts of HMG CoA reductase, we like to have as high as possible activity. Therefore we go by the cell homogenate instead of the microsomal fraction. The cells were washed twice by centrifugation at 600 x g for four minutes with a buffer at pH 7.4. The cell pellet was then swollen in the hypotonic solution and disrupted by passing 25 times in the homogenizer. The homogenate was diluted 1.5 times and assayed immediately for HMG CoA reductase activity. An aliquot of homogenate which contained about 22-100 µg of protein was incubated for two hours at 37°C with constant shaking in a final volume of 0.2 ml which contained a phosphate buffer, 2 mM NADP, 20 mM G-6-P, 20 mM β-mercaptoethanol, 1.75 e.u. of G-6-P dehydrogenase. The reaction was started by addition of 3.4×10^{-5}M of DL-(^{14}C)-HMG CoA and was terminated by the addition of 50 µl of 5N HCl. DL-(^{3}H)-mevalonate was added as an internal standard to correct for incomplete recovery of mevalonic acid. The radioactive mevalonolactone was extracted from the reaction mixture with diethylether and separated by TLC and then counted in a scintillation counter. We used various concentrations of 25-hydroxycholesterol from .5 to 1.0, 2.0, and 3.0 micrograms in the tissue culture medium. The percentage of inhibition of HMG CoA reductase activity appeared to be proportional to the concentration. Up to 3 micrograms it is almost more than 90% of inhibition. To compare relative potency, we used 3 micrograms per milliliter of each sterol.

Table 4 shows the result in which 25-hydroxycholesterol shows 93% inhibition and 20α, 92%; then 7α, 88%; 7β, 77%; 5,6-epoxide, 57%; 7-keto, 41%; and cholesterol, very little effect. Cholestan-triol is a stimulant in one occasion and an inhibitor in another. Other investigators used the liver slices and showed stimulating effect. When I tested it for the cytotoxicity study, I took a look at it every two to four hours under the phase microscope. With cholestan-triol, the effect started to show up earlier. There was 10 to 20% cell death in the first few hours. Then up to a certain time it almost remained the same percentage. But 25-hydroxycholesterol took a longer time to

kill the cell and appeared to have no effect in the first few hours--then gradually increased. So there is quite a different pattern between the cholestan-triol toxicity and the 25-hydroxy toxicity.

Let me go back to this cholestan-triol. Another peculiar finding showed the inconsistency of cholestan-triol in the result we got. This is just a preliminary study. I hope to get a little better idea later. That happened when we stored that compound in the ethanol solution for a few days. When we checked it again, it showed inhibitory reaction. So I'm not sure of the stability of the triol in solution and if its effect is a primary, stimulating, or inhibitory one. But there is some evidence showing stimulating effect. If you store it for a longer period of time, it may have an inhibitory action.

We also tested our fractions from the barrel of old cholesterol and Table 5 shows the result. Fraction one, which contains the cholestan-triol and some minor compounds, has 57% inhibitory action. Fraction two, primarily 7β and some 7α cholesterol shows 82% inhibition. Fraction three, which has 7-keto and 25-hydroxycholesterol has 93% Fraction four, which we thought might be the hydroperoxide, has 89% inhibition and also shows minimal effect on the HMG CoA reductase activity. Further beyond the cholesterol shows 62% inhibition.

Now let us go back to compare the result of cytotoxicity and that of the HMG CoA reductase inhibition. The correlation is rather loose. My speculation at the present time is that 25-hydroxy could be explained based on the HMG CoA reductase because it takes a while to interfere with cholesterol metabolism and eventually kill the cells. It is conceivable that 25-hydroxy has two hydroxy groups--one on each end. I don't know how this compound will fit the cell membrane and how interchangeable with the cell membrane that we like to study. We suggest that a good model of the membrane study may solve the question. Cholestan-triol, on the other hand, has the hydroxy group on one side and may fit perfectly to the cell membrane structure which would replace the cholesterol in the membrane and, therefore, the cell couldn't function properly anymore. This is my speculation at this point. To confirm it, we suggest studying the changing activity of the enzymes in the cell membrane which might be helpful.

DR. WATSON: Were all these experiments done with cells in serum?

DR. PENG: Calf serum.

DR. WATSON: In calf serum----5%?

DR. PENG: We shake them with serum in the incubator for a couple of hours before we change the tissue culture media, so we can maintain the same condition.

DR. WATSON: Now I think one of the things that we want to point out....this was calf serum--not fetal calf?

DR. PENG: Five percent calf serum and five percent fetal calf serum for cytotoxicity study. Lipoprotein-poor serum was used in the HMG CoA reductase study.

DR. WATSON: Well, if it was fetal calf serum, you have a total cholesterol at five percent of about 30 µg/ml of total cholesterol. In your dose-response curve and for a number of the compounds to get greater than 50% inhibition, you were inhibiting

rather or getting killing, you had to be up around 75 to 100 µg/ml of these oxygenated sterols which you are really looking at, in fact, a toxic effect when you consider that the total cholesterol that's in the medium is one-third to one-half as much as the amount of these oxygenated sterols that are being tested in the system. And I'd like to know, do you happen to have on the top of your head, what values Cook used when he showed massive inhibition of growth of aortal explants many years ago? Was he using values like this?

DR. PENG: Cook & MacDougall? You mean the cytotoxicity or cholesterol inhibition?

DR. WATSON: He was adding a variety of sterols. He looked at many of them. He's not cited much in the literature.

DR. PENG: That was a paper of Cook et al., and he used the chicken heart explant. They studied many different compounds--I think he picked up seven of them and tried them in the rabbit aortic explant again. I think I have the papers, if you wanted the compound names.

DR. WATSON: No, I just wanted to know if you had any idea of the concentration that was used.

DR. PENG: The concentration? I'd have to look that up.

DR. WATSON: Okay, I'll get that from you. The studies in which you were looking at the inhibition of HMG CoA reductase--were these cells that were grown in normal serum or had they been transferred to lipoprotein-deficient serum?

DR. PENG: They were transferred to lipoprotein-poor serum--I forgot to mention--for 24 hours before we used it and tried to stimulate the activity of the HMG CoA reductase activity.

DR. WATSON: And how long were the cells incubated in the presence of the inhibitors?

DR. PENG: Two hours.

DR. WATSON: Not the enzyme assay...the suppression of reductase by the inhibitors. Do you add inhibitors?

DR. PENG: These are sterols.

DR. VEECH: You would, I think, perhaps get more consistent data if you constructed the assay such that one had a linear.....I don't quite understand how you did the assay. You did the assay for two hours?

DR. PENG: The assay was after incubation with the sterol.

DR. VEECH: And then how long was your assay?

DR. PENG: Two hours and then you just measure the radioactivity of mevalonate.

DR. VEECH: How long was your assay linear for?

DR. PENG About one to two hours.

DR. VEECH: How long was it linear--see you've got a curve there that's nonlinear over one and one-half hours if I got it right, so when you're.....

DR. TAYLOR: It was incubated for two hours.

DR. VEECH: I got the wrong curve then. I thought your curve started going up straight for about 10 minutes and then curved off flat at about one and one-half hours. Am I misunderstanding the slide?

DR. WATSON: The curve I saw I must have misinterpreted as an assay curve.

DR. PENG: Well, I have a slide showing the result we got for the activity of the enzyme. Then we picked up a certain period of time for comparison of the inhibition of the enzyme.

DR. VEECH: In order to get...I was just asking if the assay was linear at the time of the measurement.

DR. PENG: It looked like linear but it is only three or four determinations--well that's a very arbitrary curve. I figured it's a bit more linear. The time going is long; the activity is higher, but you cannot leave it for three or four hours. I think in most of the papers it turned out to be 45 minutes to two hours. I forgot who got in 45 minutes--I think Brown tested it in the fibroblast and then Dr. Watson...how long did you use it? I think it was longer than 45 minutes.

DR. WATSON: No, my assays routinely, well, depending on what the cells are and in what state they're in, we go from 20 to 60 minutes, something like that. You did show a curve that was "S" shaped rather than being linear over the whole assay period of time.

DR. VEECH: You see, the problem is how you reconcile this data with Dr. Watson's data and (1) it may simply be a technical question and.....

DR. WATSON: Yes, after I thought about it there's really not that much that...the cholestan-triol value is the only one that disturbed me, but his incubations are only for two hours with the inhibitors and cholestan-triol depending on the concentration, is not the most potent inhibitor of HMG CoA reductase.

DR. VEECH: I just didn't think it would be fair for a pathologist...I wouldn't take those numbers too seriously, or Dr. Werthessen was asking about the possible discrepancies. I think that the discrepancies may be more apparent than real and that a little more attention to the assay might change the numbers in some degree.

DR. L. SMITH: Can you tell me what is the difference between your rabbit aorta system and that of Biswas, MacDougall, and Cook? I am not certain I understand the difference.

DR. PENG: You mean the methodology?

DR. L. SMITH: Yes, they were using the same kind of rabbits.

DR. PENG: They used the explant. We used the monolayer.

DR. L. SMITH: I see.

DR. PENG: I don't know which one is more sensitive or which one more accurate, but the explant is less homogenous than the monolayer cell culture.

DR. WERTHESSEN: Do you have any more questions?

DR. WATSON: I have a suggestion. I don't like this bantering around as a pathologist or as a biochemist. As a person interested in getting the answer, it would seem to me that the methodology for measuring death or the number of viable cells should be refined above; what I think you were doing was just counting cells that looked funny and relative to what you defined as normal. I was wondering if there was any way to quantitate, like protein measurements left on the plate, DNA left on the plate, prelabelling the cells with chromium and looking for the chromium release. Things that have a little quantitative--something to put your hat on rather than gut feelings.

DR. WERTHESSEN: John, I'll rise to the defense of the biologist there. You didn't notice a little item at the bottom of the slide, which was that they were counts by independent observers not knowing what they were looking at. And if you do this right, like measuring the number--or estimating the number of sperm that are running around or number of cornified cells in a smear, you'd be shocked at the low margin of error. It is a lot less work than DNA measurements which have to be turned over to a technician and come out.

DR. WATSON: Yes, but when you're defining a process like death, when you see a curled up cell, you may be 20,000 cells removed from when death really occurred. It would seem to me that your sensitivity would be significantly enhanced, like moved over three days rather than the day three, or something of this nature.

DR. WERTHESSEN: Sure, but it's just like the old problem we have now: when is a person dead? But if what you're looking for is a relative bioassay, you can define it in these terms and come out quite accurately. Obviously there are limits. I'm not objecting to your approach, but as a first approximation, I think this is the quickest and easiest way to get at it. Now there may be changes in the DNA and other things which in my opinion would then be cross-correlated with this, in your opinion, crude observation. There I think you could get some more information. But I will argue with a biochemist about the accuracy of the procedure.

DR. PENG: My understanding is that the relative interest of the biochemist and the pathologist is slightly different.

DR. WERTHESSEN: We'll have a good chance tonight at dinner to fight this thing out. Why don't we let Dr. Scallen give his material at the moment.

DR. PENG: Well, in the meantime I'd like to provide a handout if anybody is interested. There is a little more detail on that aspect.

DR. SCALLEN: I'll just very briefly present a study that we did with cholestan-triol It had been reported a number of years ago that cholestan-triol, when added in vitro to cell-free systems from liver, produced an inhibition of cholesterol synthesis, so we decided to start out and investigate that particular phenomenon.

We were investigating the effects of adding cholestan-triol to cell-free systems in vitro from rat liver. The incubation conditions are shown on this slide. We wanted to work on a very large scale so that we could actually isolate milligram quantities of the sterol intermediates which we were going to accumulate in the incubations. So we used 100 ml of a rat-liver S10 preparation with the appropriate cofactors and the substrate that we used was mevalonate. But we used it --50 mg was actually added to the incubation mixuture-- at about a 3 mM concentration so that we would essentially saturate all of the enzymes of sterol synthesis. And then the cholestan-triol was dissolved in a small volume of organic solvent--50 microliters of dioxane propyleneglycol mixture . Of course we are not sure what the final concentration is. If it were all dissolved it would be 0.1 mM and 1 mM. This shows the results that we obtained at the lower concentration of the cholestan-triol. Cholesterol is over in this particular portion of the chromatogram. This is a silicic acid column. Then there is a 4-monomethyl sterol labelled here 4β, but the configuration may be 4α. Then a 4-4 dimethyl sterol and radioactive squalene was also observed. This is at the higher concentration of the triol and now notice in comparison to the last slide a much larger 4-4 dimethyl peak was present and also a small peak which we have labelled "ketone." We'll say a little more about that. And radioactive squalene is also more prominent. The X's represent authentic reference cholesterol. You can see that very, very little radioactive cholesterol was synthesized by this rat-liver prep with the cholestan-triol present. We went on to characterize the C_{29} and C_{28} sterols. We isolated the 4 monomethyl and 4 dimethyl sterols by silicic acid chromatography. We obtained the infrared spectrum of the monomethyl sterol shown there and of the dimethyl sterol, and then we studied the fragmentations with high resolution mass spectrometry; C_{29} H_{50} is the formula for the dimethyl sterol. We also studied the fragmentation patterns. Here are some of the mass spec fragmentations that these sterols underwent. We won't dwell on details on these. The most prominent mass spectral peak was at 105 and actually it turns out to be the cyclopropylium cation. We also had enough to look at the NMR spectrum and one problem was to figure out whether the double bond was at 8-9 or 8-14. That can be calculated and it was observed to be consistent with an 8-9 location rather than 8-14. So the structure of the first compound, probably 4α in this location is a 4-monomethyl Δ^8-cholestenol and at the higher concentration, a 4,4-dimethyl Δ^8-cholestenol. There is also a ketone compound isolated and on mass spectrometry the formula obtained was $C_{29}H_{48}O$ and the probable formula of the ketone obtained in the enzyme incubation with cholestan-triol is shown here, ketone at C-3 and the two methyl groups of lanosterol and simply minus the methyl group between the C and D rings. Both of these compounds that were metabolically formed from mevalonate were shown to be converted to cholesterol in a 20,000 x g supernatant preparation and that's shown in this slide. This is probably difficult to make out but it simply is an abbreviation of a pathway of cholesterol synthesis showing that cholestan-triol effectively blocks the removal of the methyl groups at C-4 and that is true even if there is a double bond in the side chain--or if there is not, that doesn't make any difference. So that this location at the 5,6--these two hydroxyl groups are in the vicinity of the C-4 demethylations and it is not an unreasonable idea that one effect of this compound, at least in vitro, would be to interfere with enzymatic removal at that neighboring carbon atom. The details concerning these studies have been published previously (see Scallen, T.J., Dhar, A.K., and Loughran, E.D. "Isolation and Characterization of C-4 Methyl Intermediates in Cholesterol Biosynthesis After Treatment of Rat Liver In Vitro with Cholestan-3β,5α,6β-triol" - J. Biol. Chem., 246, 3168-3174 (1971)).

DR. WERTHESSEN: I guess in the light of all the data we've just received, my idea of a blackboard excercise isn't of much value. Because if I'm correct here, the relative potencies of these compounds just don't exactly conform up and down the line. They can't. Because the qualitative effects of each are different too. Is that correct? And I think, especially from what we saw just now, that we're going to get some very odd products floating around in the cell. To put it mildly, I'm sort of nonplussed. I don't know what to say after these three presentations. Has anybody got more sense than I have?

DR. PENG: I would say in relation to that HMG CoA activity study, I suggest that someone whose primary interests are in the cell membrane function do a study by using some kind of membrane model to test all of the compounds. This would be helpful for understanding the mechanism.

DR. WATSON: I suppose what I'm concerned about is that you might get right back into the morass that we're in now--the subtleties. The generic answer that you'll get is that they screw up the membranes but then that's as far as you can go because they all have different subtle modes of action of screwing up the membrane.

DR. IMAI: I don't think you can really compare the different effects obtained by different techniques. For instance, my bioassay result would depend not only on the injected agents. We would have to consider the possibilities of secondary biotransformation and interactions with other organ systems, endocrine system, and so on. Whereas Dr. Watson and Dr. Peng are using isolated *in vitro* systems.

DR. WERTHESSEN: Well I'll agree that you have the added complication of, let's say, your compound going down to the liver and getting transformed or going somewhere else and getting transformed and then coming back at the blood vessel. But I don't see any reason to assume that either the liver cells in John's preparation or the smooth muscle cells in Peng's preparation haven't got the ability to transform the compound too. As any pharmacologist will tell you, just because you put something in, you don't know what's coming out. In fact, most of them don't like to know.

DR. KUMMEROW: I wonder if you've measured the surface activity of these compounds and made some comparisons?

DR. WATSON: At one time when we were trying to think of ways of how 25-hydroxy was so very effective, we were thinking of it going in the membrane and laying on its side, because it has the two hydroxyl groups. Or if it went into the membrane perpendicular to the plane of the membrane with the polar group, it would potentially bring an aqueous environment in the hydrocarbon region. I had some friends do surface measurements and compare the interaction of 25-hydroxy with lecithin and get the force area diagrams and they couldn't distinguish it from cholesterol. Now that may be the wrong tool. You may have to look at calorimetry using vesicles but at that level of measurement, 25-hydroxycholesterol and pure cholesterol were indistinguishable.

DR. SCALLEN: As far as the effect on the membrane-bound enzymes are concerned, the 25-hydroxy and the cholestan-triol appear to be somewhat different. The cholestan-triol is apparently fairly specific for inhibiting the removal of the methyl groups and it doesn't appear to interfere with many other steps in cholesterol synthesis such as removal of the methyl group between the C and the D ring. So it's not a situation where all of the enzymes are being inhibited. It appears to be that the inhibition is more specific than that.

DR. WERTHESSEN: Your Chairman just doesn't know quite what to do- , I think we'd better stay on the degraded cholesterol for a moment. What I might be able to do is to get another item of the agenda done with.

The fundamental question that we wanted to answer here was, "Is it dangerous to eat prepared foods of the quickie-type?" At the same time we wanted to find one, which on the basis of the techniques then available could be subjected to analysis for the sterols present in the food. This was before Dr. Merritt developed his nice technique for separating the plant sterols from the so-called animal sterols. He found this material known as "Jello's Instant Golden Custard." It is available in the supermarket and contains carrageenan. It's one of those nice foods whereby a housewife can prepare a superb dessert by the mere process of pouring boiling water into the mixture. Now on that sheet without the graph lines (Fig. 1), you can see the legend. The base diet, 25, 50 and 100% of the custard in the diet the animals ate. And as I pointed out earlier, my background was in the physiology of reproduction, so I figured that we might add to the usual FDA and NIH systems the question of seeing if these materials could interfere with the sex life of the animals. What was done was to pick them up at the age of weaning and feed them these various things. I think it's rather obvious from that graph that, if you eat this custard, even though it's got everything in it that you're supposed to have in order to grow, and you eat nothing but it and you're a female mouse, your growth rate is impeded and it acts in good part as a contraceptive because it took them 60 days to become pregnant as compared to roughly 30 in the controls.

DR. ____: How well do they eat it?

DR. WERTHESSEN: They eat it avidly and I might add that those 100% diet females are the best looking of the lot. They're pretty.

DR. IMAI: What do the males get?

DR. WERTHESSEN: The males get the same diet. There's one male in each cage, so I haven't plotted the males.

DR. ____: Have you done the experiment with the males?

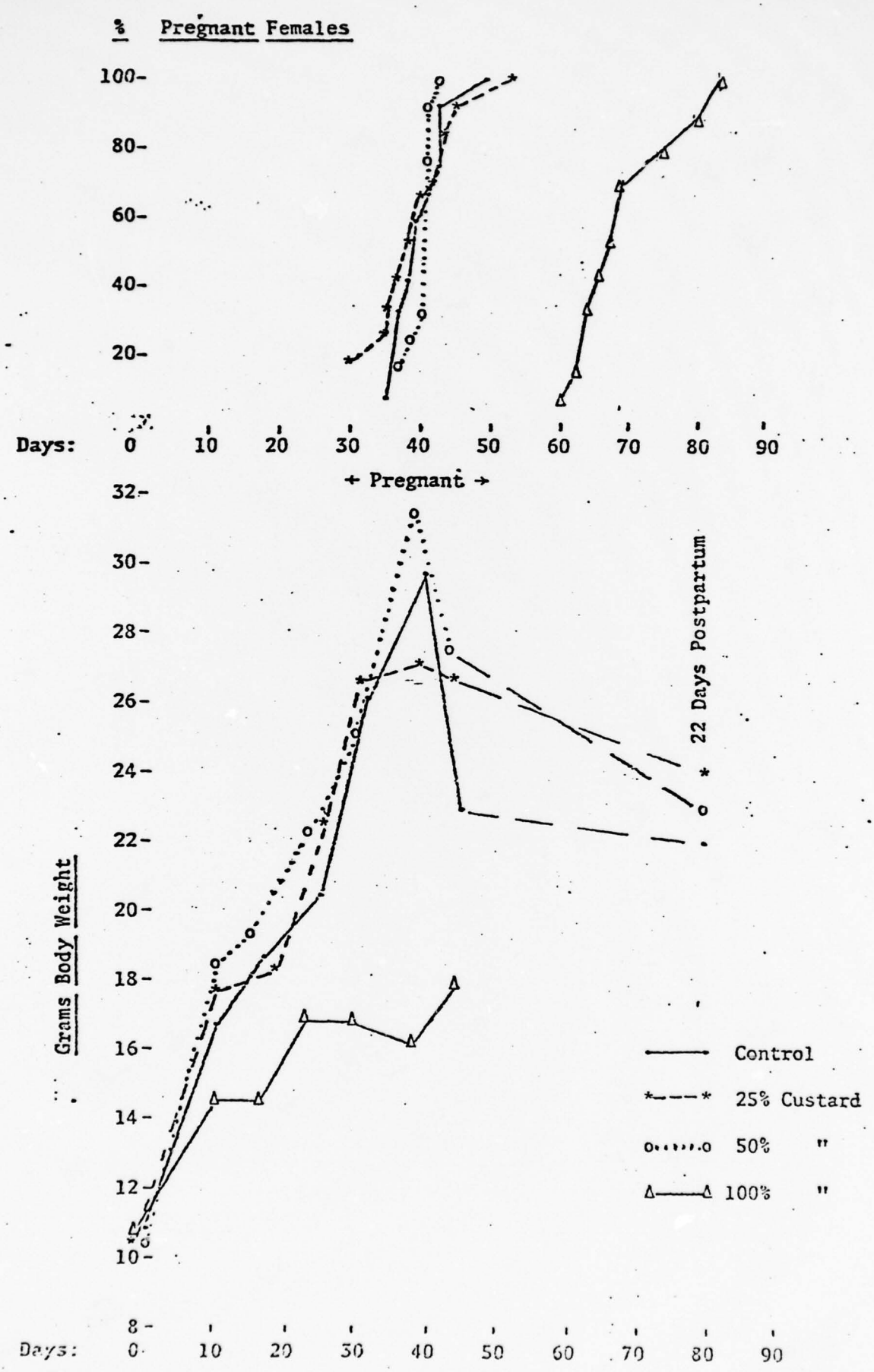
DR. WERTHESSEN: No, no. Males of this breed are hard to keep. They fight. One male and four females provide a peaceful cage.

DR. ____: What is the breed?

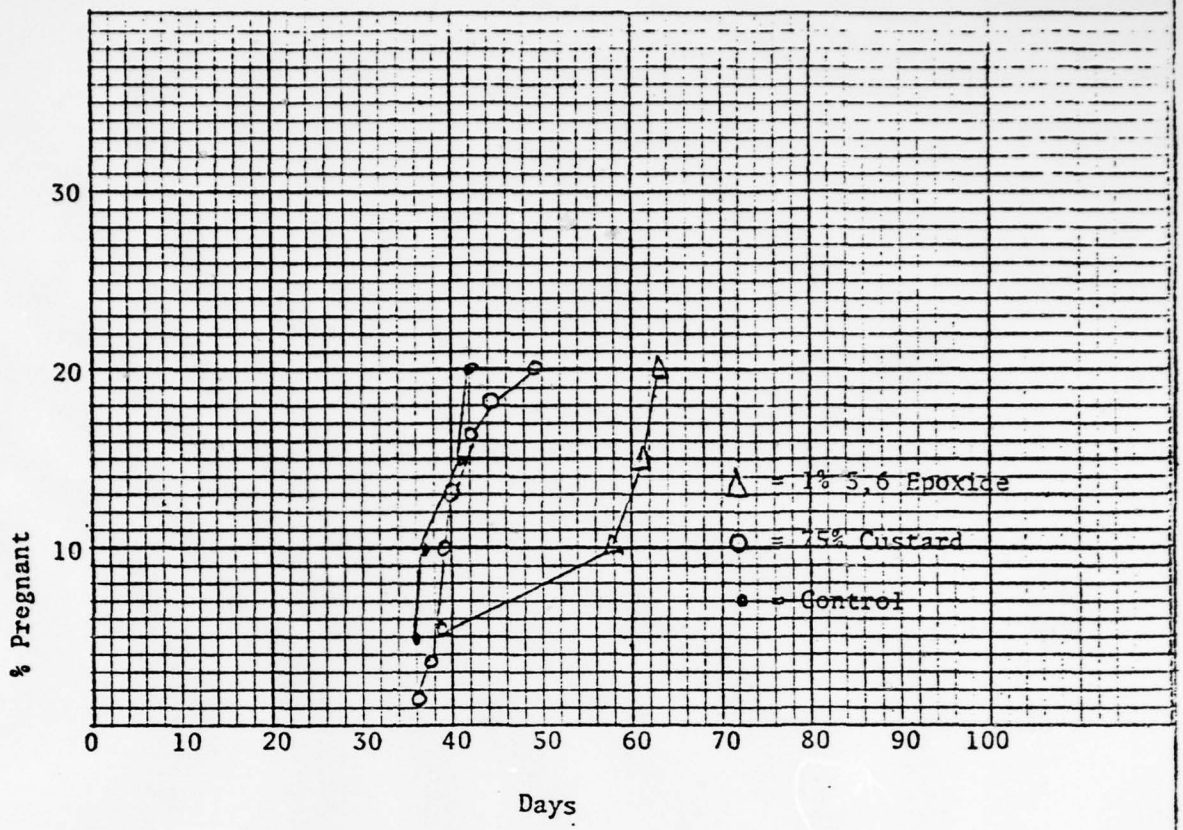
DR. WERTHESSEN: It's an F_1 cross. I can't give you the precise one, but it's provided by Charles River. They're two very solidly established strains, so the F_1 cross is perfect--I mean the mice--one is just the same as the other.

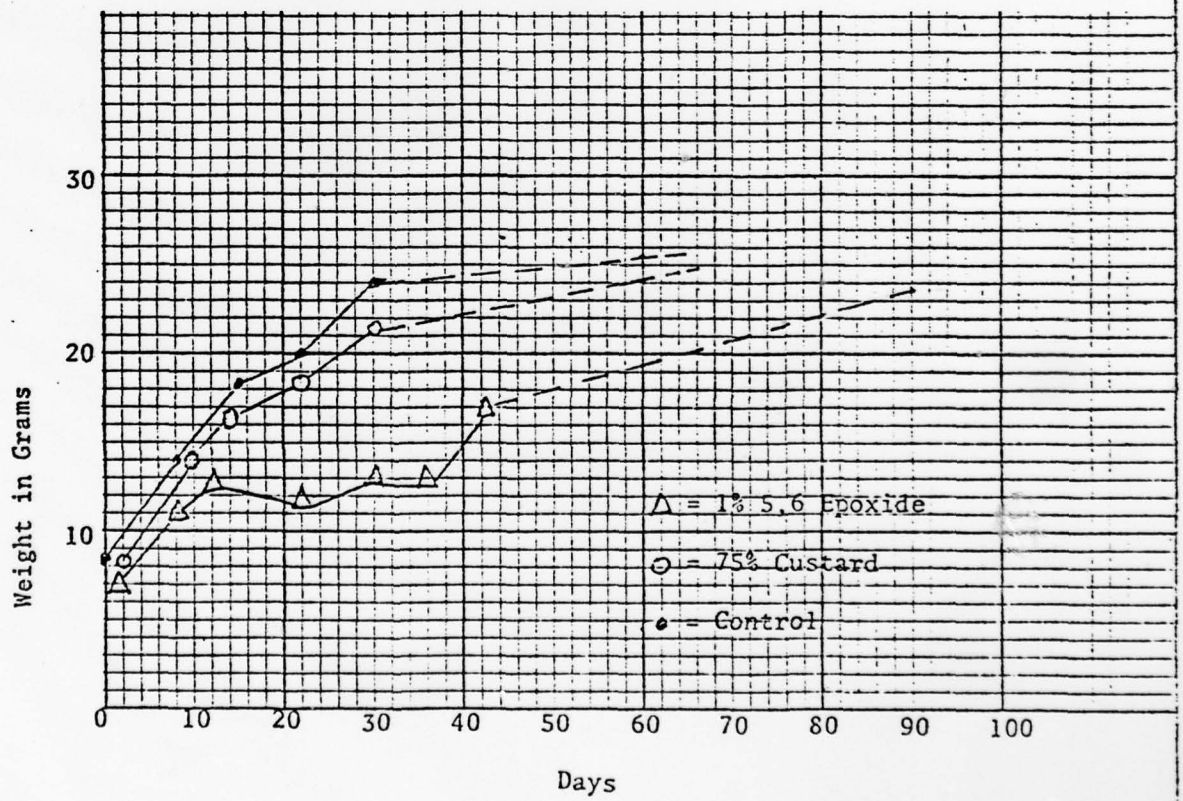
As you can see from that first graph, what we should have done was to put in a 75% diet. You'll see that 75% custard in the next plot (Fig. 2). The percent pregnant, you can see that the 75 is a little bit different from the group of controls. But here we also went to our first pure compound and we used one percent 5,6-epoxide in the base control diet. And there you can see a real shift in their ability to become pregnant. In the lower curve, you can see that their growth rate was seriously impeded. This, of course, confirms what Dr. Watson said. If you feed the animals these compounds, you can kill them.

Dr. Werthessen's Figure 1



Dr. Werthessen's Figure 2





DR. L. SMITH: Do I understand that you are feeding the male rat the same level of diet as the female rats?

DR. WERTHESSEN: You are correct.

DR. L. SMITH: You really do not know then which rat ate what portions.

DR. WERTHESSEN: No., This is just proof that it's not good to eat these prepared foods and/or add one of these agents to your diet.

DR. WATSON: You mean you can get it on the market and have a birth control agent or something?

DR. WERTHESSEN: Well I'm not going to advocate it, no.

DR. MERRITT: What's the conclusion relative to the effect of the foods, because we don't know whether or not supposed oxides of cholesterol are present in this food?

DR. WERTHESSEN: Well, the real answer here will come out.....

DR. MERRITT: Maybe just eggs are bad for them---or whatever else is in there.

DR. WERTHESSEN: Well obviously to control this thing properly, we've got to go and permit the mice to eat fresh eggs or somehow decently prepared eggs and see if they interfere. We're going to have to check out which one of the oxidation products that we eventually find in this food--whether each one of them or a combination of them are effective. But we've only been at this now for four months.

DR. LEQUESNE: What is the method of preparation of the "Instant Golden Custard" and do they use powdered eggs in making it up or do they use powdered milk?

DR. WERTHESSEN: According to the squib on the box, the preparation is made in part from dried eggs, added whole yolks, dried milk powder, and carrageenan. Now the carrageenan, Dr. Imai tells me, as far as the pathologists are concerned is not a healthy item to eat either.

DR. IMAI: This carrageenan. There are two kinds--one is the native and the other degraded, broken down into say a 20 to 30,000 molecular weight. The bad ones are these smaller molecular weight ones, and these are only used for medicinal purposes. The ones that are contained in food are native type which just go through the intestinal tract and are innocuous.

DR. WERTHESSEN: Well this carrageenan is obviously approved by the FDA at least at the present time. It does have one effect on the mice since it absorbs water. The mice eat this stuff and they get frightfully thirsty. Then of course they digest the carrageenan and they've got to get rid of all the water, so they make a mess as far as cleaning the cages is concerned.

I think we'll also find a little bit later on that their life expectancy from various disorders will be decreased.

DR. MERRITT: I don't know much about feeding studies but I've been told it's important to know whether or not they eat all the food. Is this an ad libitum feeding?

DR. WERTHESSEN: This is ad lib but we also have a pile of numbers on how much they eat in the beginning. We have a lot of numbers on how many offspring; what the weight of the offspring is; how much they grow during lactation. I have not presented all of that to you because we just don't have enough data.

DR. WATSON: Are they pair-fed?

DR. WERTHESSEN: No, no.

DR. LEQUESNE: It's true, is it not, that rats and mice are very difficult animals to induce atherosclerosis in?

DR. WERTHESSEN: You're right.

DR. LEQUESNE: Are you planning, in view of the fact that some of these animals have been fed 100% of this egg-containing diet, to look and see if you can see it?

DR. WERTHESSEN: We're all set. We haven't had any deaths yet. You can see from the numbers on the bottoms of the graphs, these animals are still young.

DR. LEQUESNE: So they're living more and enjoying it less, in other words, at this point.

DR. _____: What is the basal diet?

DR. WERTHESSEN: The basal diet is one developed by Dr. Leduc except for the fact that she used codliver oil in it. I figured that that could be a source of confusion using these various oxidized sterols. So we pulled that out of the diet and put in whole corn to provide the vitamins and they grow nicely on it. Now I think we can go to coffee.

Coffee Break.

DR. TAYLOR: This is really not related to breakdown products of cholesterol, but is something that's been bothering me for a good many years. About 1960 Irving Page wrote a review article and made a big issue of perfusion distance through the arterial wall being the cause of atherosclerosis. At that time I had a colony of monkeys, so I decided to increase the perfusion distance through which the plasma had to pass through an arterial wall. All cells of the media of monkeys' arteries were killed by hypothermia in normocholesteremic animals on a cholesterol-free diet (Taylor et al., 1963). An intimal scar was allowed to form in these monkeys on a cholesterol-free diet (Fig. 12) during the subsequent 15 weeks, then hypercholesterolemia was induced. To my great dismay, even though the perfusion distance was about twice as great as that in normal arteries, this scar which I though would surely collect lipids avidly was essentially immune to atheromatosis (Fig. 12). The only place in a few of the cases, and I kept these animals hypercholesterolemic for 42 weeks, was a minimal amount of lipid deposition near the corner of the scars (Fig. 11). Then, as I should have done earlier, I went back to the very early literature and found out that somebody had already done this in 1929. There was a man by the name of Ssolowjew (1929) who used rabbits; this

was before they did serum cholesterol levels, and these lipid-free areas were areas where he injured the aorta in a normal rabbit with a normal blood cholesterol. Then induced hypercholesterolemia after he allowed 50 to 92 days for this scar to form; Figure 11 is a drawing of a cross-section of one of Ssolowjew's rabbit's aorta. Here the scar was produced with cautery in normocholesterolemic rabbits. Then he produced hypercholesterolemia 50 to 92 days after the scar had formed. He got lots of disease in the adjacent normal arterial wall, but the healed scar was disease-free except at the edges as my monkeys were. This is the type of lesion he got (Fig. 11). When the hyperlipidemia was induced before he produced the injury, he always got marked atherosclerosis in the intimal scar (Ssolowjew, 1929). That's always fascinated and annoyed me because it seems teleologically incorrect.

DR. WERTHESSEN: What Bruce has obviously moved into is the area that I think we ought to cover tomorrow. I would like to put one point forward to perhaps confuse your thinking about the question that he just posed and that is that in Dr. Imai's three-day experiments as compared to the one-day experiment, it should be noted that the proliferating cells are not attacked by the third shot--if you analyze it that way. Dr. Merritt, you're next.

DR. MERRITT: Well this is mostly a story of how not to do it, but sometimes negative results can be instructive, I guess. But at any rate, as Dr. Taylor and many others have, of course, pointed out, atherosclerosis is to some extent at least a nutritional disease and we've become aware of the potential significance of the oxidation products in cholesterol as angiotoxic. So the question has come up: "Are these things in the foods we eat and, if so, to what extent?" About 10 years ago a fellow by the name of Chicoye did a thesis with a supervisor by the name of Powrie (I think it was in Wisconsin) in which they studied dried eggyolks and they found that the epoxide, 7-keto and some of these other oxidation products we've been talking about were formed. So we undertook about a year ago to determine whether or not these compounds were present in typical foods which contained dried eggs. In particular there is a product which the Army uses which is called Robert's Egg Mix which is basically the product from which scrambled eggs and omelets and that sort of thing are prepared in mess halls and other Army feeding situations and there's a tremendous amount of it used. Its composition is mainly--well it's about 50% eggyolk, dried milk powder, corn oil, and perhaps a little flour. At any rate this was the first product that we looked at, and it occurred to us to extract the fat, to hydrolyze, to extract the nonsaponifiable fraction, and then put the nonsaponifiable fraction on a gas chromatograph. Lo and behold, the first thing we discovered was the presence of the epoxide. So this got us really excited. Now this turns out to be probably an artifact, but at the time we were not aware of it. And before I tell you why we goofed on that, let me go to the next thing which is--oh, incidentally there were two ways of carrying out the extraction for the separation. One was to put, as we did here, the entire nonsaponifiable fraction on the gas chromatograph. The other was to separate the sterol fraction by digitonide precipitation and then, after isolating the digitonide and recovering the sterols from the digitonide precipitation, taking that up in solvent and putting it on the gas chromatograph, again the epoxide was found to be present.

This is another workup of the same material from a different can and again it was found to be present--the epoxide. And there are other oxidation products in there--the 7-keto and the triol.

DR. WATSON: The two curves you have, the top and the bottom, are they just two different attenuations?

DR. MERRITT: One is a factor of ten more sensitive. So after coming to the conclusion on the basis of these two analyses that the stuff was indeed present in the food, we thought well now let's find out if this is present in fresh eggs or not. There were two preparations of the two samples that we analyzed which I showed you on the previous graphs which were prepared in Dr. Werthessen's laboratory up to the point of the extraction. Then we took the extraction from that point and ran the GC MS. We then started doing it in our own lab and we took another can of the egg mix and this time we didn't find any oxidation products. We did find the plant sterols that you can see there. Sitosterol and compesterol. Now we have a situation where sometimes we do and sometimes we don't.

DR. TAYLOR: Could there be a difference in the age of the eggs that sat around before the egg mix was made?

DR. MERRITT: There may. The particular lot of material that we are dealing with here is all from the same production lot by one supplier.

DR. TAYLOR: He could have gotten fresh eggs one day and old ones the next.

DR. MERRITT: I'm not aware of what the quality control is on that...it's possible. But at least as far as these three samples are concerned, they all had the same production lot number on the can and they came from the same carton. Anyway we can explain the anomaly here, but before I do it I just want to point out one other thing.

DR. L. SMITH: Were these experiments conducted with the digitonin procedure?

DR. MERRITT: This here was not. This was an extract. The first lot I showed was a hexane extract of the nonsaponifiable fraction of the hydrolysis mixture. The second one was the digitonide precipitation of that. This one again is a hexane extract.

DR. L. SMITH: One can lose material from digitonin precipitations and from subsequent dissociation of the digitonide complex. There is something else missing here. Eggs contain dihydrolanosterol and lanosterol in abundance, indeed in greater proportion than sitosterol or campesterol. Does your chromatographic system resolve dihydrolanosterol and lanosterol?

DR. MERRITT: It may be under the cholesterol in this case. It could be the lanosterol is not here. In fact they may not be resolved. These are very crude. We are trying to develop better systems and improved procedures. These are the early studies and they led to some confusion. I'm approaching this historically. The next anaylses we made were of fresh eggs. The diketone appears here. We also see the main cholesterol peak and the associated plant sterols.

What put us wise to the fact that you have to be very careful in doing this was that we thought the fresh egg sample would be a good sample to test our procedure by spiking it with known's and get the retention times and evaluate our resolution and all that sort of thing, but we were very careless about this and we were also rather naive about how rapidly cholesterol will oxidize. Here we have the cholesterol fraction that had been isolated from the eggs and it was sitting around on our bench for about three weeks, but these are oxidation products. I'm sure this is no surprise to Dr. Smith, but we realized it because we had another fraction, the

nonsaponifiable fraction, from which the alpha tocopherol which was actually present in the eggs, had not been separated from the cholesterol and this didn't oxidize. Then we went back and looked at how we handled the samples that Dr. Werthessen had given us initially and we realized that we didn't analyze them the day he brought them to the lab. They sat around on our bench. At this point, as we look back on our first analyses, we have no assurance as to whether the stuff was in there or whether it was formed sitting around on our bench. We then decided to look at our procedures and to try to improve the situation. We realized we had to handle everything in a more proper manner. Moreover we realized that the sensitivity at which we were detecting these compounds--for example, in the preparation that I showed you the results of here, really the epoxide was present to about two percent of the cholesterol. Now this is a rather large amount. In the preparation we made in our own laboratory, we did not find it; we estimated that the level of detectability would be about a half a percent of the cholesterol available. But we had to face the possibility in any event that if these things were present they were probably present at levels beyond this and that the amount of cholesterol that we were putting on the chromatography column was probably overloading it and that, if the oxidation products were present to amounts less than a part per thousand, we wouldn't see them. In the meantime we had developed our experiments with the U.S.P. cholesterol and recrystallization as a concentration factor and we decided to use that. In fact we developed a scheme more or less as follow. This is a fairly abbreviated variation but for the most part this is the procedure that has been followed:

Solvent extraction, and of course there are almost as many solvents that are used for this sort of thing as there are in the Eastman catalog. But for the most part though, we have stuck to the chlorinated hydrocarbons, although in later work there were a couple of other solvents that we used--acetone alcohol extract in the cold. In any event, the lipid fraction was hydrolyzed. This is a hot hydrolysis. We had some that were done in the cold. The nonsaponifiable fraction was isolated by extracting with hexane from the hydrolysate. In some cases, taken directly to the recrystallization procedure; in other cases, put through a digitonide precipitation and then recrystallized. But the recrystallized fractions were then treated by GC-MS. Now the thought here, of course, was to concentrate the oxidation products relative to the total cholesterol.

DR. L. SMITH: Was the saponification conducted under vacuum or under inert atmosphere?

DR. MERRITT: I think not. Right, Nick?

DR. WERTHESSEN: No, it was conducted under nitrogen. And some of them were just standing overnight; others were put on the steam bath. But those that were done at my lab, all the reagents employed were first boiled, then cooled under nitrogen.

DR. L. SMITH: The temperature and the nature of the alkali are also important. However, unless air is excluded rigorously, autoxidations occur.

DR. WERTHESSEN: Yes! No doubt. That is why we were scrupulous.

DR. MERRITT: Anyway this is the general scheme with one modification or another. We performed a very large number of analyses on a variety of these products and this summarizes some of the results as to appearance of the epoxide, which from our earlier work appeared to be the most abundant of the products except for the 7-keto.

DR. L. SMITH: You have the 5α,6α-epoxide mentioned twice, but this may be a mixture of 5α,6α- and 5β,6β-epoxides.

DR. MERRITT: I don't think we know exactly which one it is. It's probably the 6 and the 5 ,6 which.....

DR. L. SMITH: The 5β,6β-epoxide predominates in autoxidation experiments, but the 5α,6α-epoxide is the one which can be bought commercially, therefore the one frequently suggested as implicated, even where both might be.

DR. MERRITT: I feel that this is loose terminology. At any rate, the peak which we identify as the epoxide by the mass spectrum, I'm sure it's the beta--was not found, except in a few cases. This is a summary of the study on the U.S.P. cholesterol. Subsequently we analyzed the Robert's egg mix, the custard mix, which is the Jello product that Dr. Werthessen has described, a pancake mix and dried milk. Now, I think you can dismiss the dried milk since it contains only about one percent fat to begin with. It would take a tremendous quantity of it in order to get enough lipid to perform well. But it is interesting, that at the level of detection we establish here, i.e., about 0.1 percent of the level of cholesterol, we found only the epoxide in one of the two samples of custard where the sterol fraction was isolated by digitonin precipitation, and one from the dried milk. So at this point we don't really know, I think, with any degree of assurance that these products are in fact present in the foods, but we're doing a number of things which makes this result uncertain because all these products are protected by anti-oxidants. To begin with the egg contains natural anti-oxidants in the form of alpha tocopherol and most of the products have BHA or BHT added. Moreover, in connection with the analytical procedures, there are two things which I think are suspect. One is that when we do the digitonide precipitation, we get a precipitation which could possibly occlude these compounds and perhaps they could be lost. We do get those too, at times, so I don't know about that. The other aspect is that we have appreciable concentrations of the plant sterols. These are eluted at the same time as the oxidation products. For example the epoxide that I've spoken about mainly, elutes on the gas chromatograph at the same time that β-sitosterol does and, in general, whenever we have identified the epoxide in these products, it has always been by the appearance of peaks in the mass spectrum of that fraction of that chromatographic peak that corresponds to the β-sitosterol. These are not separated and identifying the compound as a component of the binary mixture which was eluted at that time was difficult. We can see this example here--the mother liquor from recrystallized nonsaponifiable fraction of the pancake mix--and here we have some campesterol and another peak and lanosterol and so on.

Plant sterols are present whenever we have corn oil in a product in an abundance, which is generally larger than we expect to find in the oxidation products. Now to overcome this we decided to develop a new scheme where there would not be any hydrolyses, in which there would not be any precipitations--in which we would not use recrystallization. This is an outline of the procedure we expected to follow and we tested most of the steps of this procedure. The main step after extracting the lipids was the separation of triglycerides by the liquid chromatographic procedure. The one we chose for this was size exclusion initially because this had worked rather well for us in other studies in which we planned to separate minor components from bulk triglyceride. One thing, we separated the sterol fractions from the triglycerides and got a sterol fraction from the bulk lipids on a 100 micron column. This worked well for us in a modern system. Then the next step we thought we might want

to separate the sterol esters and the free sterols and we were going to use enzymatic hydrolyses, which we never did because in eggs the sterols are about 80% in the free state, so we decided we wouldn't worry about the esters at this time. So this step we eliminated. And then after getting a free sterol fraction, we decided that we could separate not only the bulk cholesterol but also all the plant chromatography column and then this would give us an oxide fraction. Then this is what we would use for GC-MS. So we have been undertaking to do this just to give you an idea of our model system. This is the silica gel preparation, that we were able to separate the neurtral sterols and the oxides. I showed earlier some of the plant sterols elute on gas chromatography very close to the cholesterol.

DR. L. SMITH: If you can separate cholesterol from desmosterol in this system, you should be able to resolve dihydrolanosterol and lanosterol if they are present. Dihydrolanosterol and lanosterol would probably be resolved and both have been reported present in hen eggs.

DR. MERRITT: I'll tell you what this is exactly. This. fraction, according to my notes here, was prepared from cholesterol--U.S.P. I suppose--and it contained the amount of the oxidation products that we could clearly see. Now we also have a similar situation with the nonsaponifiable fraction from the custard. The objective was to test the silica gel separation of the sterols from polar compounds and to see if it works. So we started out to implement the procedure and we found out quite soon that when you calculate the amounts of sterol in these products, for example, you have to take an amount of the Robert's egg mix that requires the handling of about 50 grams of fat, i.e., the lipid fraction, or the bulk lipids, because there's a substantial amount of corn oil in this product. And to handle 50 grams of fat on the size exclusion column we were using, it would have to be done in about 25 multiple injections, and collection fraction. You can only put 2 grams of fat on the column at a time. Well, to prepare a large column and at $10 a gram, this would require an investment of a couple of thousand dollars, I think, for the stationary phase, and our budget wasn't able to stand that. We had to seek an alternative solution to handle these large amounts of fat, so we decided to do a class separation instead on silica gel and this is the scheme which we are working with now. And in this case we again start with an extract of the lipids. With silica gel and a non-polar solvent such as hexane or a chlorocarbon, we can eliminate the separation of triglycerides and then after they've been washed off, start programming with isopropanol and take out the sterols and then have them eluted to get the polar lipids. Now these polar lipids may contain some of the mono and diglycerides and other compounds of that nature. And this fraction here did not permit the size exclusion chromatography which would separate the oxides from the other glycerol types of components. Hopefully then we get a fraction which contains the polar sterols and which we would put on the GC-MS. So we've taken it down to this point and Murphy's Law being what it is, I had hoped to present this data at the meeting here, but at this moment the chromatograph is on the fritz so I'm not able to tell you any more.

DR. WATSON: Just a methodological question. I'll direct it to Leland because he might be able to answer it for me. Considering the wide range of polar sterols that can be formed, is it better to run a mixture on the column or to make derivatives in the GLC work, because I thought I read somewhere that some of these compounds are just degraded on the columns and you never see them?

DR. L. SMITH: We do not make derivatives directly. Some autoxidation products are degraded and derivitized too, so one does not always know what has occurred when half is derivitized, half is not, some is degraded, etc. One could approach the problem either way, but methods for analysis of the underivitized sterols are available, particularly if one knows what sterol is being sought. If looking strictly for the 5,6-epoxides, these are fairly stable and they can be detected.

DR. WERTHESSEN: I'd like to raise a question to both Leland and Elspeth. When we started off on this dried egg custard, it was commonly assumed that it was easy enough to get the lipid out. But the yields from the Soxhlet extraction were atrocious. Finally we went at the problem doing a clinical cholesterol determination on the stuff and we found that the concentration was roughly 2.2 milligrams per gram of dried custard. Now would you be surprised to learn that the lipids don't come out with any of the expected solvents to take them out and that the only one that we found that would work was a mixture of acetone alcohol, 95% ethanol for the alcohol? Then we finally got up to the proper number. Does that surprise you at all Leland?

DR. L. SMITH: Are you talking about egg products--dough, custard, etc.?

DR. WERTHESSEN: I'm talking about this product which is supposed to be nothing but dried eggs with carrageenan added.

DR. L. SMITH: It is known that sterols form tenacious complexes with some carbohydrates, and this might be one of those situations where sterol-carrageenan complexes might be formed and the sterol may not be easily extracted from the mixture. I have not looked into this prospect, but it is not surprising that some materials might not be easily recovered.

DR. E. SMITH: I've always found for artery wall one needs a very polar solvent, and we start off with 75% ethanol to 25% ether for the first extraction. We extract for a fairly long time, at room temperature in the dark. Then we go on to a 50/50 chloroform/methanol mixture. Using less polar solvents we got pretty inadequate extraction. I frankly don't know how much we're leaving behind. We subjected some samples of residual arterial tissue to digestion with sulphuric acid and we got about another one percent out. I suspect that we're not getting complete extraction in any analytical sense, but the amount left in is not significant in terms of atherosclerosis as we're looking at it.

DR. MERRITT: I think there are two things that should be stated in this connection. The first is that cold extraction of all the lipids is not necssarily desirable. I mean there are solvents which could be employed here which would take in addition to the triglycerides and the sterols and all these other things--the phospholipids. But we have tried to select solvents and, while they may not completely exclude the extraction of phospholipids, at least don't take them all out. Eggs contain something like 30 or 40% phospholipids. If you extract all of this along with the triglycerides and other lipids and triglycerides that are there from added corn oil and other things we

have not only more fat but we have a larger fat fraction to handle and from which the sterol fraction is a still smaller percentage. But in addition, the procedures for removing the possible lipids involving precipitation give you tremendously flocculent precipitates that lead to occlusion and co-precipitation and other possible losses of a trace component, so that's something that we wish to avoid.

DR. WERTHESSEN: Well this has been a slight bone of contention 'twixt my colleague and myself and my hunch is that you can't get the sterols out without taking out the phospholipids. What's your feeling on that, Leland?

DR. L. SMITH: We have tried to do total lipid extractions on a worse case: human brain. We try to extract all lipids and then worry about resolving the lipids later. Prior removal of unwanted protein might aid; prior freeze-drying to remove moisture might aid. I do not have any direct experience in foodstuffs' analysis, but repeated chloroform-methanol extractions in the cold usually suffice to remove total lipids and sterols from tissues which we have examined. We tend to ignore lipids not extracted by three to four such successive extractions as not likely involving cholesterol derivatives.

DR. MERRITT: The phospholipids also give you problems on a column and, insofar as chloroform/methanol is concerned, it's a good solvent, no doubt, but the methanol is present primarily when you're working with wet substances and we have, in almost all cases, dried products to deal with. Even when we're working with eggs and so on, we use additives to go with fat. In general we find, at least for the moment, our solvent of choice is something like chloroform or methylene chloride.

DR. WERTHESSEN: Do you have any further comments?

DR. WATSON: Should we conclude from this talk that in fact the oxygenated compounds have a high probability of not existing in common foodstuffs and that maybe our initial concerns may have to be turned down?

DR. MERRITT: I think it's a problem of how the food is treated. I would say that in general if things are handled well that the foods are protected to some extent from oxidation by the presence of the anti-oxidants. Now if this system goes haywire somewhere, either because of long storage, or they get overheated, or get a lot of oxygen in the package--the egg mixture, for example, is packed in cans under vacuum, so there's not much oxygen present in the product as it's stored. If oxidation occurs, it would most likely occur during the spray-drying in which you're atomizing these things at a high temperature right in the air. There's a good possibility for oxidation to occur, but whether it does in fact, you would have to go back to the product before it's packed for comparison. We have not done that. But again, as I mentioned earlier, all of these products contain anti-oxidants to begin with, so it may not happen. This is what we have to find out. But at the moment I would say we have no need to become terribly alarmed about these things being present in food because, if they are present, it may be a random occurrence. It may be that the extent to which this occurs probably is at a very minor level.

DR. WERTHESSEN: This is why we make a good team. You see he's a food technologist fundamentally. I'm not. Of those numbers we had up there, for example, there were five experiments with no epoxide. It wasn't until the last one where it was shown, as positive, that we were satisfied with total extraction.

DR. MERRITT: I have some figures on the amounts, on the analyses that we've performed to date on the extractions and so on. If the oxides are present, they would have to be present in amounts less than about a tenth of a percent relative to the cholesterol. And if the level at which we're shooting for now based upon the cholesterol and the spiked sample, we can detect about one part per ten thousand relative to the cholesterol. That, if you take it down further, means only about one percent cholesterol in the product, would mean about in the part per million range for the--excuse me--for the fat, it would be about one percent of the cholesterol in the fat--maybe up to three percent. Anyway this puts it in the part per million range for the oxides relative to the fat and then, depending upon the fat in the product--for example, in the custard is about five percent and in the egg mix about 50 percent--puts you somewhere in the 10 to 100 part per billion range for the amount of these compounds in the product.

DR. WERTHESSEN: Agreed, but that's also about the concentration of the oxidation products in the rabbit's diet.

DR. MERRITT: Well, this might be enough to do you a lot of harm.

DR. FRANK: Does this perhaps suggest on the basis of the custard information that the cooking process may be the resulting producer of these products, particularly if you're mixing the air and moisture and heat and letting it bake or something for a period of time?

DR. MERRITT: That's something we haven't looked at at all, but it's on our agenda and it's a very important aspect. If you take a fresh egg right out from under the hen and put it in a frying pan, what happens to it? That we don't know.

DR. L. SMITH: There is one report of finding 7-ketocholesterol in pork fat (Williams and Pearson, J. Ag. Food Sci., 13, 573 (1975)) and one where neither 7-ketocholesterol nor the 7-hydroxycholesterol were found in cooked meats (Tu, et al., J. Food Sci., 32, 30 (1967)). Neither report can be considered definitive. One needs to look at the treatment of foodstuffs under heat in air in the frying pan in your own kitchen as well as in food processing. One more point, if one is searching samples for the cholesterol 5,6-epoxides, they do not exist by themselves. In order to derivve the epoxides by autoxidation, one requires initial cholesterol (or other lipid) hydroperoxide formation. Thus, these products of autoxidation will also be present. Were one seeking strong evidence that autoxidation had occurred in a sample, these other autoxidized materials should be sought as well as the cholesterol epoxides.

DR. MERRITT: I hope I didn't give a misimpression here. I'm only using the epoxides as an example. But in fact one should expect probably to find an abundance of the oxides other than the epoxides. I think the 7-keto is expected to be the most abundant, but probably the 25-hydroxy and the triol would be expected to be more abundant than the epoxide. But there's another aspect of the epoxide which is of some concern in proving this. If the epoxide is a carcinogen, it comes under the Delaney law, whereas the other compounds we've been talking about here at least have not been legislated against.

DR. L. SMITH: Regarding the literature which claims that cholesterol 5α,6α-epoxide is a carcinogen, this claim is repeated often but the evidence is not really very convincing. I believe that the question of carcinogenicity for cholesterol 5α,6α-epoxide should be reexamined by experienced investigators to set this matter straight.

DR. WERTHESSEN: That's one reason why we're re-testing it, because this assay procedure that we're engaged in is fundamentally for carcinogenesis.

DR. L. SMITH: Neither cholesterol 5α,6α- nor 5β,6β-epoxide is a mutagen in the Salmonella typhimurium (Ames) test in our hands.

DR. SCALLEN: This is a methodological question. Can you spike your biological samples with 7-ketocholesterol or with 25-hydroxy and therefore quantitate recoveries through the process?

DR. MERRITT: Yes.

DR. SCALLEN: You are doing that....what kind of recoveries are you getting?

DR. MERRITT: I can't answer that offhand.

DR. WERTHESSEN: We've got another problem there too. For example with this custard. Let's say you mix some 25-hydroxy into the custard in its current dried state. That would not necessarily bind it to the carrageenan.

DR. MERRITT: One of the reasons why I can't answer the question is because the spiking that we've done so far has been done with these cholesterol fractions, not with the fats. I haven't dealt with them initially. If you're going to do a recovery study, you have to start from the beginning.

DR. WERTHESSEN: We should go to the manufacturer and start.

DR. SCALLEN: That's right, you'd have to do it during manufacturing process.

DR. WERTHESSEN: It's a very rough problem. But my hunch, since I love to cook, is that we're going to shock some housewives about the beating up in air some six-month old eggs to make a souffle. That would be just about the ideal condition.

DR. SCALLEN: It might be important also during the the cooking process to know what kind of a pan you're using.

DR. WATSON: Leland, is there evidence that aged-intact eggs accumulate auto-oxidation products?

DR. L. SMITH: We have no evidence to suggest that cholesterol autoxidation products are in eggs. We have not examined eggs systematically with this point in mind, but we have examined eggs for their possible content of 26-hydroxycholesterol, thus as a possible dietary source for the sterol. We did not demonstrate to my satisfaction that eggs contained 26-hydroxycholesterol. Cholesterol autoxidation products were found, but we cannot state that these products were present in eggs with intact shells. In spray-dried egg materials that is another matter. A report by Acker and Greve, Fette Seifen Anstrichmittel, 65, 1009 (1963), showed the unmistakable presence of cholesterol hydroperoxides in egg-containing foodstuffs subjected to irradiation in air. Other (Chicoye, et al., J. Food Sci., 33, 581 (1969) have found cholesterol autoxidation

products in irradiated spray-dried egg but not in fresh eggs or in unirradiated eggs. It appears certain that cholesterol autoxidation products will be formed in egg-containing products where light or heat is involved in the presence of air. With no air, autoxidation cannot occur.

DR. TAYLOR: It was my understanding that the eggshell transmits oxygen and CO_2 back and forth with great facility and that oxidation of cholesterol in the egg yolk can occur.

DR. L. SMITH: There are natural antioxidants and phospholipids present which appear to protect cholesterol against autoxidation. In order to have autoxidation a free radical process must be initiated by some agent in the presence of air. Autoxidation is slow without an initiation event.

DR. TAYLOR: Well, what happens when the egg rots and the shell is intact? Is there any oxidation that takes place then?

DR. L. SMITH: I do not know. However, rotten eggs appear to involve reductive processes, hydrogen sulfide liberation, etc. and not autoxidations. I have no interest in such matters.

DR. WATSON: But considering that with every reduction you have to have an oxidation, there must be something going on. Right?

DR. L. SMITH: But if there is an overall reducing system, limited in oxygen, cholesterol does not oxidize. Other oxidations (electron removal) may occur.

DR. WATSON: Is it possible, Nick, that Leland could give us a five-minute dissertation on this business of singlet oxygation? I'm always confused on this radical formation.

DR. WERTHESSEN: I think it would be very worthwhile.

DR. WATSON: Well, then, would you accept giving us the five minutes then?

DR. L. SMITH: I am presently battling with the literature trying to prepare a suitable description of these processes for the monograph I am preparing on cholesterol autoxidation. Perhaps the monograph can serve this purpose best.

DR. LEQUESNE: Just a comment. It's generally true, I believe, that the evidence for the involvement of singlet oxygen in a chemical reaction, such as the one with cholesterol, is the stereospecificity of the addition reaction, and this is what you see when you are reacting cholesterol with singlet oxygen and getting the hydroperoxide group going in stereospecifically at the five position and the five-alpha orientation and that's in contrast, in stereochemical contrast, to the observation that with the ordinary radical attack of regular oxygen at the 7 position where you get both the hydroperoxides, and there's mechanistic argument about the exact way in which the singlet oxygen does attack and the exact pathway but the evidence that it is involved comes from the specificity and I think that that is fairly certain.

DR. L. SMITH: For cholesterol and some other substrates, this is correct. However, for some cyclic olefins a migration of the double bond occurs in free radical oxidations.

DR. LEQUESNE: No, no, but the reaction should be stereospecific in the sense that only one stereoisomer is formed. The hydrogen which is sitting below the surface or below the plane of the molecule is abstracted and the new group--the peroxide group that comes in, comes in from the same face of the molecule. And you say that the stereochemistry is transferred from one side to the other and when that takes place, cleanly and unequivocally, then we know it in the steroids and you can demonstrate it if you use deuterium or tritium in the right way in other simpler systems, but the steroids are a beautiful substrate for testing these kind of things and that's some of the best evidence I know for assuming the involvement of singlet oxygen here.

DR. L. SMITH: One really has to examine the proposed reaction mechanism by several arguments in order to be certain. In a general case if there is only one product formed, it may be difficult to recognize the pertinent mechanism unless special methods, kinetics, stereospecific isotopic labeling, etc. be examined.

DR. LEQUESNE: Right, but all I'm saying is that if you do get, say, both hydroperoxides, then it's dollars to doughnuts that it's not a singlet oxygen process going on unless the oxygen can approach from both sides of the molecule in which the double-bond is moving.

DR. L. SMITH: This is just what happens with cholesterol and its minor singlet oxygen oxidation products. An abstraction of the 4β-hydrogen (axial) leads to 3β-hydroxy-cholest-4-ene-6β-hydroperoxide, whereas abstraction of the 4α-hydrogen (equatorial) affords the epimeric 3β-hydroxycholest-4-ene-6α-hydroperoxide. Both are formed in photosensitized oxygenations of cholesterol in which 3β-hydroxy-5α-cholest-6-ene-5α-hydroperoxide is the major product (by far). These 6-hydroxperoxides may not be singlet oxygen products, but they correspond to some of the detailed mechanism requirements of the singlet oxygen reaction with cyclic olefins.

DR. WATSON: **Would you please explain further the basis for the distribution of oxidation products obtained?**

DR. L. SMITH: 25-hydroxycholesterol is a free radical oxidation product too. The B-ring of the cholesterol molecule is the most sensitive to free radical oxidation by air, with attack at the C-7 positions predominating. However, some attack at the tertiary carbon C-25 also occurs. Electron spin resonance spectra for the cholesterol C-7 radical have been recorded, but such spectra for a cholesterol C-25 radical have not been. A similar radical at the C-20 position also forms. Radical attack at the C-17 position must also occur, although we have not found the requisite primary product cholesterol 17-hydroperoxide. We have products of direct autoxidation at the secondary carbon C-24 and at the primary carbon C-26 as well. Thus, primary, secondary, and tertiary carbon atoms are oxidized to the corresponding hydroperoxides, all in free radical oxidations. These reactions occur in crystalline cholesterol, but we have not found similar evidence of such oxidations in systems where cholesterol is dissolved in solvents or dispersed in water. There may be a crystal or solid state effect in formation of these side-chain oxidized derivatives.

DR. WERTHESSEN: May I ask a question now, Leland? If I understood you properly, you said that down there in the seven position is where all the action is?

DR. L. SMITH: Quantitatively, most products are oxidized at the C-7 positions.

DR. WERTHESSEN: All right. Now in these U.S.P. cholesterols, Dr. Merritt has consistently found the 25-hydroxy, not the 25-7-hydroxy. If the action starts at 7, how come you can have 25-hydroxy, by itself?

DR. L. SMITH: Oxidation at the C-7 position appears to occur predominantly, but once initiated, some oxidation at the other sensitive positions does occur by free radical processes, probably involving hydrogen atom abstraction from the C-25 or other positions by peroxy radicals formed from the initially formed products. A detailed explanation of how attack at the C-25 position occurs in the face of more extensive oxidation at the C-7 position cannot be given except speculatively.

DR. WERTHESSEN: Well, how do you know you do?

DR. L. SMITH: By chromatography.

DR. WERTHESSEN: No, no, no. What I mean is what you would have to do, it would seem to me, to prove that an isolate of the 25 hydroxy was actually due to spontaneous oxidation would be to start with the material which absolutely contains none. Have you done that?

DR. L. SMITH: All of our results are based on pure cholesterol being heated in air.

DR. WERTHESSEN: Ah, heated!

DR. L. SMITH: In short-term experiments products are not formed unless cholesterol is heated in air.

DR. WERTHESSEN: Will it happen in the cold?

DR. L. SMITH: Cholesterol stored in a bottle in the cold and in contact with air will slowly autoxidize, and 25-hydroxycholesterol will be a prominent product.

DR. WERTHESSEN: Yes, I know it

DR. L. SMITH: Perhaps as much as ten years may be required for samples kept cold.

DR. WERTHESSEN: Okay, but then if you find it in a tissue that you've handled with care, if it takes ten years in the cold, why do you say it's an error of your extraction procedure?

DR. SMITH: Autoxidations may occur at any temperature given enough time or the presence of unrecognized catalysts, such as iron from hemoglobin, etc. One appears to balance time against temperature; samples warmed or heated autoxidized more rapidly than samples kept cold, but given long enough periods of time, the cold samples will autoxidize some. We attempt to freeze-dry tissues directly or store them in vacuum or under nitrogen in the cold. Extractions are generally conducted under nitrogen in the cold.

million. Now in this case I think we have the work of Dr. Smith and the work of Dr. Merritt who have analyzed oxidation mixtures of cholesterol and this is the foundation for an extension of analytical work. But what we need here is an activity-directed fractionation. And this is the way I would say that the problem of MP4 and food sterols must be approached. I'll add to this, if I may, that once one has obtained the active compounds, if there are a significant number of active substances, then there comes the problem of synthesis. And one must then devise synthetic methods which are appropriate to the scale of material which is required for testing. If Dr. Watson wants so many milligrams and Dr. Imai wants so many milligrams, then one has to develop a synthetic method for these substances which will allow for the production of these quantities, if they are present in small amounts in the natural source. And essentially what I'm doing is encapsulating the methodology which is used in drug development in other areas and applying it to this particular problem. I think, as I say, the foundation is the oxidation studies which have been conducted by Dr. Smith and others over many years and this is the basis for a logical future program.

DR. L. SMITH: We have no funds to support such a program. To do this properly will cost a good bit of money. We are starting such a modest examination of mutagenic sterols using Salmonella typhimurium as a test system, but we cannot entertain other bioassay approaches at this time. Bioassays requiring milligram amounts of pure sterol are already outside of ready access unless larger scale isolations are conducted, which matters unfortunately are very costly.

DR. LEQUESNE: I think the point that you make is good when you say that when you're looking for activity it's not quite the same thing as doing an analysis without an activity direction in mind. I think that one way in which we would approach this would be to look for the active substances and focus in on them for fractionating until the activity rises to a high and constant level and then try to isolate pure substances from these materials. But then one also along the way in our experience isolates other substances which are interesting and which are worth studying in their own right. And one need not necessarily sacrifice the goals of one approach in the pursuit of the other. Although in our opinion the activity direction would give the focus to the effort.

DR. TAYLOR: I just wanted to repeat that as Leland said, money is a big thing and the quantities of the various breakdown products one is going to get are going to be miniscule. I would add that I think Dr. Peng's smooth muscle tissue culture is a good test and it certainly is better than trying to get enough of these compounds to give to a rabbit, a pig, or a cow or some other large animal. I also think that our plan to move into smaller susceptible animals such as the squirrel monkey, the ground squirrel and the prairie dog which are much smaller than a rabbit are other serious steps which should be considered.

DR. WERTHESSEN: Well, I would like to take the Chair's perogative here for a moment and disagree with a couple of the people. It seems to me that what we have here, right at hand, particularly in Dr. Peng's system, is a very quick assay system which can be quantitated out on a log unit dosage with extreme rapidity and done by an expert. And I would suggest, Leland, that if you wanted to get into this that it would far more efficient for you (and I don't want to sound rude, but it's easier said that way) to stay away from the biology and restrict yourself to the chemistry and let Peng do the assays.

DR. L. SMITH: We cannot rely on the usual mail services for such matters, as the loss of one sample sent for testing elsewhere could be a serious matter.

DR. WERTHESSEN: You are quite correct, but there are other means of transporting things from place to place. There's a very simple method and I used it for years and I had collaborators in England and New Orleans. We collected aortae in New Orleans; we worked them up in San Antonio and they were analyzed in London. The airlines are the secret to this. It's a little bit expensive. We had a recent example of this, Leland. Dr. Subramanyam sent three samples out to Dr. Watson by mail and as far as I know they are not there yet. I got fed up and went over to Flying Tigers and used their "special handling, pilot-supervised service" and it was there overnight and it was under guard. You just put it in the pouch. And the best one I pulled that way was when Florey was in our lab with Poole. He wanted an EM preparation of an aorta to be completed by his technician in Oxford. I worked out the schedule. We killed the baboon at the right time; put it in the fixative; his technician was at Heathrow. When he got it back to Oxford, it was time to change the sample. There's no problem here. You can get them from one place to the other and ....

DR. TAYLOR: The only inconvenience is the problem of taking it out to the airport yourself.

DR. WERTHESSEN: That you have to do.

DR. FRANK: One comment. We've had extremely good results with Federal Express or Airborne. They service many cities and they actually pick up by a bonded carrier in their own company and guarantee overnight shipment anywhere in the country. I've sent things in dry ice and had them arrive on the West Coast untouched.

DR. TAYLOR: What time of year?

DR. FRANK: This was done in the springtime.

DR. TAYLOR: You mean the old American Express Company? That was an air express and has gone out of business.

DR. FRANK: This is called Federal Express. It is a new company.

DR. TAYLOR: That used to be a good system, the air express system but...

DR. FRANK: No, I think, Bruce, we're talking about two different things. Federal Express has their own fleet of planes. They run Lear jets and it's all centralized in Memphis. They send everything there and then send it out. We've had extremely good results sending our samples. If they pick it up at my lab by five, it's delivered in San Francisco by eight the next morning...by their courier.

DR. LEQUESNE: I think that again the word that Leland spelled out is the operative word in this case: money. And again what makes me think of this is Dr. Taylor's comment about quantities. Now in a large-scale effort of the sort that would be needed to isolate every active substance and to do this correctly, one does not know how much of the active substance one is going to get. In one of the drugs which is presently being developed for use in the clinic in cancer work, the initial concentration of the

compound was one part in 10^6. The compound is extremely active, but to get one gram, one requires 10,000 kilos of a certain plant which has to be shipped from Africa. Now we're not talking about a rare plant, we're talking about foodstuffs or MP4 or something like this. But one is going to have to start with 50 kg and one is going to have to buy silica gel and one is going to have to fractionate on that scale in order to get a true picture of what's going on here. So I think that time and planning and money are what is required and this is not going to be the sort of effort which one can mount next month.

DR. WERTHESSEN: I'm all too cognizant of the fact that this requires money, but by the same token, I have a rather strong feeling that once we start getting some sound results, funding will increase.

DR. WATSON: The primary question here becomes what is defined as sound results?

DR. WERTHESSEN: Well, I think that one of the first problems that we have to settle is...well go ahead, Elspeth, you usually speak better than I do.

DR. E. SMITH: As I see it, there are a number of different aspects to this problem. The first one was very clearly outlined in terms of whether, in the cholesterol-fed animal model, the atherosclerosis is produced by cholesterol or by the oxidation impurities. I think that that is a very important problem to solve, because at least it may tell us whether the cholesterol-fed model has any validity. The second aspect is: Is this relevant to human atherosclerosis? This is perhaps the more important problem and falls into several parts: Are these compounds present in significant amount in the food that the ordinary chap eats? Are they present in significant amount in the plasma lipids, and, if as apparently they are, they are present in significant amounts in atherosclerotic lesions, did they get there in the first place from the plasma lipids or have they developed there during the cholesterol's sojourn in the artery wall? Then we have yet a third area: the implication that they may be produced during biosynthesis of cholesterol. It appears that the major part of the cholesterol that accumulates in lesions is plasma cholesterol, so that their production during biosynthesis of cholesterol in the artery wall itself is unlikely to be significant. I assume that in human atherosclerosis, it is their cytotoxicity which is likely to be the important factor rather than their regulation of cholesterol metabolism in the artery wall, because my guess is that this plays a fairly minor role in the development of human lesions.

DR. WERTHESSEN: You have expressed very beautifully the horrible thinking that I have gotten into since we got these positive results. Because they make a mess of all our preconceived notions, and I couldn't agree more with what you said. The only difficulty that I see at the moment is that--well, let me put it this way. Let's assume that we could go ahead and take an animal model, give it more than enough cholesterol, and nothing else but cholesterol, and prove that the animal did not become atherosclerotic. We'd have a nice negative result. But fundamentally it's an almost impossible thing to do, isn't it?

DR. L. SMITH: An experiment I would like to see conducted would involve feeding pure cholesterol to test animals under loading and time conditions known to lead to atherosclerosis under less controlled conditions using impure cholesterol At the same time control experiments involving feeding of mother liquor (autoxidation) products and/or

individual oxidized sterols, etc. and the standard U.S.P. cholesterol, etc. should be conducted. A differential response to these insults may be expected. One might predict that the mother liquor materials may induce atherosclerosis more rapidly, more extensively, or at a lower level of feeding, etc. The samples used should be fed the animals under conditions where the stability of each preparation against air oxidation or other alterations is secured. A convincing statistically significant experiment seems overdue.

DR. TAYLOR: I mentioned the first day that that is what should be done. I think that we should make a circulating lipoprotein milieu with some low-density lipoproteins which are all pure cholesterol, and then damage the arteries with some of the oxidation products. That's step number one in atherosclerosis as far as I'm concerned. Step number two in the study should be to have a lot of lipids of dietary origin (which would contain auto-oxidation products) that are in the low-density category which are probably ready to fall out of solution. This would be particularly striking when you have a repairing arterial wall (damaged by auto-oxidation products of cholesterol).

DR. WERTHESSEN: Well, in good part the experiment that Leland requested has been done. And I refer to the November paper where equal doses of the oxidation product in purified cholesterol were given.

DR. TAYLOR: But those animals didn't have elevated circulating lipoproteins. They didn't have any. They had almost no low-density lipoproteins.

DR. WERTHESSEN: What would you think then if you repeated that experiment and induced hypercholesterolemia by endogenous means?

DR. TAYLOR: That's why I mentioned that I thought that chickens that we have would be a good animal or use distylbesterol. Then you'd have endogenous cholesterol, which according to Dr. Leland Smith doesn't have any oxidized sterols in it. (Smith et al., 1967.)

DR. WERTHESSEN: You see the thing that I'm concerned about is, if you want to do this experiment properly, you'd have to use 10 rabbits at least on each side. Feed them a gram of purified cholesterol a day each and do it for at least four months. And that turns out to be a horrendous job. Did you want to say something, Charlie?

DR. MERRITT: I was just wondering in connection with the problem of feeding so-called pure cholesterol, everybody seems to be pretty well agreed that it's difficult to get cholesterol free of the oxides. At least you can prepare it, but you can't maintain it. I just wondered if there's any objection to feeding it in conjunction with an anti-oxidant? I mean if you make a clean preparation of the cholesterol and then protect it from further oxidation by adding alpha tocopherol or something to it and then use that as your pure cholesterol standard and then go on from there by adding oxides to it if you wanted to test the effects of oxides.

DR. WERTHESSEN: The only difficulty would be you've added 20 more rabbits to the experiment.

DR. WATSON: I'd like to respond to that, Nick. It seems to me, considering that the last 40 years have probably consumed millions of dollars trying to get at this question of atherosclerosis, 20 or 30 more rabbits for the experiment are a small matter--and whatever is entailed with 20 more rabbits--more cholesterol, etc. It seems if you're going to do it right, you've got to do it.

DR. WERTHESSEN: I'm not arguing. It's just a question of feasibility in anyone's laboratory.

DR. WATSON: Do the people up in Albany have good rabbit hutches?

DR. WERTHESSEN: Yes, but to do this one to perfection would take seven days a week with 20 animals by gastric tubing and that's one tremendous job.

DR. FRANK: As a corollary to some of this, we ought to consider also quantifying the transport of cholesterol and its products across the gastro-intestinal mucosa. Its ability to pick up in the serum and stay in the serum, as well as the storage within the mucosa, I think are things that should be evaluated in conjunction with our animal model.

DR. WERTHESSEN: The coffee is present if you want to take a break.

COFFEE BREAK

DR. WERTHESSEN: One of the problems in a discussion of this sort is that you forget to cite critical data until after it's over--the particular discussion is over. One of the things we were discussing at great length, as my colleague on the right here pointed out, the experiment ought to be done. But the point at issue is, the experiment of feeding pure cholesterol has been done. It was done as well as I think any one of us could do it without intubation. And that is the experiment that I cited that was done by Erwin Schwenck. He purified the cholesterol daily. But the lab technicians were not competent to do it in the manner that Imai did it. Dr. Leland Smith, I think, is the only man here who knew Erwin Schwenck.

DR. L. SMITH: If I recall the paper, it was in the Proceedings of the Society for Experimental Biology. (102, 42 (1959).)

DR. WERTHESSEN: Right.

DR. L. SMITH: He purified the cholesterol by a different method, through oxalic acid complexation in which he was interested at the time. There is a queston of just what purity of cholesterol he did in fact use at that time or whether adventitious impurities from the procedures used may have influenced results. Today we would want to have cholesterol purified by the bromination and debromination procedures but recrystallized repeatedly and demonstrated pure by a variety of reliable chromatographic procedures as well as by melting point.

DR. WERTHESSEN: Well if I recall it properly, the cholesterol that was administered to the animals was dibrominated, prior to giving it to them. At the time, you most certainly recall, that was considered the best you could get.

DR. SHEPPARD: It seems to me that if you're going to do feeding studies and you want to maintain the integrity of the cholesterol in the pure form, it's going to be almost a hand operation and the diet has got to be mixed under anaerobic conditions at the time it is given. But this is going to be a labor-consuming, very deeply detailed process.

DR. WERTHESSEN: Right. And about 15 to 20 animals on a side.

DR. SHEPPARD: Oh, yes, and I think that you'll have to run a pilot study. Then once you do that, you're going to have to go back and do your statistical feasibility and decide what size population you've got to have. Otherwise, develop your fiducial limits and...

DR. WERTHESSEN: I personally think it would be a superb problem for the FDA to handle.

DR. SHEPPARD: Well....

DR. WERTHESSEN: You've got the manpower.

DR. SHEPPARD: No, I've only got seven people. And I also run an operation in Cincinnati. It is a greenhouse operation in a very interesting nutritional area. We produce the plant materials and then bring them in and feed them to the animals. This is in the mineral area. But, unfortunately, ever since the advent of the FDA in 1907 in the old Department of Chemistry of Agriculture, they have hung a badge on us, so to speak, and this means you are primarily concerned with protecting the public and this, by necessity, means a lot of effort devoted to methodology. This rather narrows the approach to research. During the past 20 years there has been a gradual realization that we've got to do more research than just methods. So I'm getting ready to study the movement of the trans-fatty acids across the placenta. I've told them that I'm developing methods for biological tissue. Well, true, I'll have a method for it, but I'm really finding out what I want to know.

DR. WATSON: I'd like to ask Leland a chemical question. I was thinking about this problem a little bit last night of the stability of cholesterol. Is their intrinsic stability of cholesterol esters as opposed to free cholesterol in terms of auto-oxidation such that if you once went through the dibromination step etc. and had the cholesterol ester of some sort, would that be any intrinsic, more stable compound?

DR. L. SMITH: A systematic study has not been recorded, but impression is gained that cholesterol esters are more stable. Certainly cholesterol esters (saturated fatty acids) in bottles on the shelf do not develop the odor of autoxidized cholesterol nor are peroxidic impurities readily detected. However, cholesterol esters do autoxidize under controlled conditions, and the unsaturated esters such as linoleate are quite unstable. I do not recall feeding experiments involving cholesterol esters.

DR. SHEPPARD: I think also, Leland, what you say is true, but I think it also determines what is very important as to what happens in metabolism as to what the cholesterol is esterified to. Because this starts influencing the pathways. If it's to a simple phospholipid, for instance, this would change the ballgame.

DR. L. SMITH: Sterol esterases of the upper intestine supposedly hydrolyze dietary sterol esters before they are absorbed. As I recall dietary cholesterol esters are not absorbed by the intestinal mucosal cell as such.

DR. SHEPPARD: Yes, but on the other hand, if you use Imai's technique where you inject, you bypass that system.

DR. L. SMITH: Injection into the vein would be another matter.

DR. SHEPPARD: Well, you bypass that and that could influence the test considerably.

DR. WERTHESSEN: On the other hand for a feeding experiment it might be ideal.

DR. MERRITT: I'd like to ask Leland a question about the stability of the cholesterol esters. If you describe their deterioration in terms of color and smell, but if the oxidation were to occur only, for example, in the formation of a 25-hydroxy or some other first step, this wouldn't, probably, be detectable by odor or color. Is that so? In other words you could have 25-hydroxycholesterol acetate and it wouldn't be colored and it wouldn't have an odor.

DR. L. SMITH: Shelf stability of a sample in a bottle may be quite good. The auto-oxidation of cholesterol esters still may occur in the B-ring C-7 position in enzyme or chemical systems. Once free radical autoxidation occurs at the C-7 position, side-chain oxidations may follow. The odor of autoxidized cholesterol appears to come from the side-chain fragments which are volatile.

DR. SHEPPARD: Well, do you think those are peroxides of some form--part of them?

DR. L. SMITH: The odor is probably from C_1-C_8 aliphatic alcohols, ketones, aldehydes, olefins, and hydrocarbons formed by side-chain scissions of initially formed side-chain oxidized sterol hydroperoxides.

DR. SHEPPARD: In other words you think the thing is being decomposed down to many more fragments then.

DR. L. SMITH: Yes, we know that. We have isolated a dozen or so of these aliphatic compounds, including ethanol, acetic acid, acetone, etc. up to C_6-olefins. These components are easily recovered from air-aged cholesterol by trapping them in a cold trap with the sample under vacuum.

DR. SHEPPARD: Are the A, B, C, and D rings still intact?

DR. L. SMITH: I misunderstood.

DR. SHEPPARD: The ring structure.

DR. MERRITT: What he's saying, if it starts, does it have to go all the way?

DR. L. SMITH: In analysis of the volatiles, the C_2-C_6 components, one has no certain idea where they come from except by deduction based on their structure. Most probably they come from the side-chain, but acetic acid or ethanol could derive from many other sites as could acetone. It would be less likely to obtain a branched chain C_6 olefin from other places.

DR. SHEPPARD: Yes, because I would think if the basic four rings were still intact with the hydroxyl at the 3 position, it should give a digitonin precipitate. I don't think that would be dependent on the sidechain.

DR. L. SMITH: I would not recommend the use of digitonin for this work.

DR. SHEPPARD: Well, I can understand that. But I was just wondering whether all the action was strictly on the sidechain in this case or whether it also affected the ring structure.

DR. L. SMITH: It is unlikely that oxidations at the C-7 position and also in the side-chain would occur in one cholesterol molecule, although under special conditions both oxidations might be accomplished. There are many unidentified more highly oxidized sterol derivatives in the brown goo encountered in these studies after the known sterols are removed.

DR. SHEPPARD: One reason I'm asking all these questions is because we're quite concerned since we're driving toward a more fabricated food society. There's always the possibility that during extensive processing used producing highly fabricated foods that the oxidation products of cholesterol could be formed.

DR. FRANK: Do you think it might be appropriate at this time for me to take a minute or two and describe some of the techniques for looking at gastro-intestinal absorption?

DR. WERTHESSEN: You've got only one other competitor. Do you want to do those slides? Bruce has got some slides he wants to show and let's have your material after that, all right?

DR. TAYLOR: I think we have to get back to the target organ now and then, so I'll give you a little bit of target organ talk. (Economou et al., 1960). The reason we did this is because at one time they were doing renal angiograms by injecting Urokon (70% acetrizoate) through the abdominal aorta and sometimes the operator didn't get the needle all the way through the aortic wall; they wound up with an injection of Urokon in the middle of the aortic media. This causes a slow indolent necrosis and patients developed permanent aortic aneurysms after this misinjection of Urokon into the medial portion of the aorta. Figure 18--this Urokon caused a necrosis with hemorrhage with very little inflammatory reaction, but there was a permanent aneurysm. We decided to explore this further and tried several approaches for damaging the aorta. Figure 19 demonstrates a method for transmural damage--in (A) is shown a carbon dioxide freezing device which causes transmural freezing of the whole aorta and at (B) you have an instant aneurysm since you've killed every cell in the media. The only surviving cell is the endothelium and in three weeks (as shown at D) you have a lot of proliferation of intimal multipotential cells. These cells have a capacity to divide and fill in defects to compensate for weak areas in arterial walls due to medial cell death. At D you get correction of the aneurysm at about six weeks. It goes on to make elastic tissue, collagen, and a new internal elastic membrane, and the aneurysm is corrected. As shown in Figure 19, if one opens the aorta and uses a cork-borer (1/2 inch diameter) to remove a piece of the endothelium and some of the inner media (Fig. 20A), including the internal elastic membrane (Fig. 20B), one stirs up enough reaction of these multipotential cells in the subendothelial space so that it will proliferate as shown in Figure 20C. This will take place in three weeks and in three months you have total repair of the defect you created (Fig. 20D). This, I think, is very interesting; if you attack the vessel from the outside and remove the outer half of the media or the outer two-thirds of it surgically (Figs. 21A, B, and C) one gets an aneurysm (Fig. 21D). Since one is not disturbing the subendothelial multipotential cells nor the inner elastic membrane, one has a permanent aneurysm. At autopsy you can examine a dog 30 weeks later, and he still has a persistent aneurysm. There seems to be something very special and magic about the area between the endothelium and the internal elastic membrane (Fig. 22). This is a group of three persistent aneurysms 30 weeks after they were produced by removing the outer half to two-thirds of the media. This is an open aorta with three persistent aneurysms that didn't repair at all. Now if you want to overcome this persistent aneurysm, you can use this technic as is shown in Figure 23A and B; you can freeze half of your persist-

D . Taylor's Figure 18

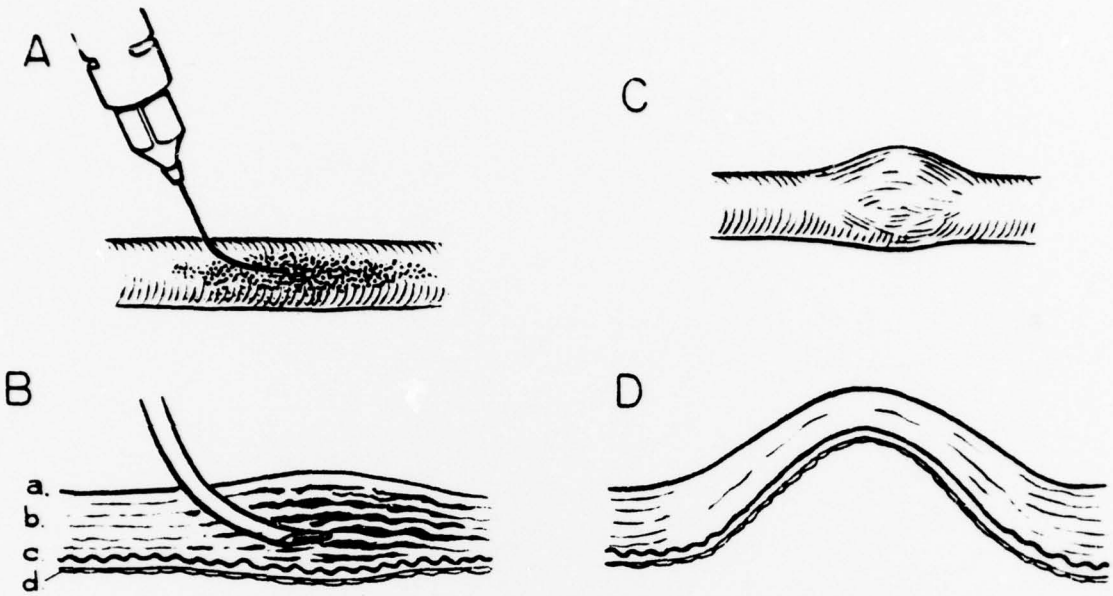


Figure 18

Diagrammatic illustration of method of intramural injection of 70 percent acetrizoate. A: a 45 degree curve in the needle makes it easier to maintain the orifice of the needle in an intramural position. B: the contrast material dissects laterally and separates the elastic lamellae. a: adventitia; b: media; c: internal elastic membrane; d: endothelium. C: aneurysmal dilatation observed as early as three weeks after intramural injection of the contrast material. D: microscopically there is destruction of the central portion of the media. There is no apparent injury to the internal elastic membrane and subendothelial layer. No subendothelial reparative scar was noted as long as 30 weeks after intramural injection of acetrizoate.

D. Taylor's Figure 19

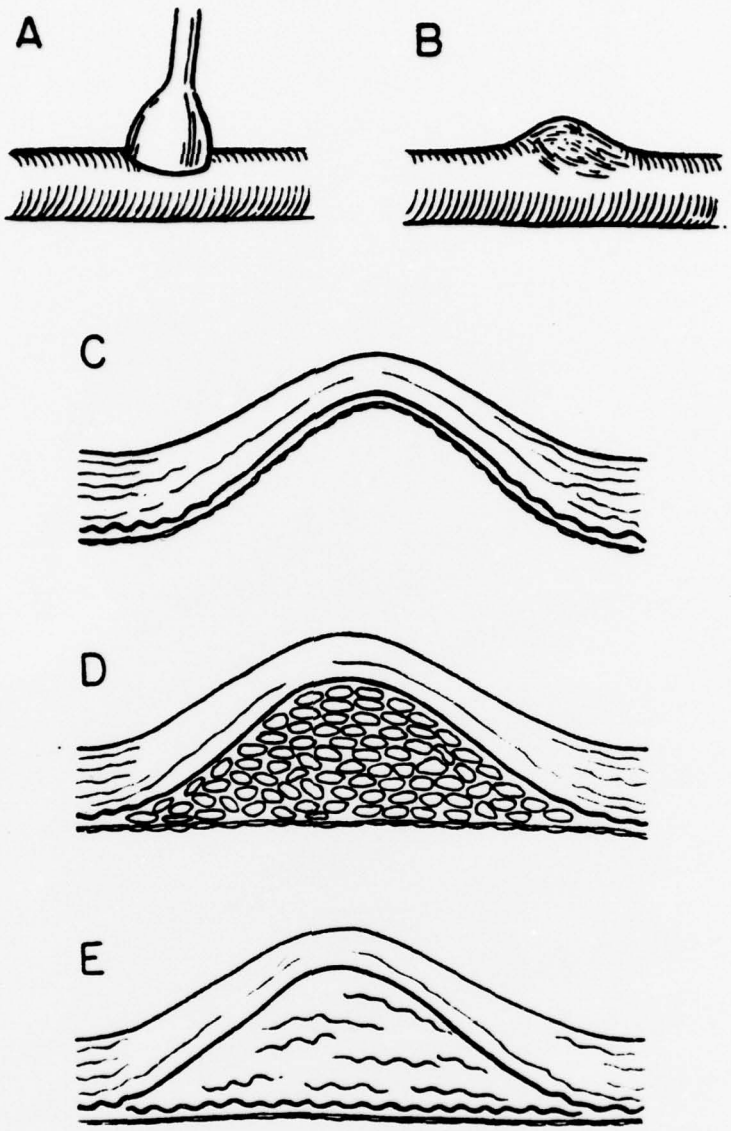


Figure 19

Diagrammatic illustrations of effect of transmural freezing (A) on arterial tissue. Immediately after freezing aneurysm develops at site of freezing (B) and persists for several weeks. C illustrates straightening and disruption of elastic lamellae and dissolution of killed cells in area frozen. During the first two weeks there was no proliferation of multipotential subendothelial cells (C); however, during the third week these cells proliferated abundantly and essentially filled the aneurysmal defect (D). The multipotential cells differentiated and formed an essentially new vascular wall at about the sixth week (E). After six weeks, mature cells and new elastic and collagen fibers have restored the artery to its original caliber and repaired the aneurysm.

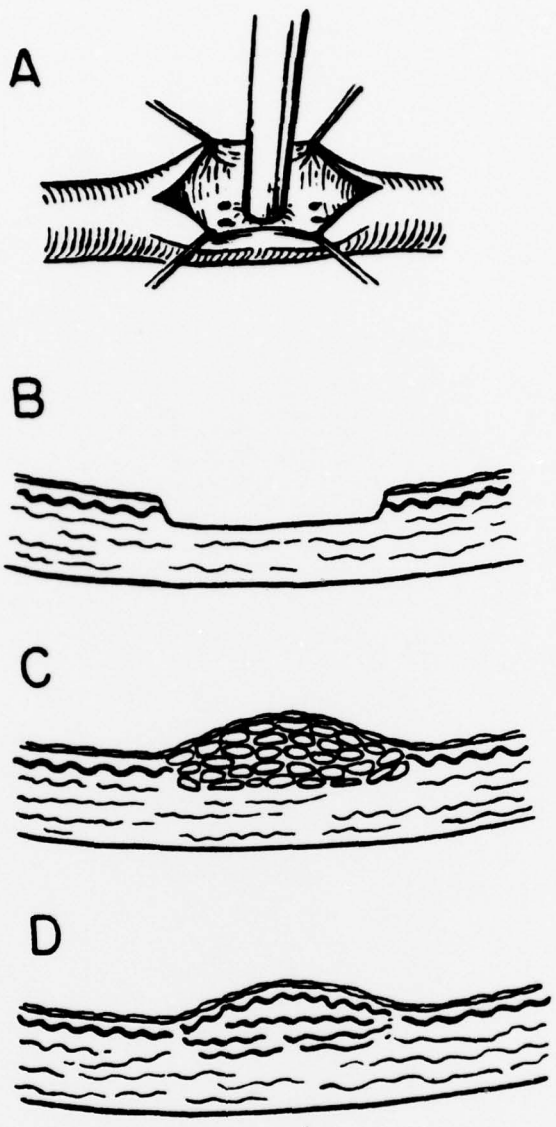


Figure 20

Diagrammatic illustration of response of aorta to stripping of endothelium and subjacent internal elastic lamella. A shallow circular cut is made by gently rotating a 1/2 inch diameter cork borer (A). Endothelium, subendothelium, and internal elastic membrane are then dissected away leaving shallow circular defect (B). As shown in C at about 3 weeks, a thick tuft of subendothelial cells and a thin layer of endothelial cells cover the defect. At about 6 weeks the intimal scar covering the small area of injury has differentiated into mature vascular scar tissue with elastic and collagen fibers (D).

Dr. Taylor's Figure 21

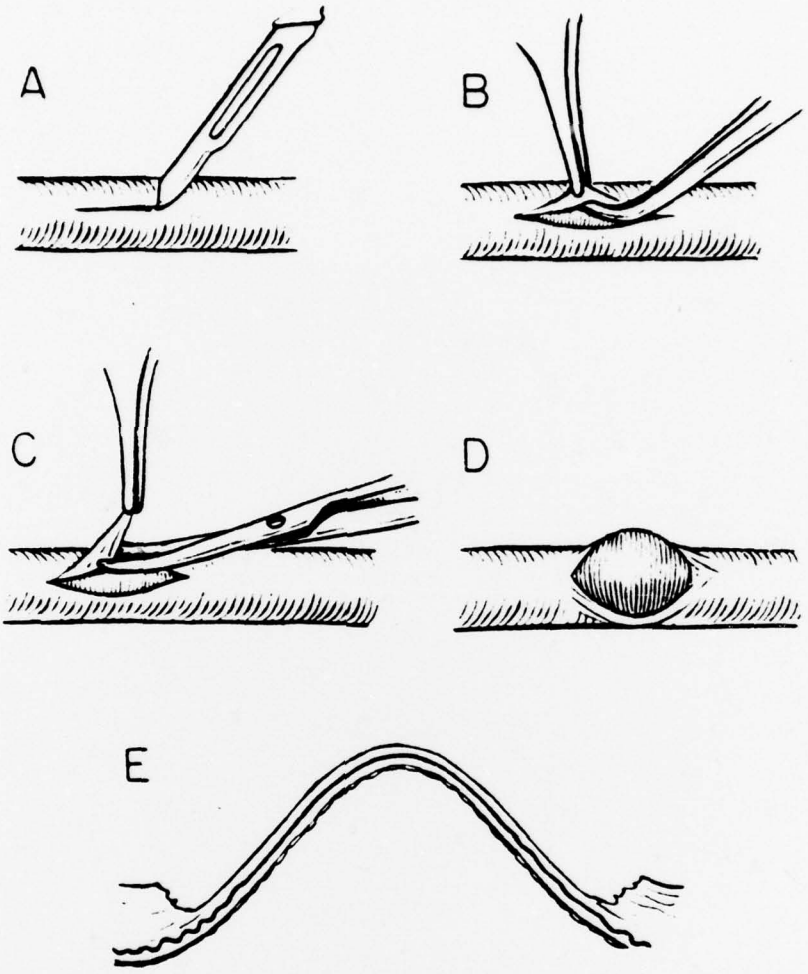


Figure 21

Diagrammatic presentation of formation of aneurysm by stripping of outer 70 percent of the media. A: with gentle strokes of the scalpel the desired depth of dissection is reached. B and C: at the depth of the incision the media could be peeled along a cleavage plane. An elipse of the dissected media is excised, allowing full development of the aneurysm (D). With removal of more than 70 percent of the media, acute rupture of the resultant aneurysm occurred. E: aneurysmal formation showing stretching of internal elastic membrane. Even though 70 percent of the media had been removed without injury to the subendothelial space and internal elastic membrane, there was no reparative response.

D Taylor's Figure 22

Figure 22

Photograph of three persistent aneurysms in abdominal aorta, 30 weeks after outer half of media had been surgically excised as diagrammatically shown in Figure 21. Central aneurysm has a smaller diameter than the two aneurysms shown at two ends of specimen. Without disruption of subendothelium and underlying tissues including internal elastic membrane, there was no stimulation for repair of these aneurysms.

D. Taylor's Figure 23

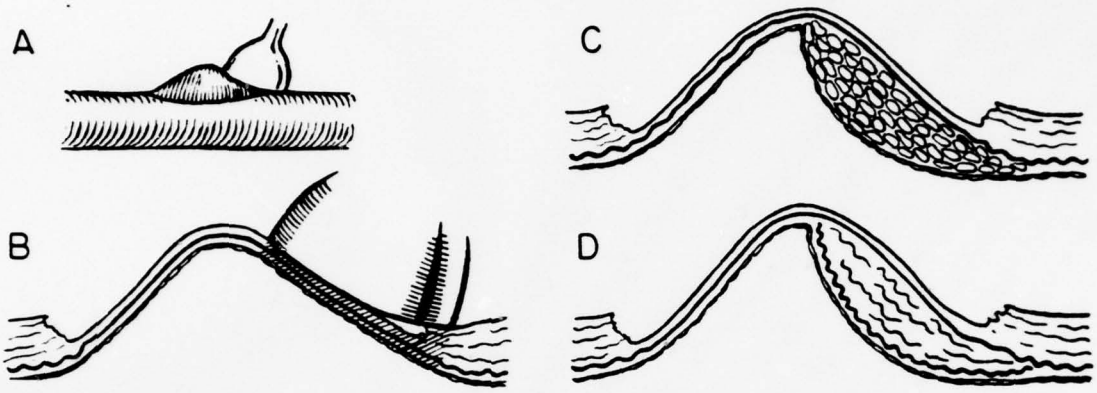


Figure 23

A and B: transmural freezing of one-half of aneurysm which had been produced by excision of 70 percent of outer media. The other half was left undisturbed. C: whereas there was no reparative process in the portion of the aneurysm which was left undisturbed, there was brisk proliferation of subendothelial cells in the portion of the aneurysm that had been transmurally frozen. D: the multipotential subendothelial cells have differentiated and formed an essentially new arterial wall in a thickened intima at the site of transmural freezing.

ent aneurysm and cause death of the subendothelial multipotential cells and other tissue near the internal elastic membrane, then one gets repair as shown in Figure 23C and D. Figure 24 shows the result of freezing two-thirds of an aneurysm produced by excision of the outer half of the media. We removed the outer media from near each margin of the illustration, then we transmurally froze the right two-thirds of the artery; this intimal scar has formed only on the side where we damaged the multipotential cells and subendothelium.

I promised Dr. Sheppard that I had a few things for him. He's with the FDA. One year just before Thanksgiving, as I recall, there was the cranberry scare and the activities that made national headlines practically ruined Massachusetts and the cranberry bogs. We call that one of the first ones, don't we Dr. Sheppard? It was supposed to poison everybody.

DR. SHEPPARD: I have a rebuttal to that.

DR. TAYLOR: Well, let me finish. Then we had the mercury scare. This is a factor here and it's got effluents which contained mercury. There was supposed to have been a lot of mercury in tuna fish. Manufacturers of tuna fish aren't as stupid as you think they are. They kept cans from the day they first canned tuna fish and they analyzed the tuna fish that was 50 years old. It had the same mercury as it has now. I work on the same floor with a man who got an old tuna out of a museum and it was 50 years old and it had the same amount of mercury as the present day tuna fish.

Then there's the famous Red Dye No. 2. I submit that these are peanuts compared to what we should be concerned about which are the breakdown products of sterols. The sources of them, as has been mentioned, are powdered eggs and powdered milk. We found it in "Slim Jims;" you found it in "Custard Jello." So I think there needs to be a shift in direction of enthusiasm.

DR. WERTHESSEN: The reason I wanted to have Bruce go through this is that it appeared to me that a repetition of this injury experiment of his--but instead of feeding cholesterol, use it as a control, and see what effect these oxidation products would have on the regenerating tissue--might be of acute interest. And here you could probably get away with fewer animals and perhaps use an injection route.

On the possibility of getting some of the things into proper solution, I'd like to have some words from Dr. Frank. That was what Dr. Soloway promised me. Did you see my point there, Dr. Imai?

DR. FRANK: I'd just like to take a few moments and reflect on a few things that I've mentioned and that others have mentioned. Getting to the problem of presenting the rather insoluble derivatives to the body in a fashion which might have some biological relevance and also the testing of these for intestinal absorption. Now let me start with the latter.

With regard to quantifying the absorption of drugs from the gastro-intestinal tract, we commonly employ several techniques. There are two techniques described on the slide here. The first of these is the everted gut technique. Now this can be done very easily by simply taking a section of intestine, usually from a rat or whatever animal of your choice, and then the intestine is preserved in viable condition and simply everted and attached to a glass tube. It's tied off at one end and solution of whatever material you're interested in is placed in the external compart-

[. Taylor's Figure 24

Figure 24

Photomicrograph of Weigert's elastic tissue stain of lesion produced by excision of outer media. Cut ends of outer elastic lamellae are visible on the lower surface of the aorta near each lateral margin of the photograph. After excision of the outer media, two-thirds of the aorta on the right was transmurally frozen; two months later this right-hand portion of the aneurysm showed a thick intimal scar containing a rich elastic network. The aneurysmal dilatation on this side of the aneurysm was markedly corrected by this thick intimal scar. Technics employed and reaction of frozen portion of aortic defect are diagrammatically presented in Figure 23.

ment on the internal compartment sample. Now it's a very simple technique and as long as the section of gut remains viable, which it usually does for extended periods of time under these conditions, we can measure in a quantitative sense the transport of mucosa serosal for a particular drug substance. Now this has some additional advantages to the studies that I might project and that is the interest I've expressed at several times when you've told the mucosal stories because obviously the mucosa in this particular in vitro situation can be assayed, histological examples taken, and appropriate information derived therefrom. I have indicated a thickness here which has a lumen. This is really a sack; it's the intestines hanging vertically off of the tube much like a dialysis bag would do, and this is essentially the same type of technique. Now an alternative to that, and I unfortunately don't have a sufficiently good example to illustrate to you, is a regional ligation technique which we also use. In this technique we simply tie off a portion of the gut and cannulate the gut and then perfuse in a slowly washing-type fashion from two syringes attached to the cannula. I've indicated two types of implantation of the cannula there. The lines here simply indicate the tie-off points. And from this information we can also do the same sort of thing except we have to have an assay. And the assay can either be total gut contents, which you can extract from the cannula or else simply, if you're working with a larger animal, tap the ear vein of the rabbit for example, and do blood-level studies. So that this is in the intact animal and the everted gut is in the in vitro situation. There are a number of other tests and examples that could be used, but I think that these sorts of simple studies here, if we can come with a suitable solubilized and presentable form of the oxygenated cholesterols and cholesterol itself, which really hasn't been documented to any great extent with regard to its quantitative absorption, although there have been thorough studies directed at the mechanism by which it goes, could be useful.

Now I would like to bring up the second consideration and that is the question of solubilized cholesterol and cholesterol derivatives and I think that it might be possible to prepare the oxygenated cholesterols and cholesterol itself into suitable forms for various types of administration. You know the kinds of administration that are ideal would be, for example, intravenous administration which would be a very useful presentation. And we could also consider IP (intraperitoneal) or implantation techniques.

Now looking at just three different types of techniques that can be used, I'll discuss the liposome very, very briefly. I'm sure you're all familiar with it, but I'll just review it for a moment because it has some additional virtues that I'd like to elaborate on. Microemulsions are very highly concentrated, fine particulate, fine droplet systems and could be conveniently prepared. They are particularly useful for highly hydrophobic materials, as is micellar solubilization. The solubilization of the cholesterol derivatives in a micellar form would present sufficiently small particles--I say particles, but fluid droplets--which could be used intravenously and pass the capillaries and perhaps not present some of the problems that I might feel would happen on the administration of solid material. Now looking at the liposome as an example, in my crude artistic attempt here I have illustrated the classic liposome. The kind that we are most enamored by at the present time are what we call the "Love" liposomes, the unilamella liposome in this particular case. Unilamellar liposome. This particular liposome has a rather large structure and what I've indicated here is the pairing of the phospholipid groups around an aqueous core. The way I view this type of structure in the interests we have in a common share here is

that the liposome could be used as a delivery system to be injected intravenously and, in this particular case, I'll just simply insert in here one of our "S" components such as 25-hydroxycholesterol. Now cholesterol forms mixed liposomes with the various phospholipids and we do this routinely in the laboratory. There have been in the literature numerous studies of the permeation of the liposome as a result of the presence of strangers within the phospholipid matrix. I needn't go into those things, but there have been numbers of studies, for example, of calcium ion permeability and so on. Now I propose a liposome as a mechanism, both to administer the cholesterol derivatives; that's on the one hand. In other words, very small liposomes of the dimensions of red blood cells can be prepared conveniently. The systems are virtually transparent because of the small size of these particles and that they be prepared from a mixture of phospholipid and cholesterol or cholesterol derivative--the 25-hydroxy may be an example and an epoxide or whatever we have--and that that actually be a component of this system and that that be injected intravenously. Now in an essence it becomes a model for a lipoprotein and, by circulating through the bloodstream, provides the source in a soluble fashion and an easily-transportable fashion of the compounds of our interest. These structures as usually presented are scavenged by the liver and spleen and for hepatic studies, of course, they offer some very interesting observations.

Now there is a problem. The uptake by the liver and spleen is not very, very rapid, but it does occur with a degree of tenacity and it might be necessary to perfuse the animal over a period of time with liposomal preparations. This can be done by several methods--I was going to say convenient, but I'm not absolutely sure this is entirely true, but it's certainly feasible. Liposomes can be injected intravenously, periodically. That would be one thing. But Alza Corporation, of course, has an intravenous pumping device which is made for wearing on the arm, for example. It's a small device which has an air cartidge which has a compressed air cylinder in there. The idea with this is for continuous infusion of anti-cancer drugs particularly over long periods of time. Patients can be connected and walk around and go back to their normal daily tasks and receive a constant intravenous infusion of the material. I propose that animal models could be similarly constructed where the animal has strapped to its back or some convenient place one of these small cartridge-filled devices appropriately tunnelled under the skin with an intravenous line. By simply changing the cartridges at 12, 24, and 48 hour intervals, one can provide a continuous and long-term infusion of a suitable system containing the oxygenated cholesterol. Another variation on the theme might be to use an osmotic pump device which is implanted intraperitoneally. The Alza device, which is an osmotic pump--it is a small unit about the size of a large pharmaceutical capsule, perhaps a little bit bigger--could simply be filled with this device and then implanted IP under the tissues for constant release of the material over a long period of time. So this, in essence, is a way of presenting and solubilizing it in a nutshell. I probably could talk an awfully long time about it and I certainly don't want to at this particular time, but we'll develop these concepts and present them for appropriate review.

I might add, before I finish this, an additional advantage to liposomal models, and that is throughout our discussions I've been intrigued by the fact that the words "injury" and "cell death" and so on have been used in discussion. To me an injury and a cell death are related, but with perhaps different results. I wonder about acute and chronic administration of such things as 25-hydroxycholesterol or other compounds. The liposomal model might offer us an in vitro situation where we could take a look at the effects of these compounds on the integrity of the cell wall and if, for example, mucosal storage is a problem, or transport is peculiar, or we see some interest-

ing results--even if that doesn't occur--we'd like to know what happens with the cell wall. The biolayer structure of phospholipid with interdigitated compounds--well let's say 25-hydroxy--could provide us, if we measure permeability of a marker across this interface, useful information on the fate of the membrane strictly in an in vitro situation obviously. And that by altering, for example, using cholesterol, 25-ydroxycholesterol, epoxide or whatever we care to in a series of compounds of interest to us, try to quantify and study the effects of these compounds on the integrity of the biolayer structure. I think we might learn some interesting aspects about this and have some ramifications to use in understanding how these compounds enter the cell and whatever particular damage they happen to produce therein.

DR. WATSON: I think the liposome model is a very viable model with some sterols. It's been shown by Brooks, Doffer, and Green and others that a number of oxygenated sterols will not form stable liposomes if they are used purely with the phospholipids. Their results were of such a nature that to maintain a sterol or phospholipid ratio that was meaningful and to get viable liposomes they had to always have cholesterol in for some sterols, Sometimes 80% of the sterol content was cholesterol, so you never got an opportunity to do the clean experiment where you had a liposome that was just phospholipid and the 25--or phospholipid and epoxide-- which made it difficult.

DR. FRANK: We have made liposomes with pure cholesterol. I haven't attempted to make them yet with the oxygenated derivatives. That could be so. He's saying the liposomes won't form, but there are a lot of liposomes that won't form. In many ways it depends upon the way you treat these systems. We found, for example, that by varying the amount of cholesterol in the system, we can get a liposome by one preparation technique and not by another. For example, the conversion I have here of multilamella. Initially one forms the liposomal preparation by hydrating a phospholipid mixture with whatever other materials that you can introduce into the system. One can produce what are called macrovesicles that are very large floppy vesicles of several layers or you can produce multilayer vesicles--those are the easiest to form--or unimolecular--unilamellar--it's sometimes very difficult to go from a multilayer system to the simple bilayer system and that may be where the problem was created in handling the systems. I'd take a look at how they handled the systems and what they attempted to do before we could isolate out.

DR. WERTHESSEN: Well I've got an obvious question. Would you be in a position to try some of these things?

DR. FRANK: Sure. I've got people making liposomes right now.

DR. SUBRAMANYAM: I have just one question. There are some studies made on the release of compounds when they use the time-release methods. Are there any studies done in this and how easy are these sterols released from the liposomes?

DR. FRANK: How easily are the sterols released? Well there are two mechanisms by which we can release. There is the possibility--well, there's no possibility--but

there is the system of equilibrium set up between the material in the liposome and circulation within the serum. And this involves some breakdown of liposome structure and loss of the compound as the circulation is in serum. Most of the material which is lost, we're reflecting on the ultimate fate of the liposome, is by its scavenging in the liver and spleen where it's phagocytized and the lysosomes eventually diperse the lipids and release the contents. Now there have been some studies done--in fact this is a direction that we're heading in one of our projects, and that is to look at intramuscular injection of liposomes. In this particular case we believe that there is an actual perfusional release of the contents of liposomes using liposomes simply as a carrier. Now there's no reason why this shouldn't happen in the bloodstream, and I think that there's no evidence to support or deny. It's a current suspect. Within the muscular tissue there is evidence to indicate a diffusional structure that is essentially "capsulation--encapsulation" and that a diffusional equilibrium does exist and that the liposome itself in a muscular environment can persist for long periods of time since that tissue doesn't phagocytize it. The liposome releases its contents by diffusional process. This would be ideal--absolutely ideal for the kinds of considerations we are looking for. I mean us here and also our own interests.

DR. WERTHESSEN: Do we have any further comment? If not, I'm pretty sure that the sherry is ready. Thank you much.

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