HISTOLOGY AND HISTOCHEMISTRY OF ATHEROSCLEROTIC LESIONS IN THE PIT FOWL HEN VASCULAR SYSTEM

by

Peter F. Gerity

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APPROVED:

H. R. Steeves, III, Chairman

R. A. Paterson

R. G. Saacke

P. B. Siegel

D. G. Benson, Jr.

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Blacksburg, Virginia

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INTRODUCTION

The occurrence of spontaneous atherosclerosis in White Leghorn chickens was first reported by Dauber (1944) and Horlick et al. (1948). Dauber demonstrated that the frequency of spontaneous atherosclerosis becomes progressively higher after 5 months of age. Weiss (1957) indicated that White Leghorn stock from a closed breeding flock, exhibited increased incidence of atherosclerosis in females up to 5 years of age.

One of the major barriers to the study of spontaneous, or naturally occurring atherosclerotic lesions, has been the difficulty in obtaining a species which exhibits an adequate frequency of spontaneous plaque development. Krista et al. (1961) and Pritchard (1958) implicate the sporadic nature of spontaneous plaque formation coupled with a lack of preliminary symptoms as factors contributing to the difficulty of the study of this problem. Gerity and Steeves (1970), however, demonstrated an incidence of iliac complex plaque formation as high as 75% in Pit Fowl hens 4 years of age. Pit Fowl, thus, exhibit a sufficiently high incidence of spontaneous

plaque formation to render them ideal for histologic and histochemical investigation.

Dramatic differences between naturally occurring and induced plaques have been demonstrated by Weiss (1959) and confirmed by Gerity and Steeves (1970). Since the plaques which exist in Pit Fowl are strikingly similar to those found in man, it would be extremely beneficial to examine the etiology of these spontaneous lesions in Pit Fowl.

Many factors are cited by Krista et al. (1965, 1969b) as contributory to the etiology of atherosclerotic lesions: (1) strain, (2) blood pressure, (3) dietary energy levels, (4) infectious disease, (5) growth rate, (6) medication, (7) crowding, (8) temperature, (9) toxic substances, and (10) various stress factors. Weiss (1959) further indicated that a concomitant increase in total plasma cholesterol was not necessarily associated with the observed frequency of atherosclerosis.

Earlier dietary and hormonal studies involving induced lesions have, in most cases, focused their attention toward dietary energy levels, lipid supplementation of the diet, and genetic selection as a basis for investigation. (For a more

complete review of induced plaque literature, see Gerity and Steeves, 1970). A primary histologic and histochemical investigation of spontaneous lesions in Pit Fowl was, however, not initiated until 1970 (Gerity and Steeves).

In that preliminary histochemical investigation, alterations in chondroitin sulfate matrices present in the Pit Fowl hen tunica media, degeneration of muscle tissue, fragmentation of the elastic lamina and elastica interna were found. Coupled with this, additional alterations in protein-polysaccharides were noted in the tunica media of atherosclerotic vessels. Kresse and Buddecke (1969) reported similar findings in the aortic complex of beef cattle.

There seems to be increasing evidence, therefore, which indicates that modifications in the chondroitin sulfate complexes, protein-polysaccharides, and elaboration of these compounds, are contributory to the etiological advancement of spontaneous atherosclerotic lesions.

Work conducted in this lab for the last 3 1/2 years has demonstrated a total lack of aortic ruptures in Pit Fowl (Steeves and Siegel, 1968 and Gerity and Steeves, 1970). It was, however, quite striking to discover a 75% incidence of

plaque formation in Pit Fowl hen iliac arteries compared with 57% incidence in White Rocks. The 50% incidence of plaque formation in Pit Fowl brachial arteries was significantly higher than the 16% incidence for White Rocks. A 50% increase in arterial wall thickness of Pit Fowl over White Rock was apparent, as manifest predominantly in the tunica media. This factor coupled with an increase in protein-polysaccharide content of the tunica media, may be involved in both prevention of aneurysm and predisposition to atherosclerosis. Iliac arteries of White Rock and Pit Fowl differed significantly in tunica media thickness as indicated earlier. Iliac artery diameters and brachial artery mean bores were significantly reduced in plaque containing vessels of Pit Fowl (Gerity and Steeves, 1970). Histochemical differences between White Rock and Pit Fowl arteries consisted of a marked increase in gamma metachromatic response to toluidin blue-● stain, pH 4.9, in the tunica media of all Pit Fowl vessels which were examined. Chondroitin sulfate and extensive protein-polysaccharide changes are evidently involved in this staining response. This early investigation indicated no lipid associated with any of the plaques when using conventional

frozen sections and lipid histochemical techniques. This is most probably due to the fibrotic, rather than lipoidal nature of spontaneous lesions. Similar findings in spontaneous lesions were reported by Weiss (1959), Ball et al. (1965), and Krista et al. (1969a).

Based upon these earlier investigations, it becomes apparent that it is necessary to develop a more thorough quantitative method to expand our knowledge of protein-polysaccharide involvement in the etiology of spontaneous atherosclerotic lesions.

The primary objectives of this current research are directed toward: (1) expanding the histologic data with regard to anatomical features of the Pit Fowl iliac complex and to obtain estimates of plaque size, (2) the demonstration of some correlation between age and etiological advancement of plaques in Pit Fowl, and (3) conducting qualitative and quantitative histologic and histochemical estimates of those parameters observed to differ in previous work in this laboratory; namely, collagen, elastin, and chondroitin sulfate matrices.

MATERIALS AND METHODS

Experimental animals consisted of 24 pit fowl hens. These animals were divided into 3 groups of 8 animals each, having mean ages and weights of 1.0 years and 1.48 kg, 2.5 years and 1.45 kg, and 4.4 years and 1.71 kg, respectively. All animals were bred and raised at the Virginia Agricultural Experiment Station. These pit fowl hens have undergone only random selection with no attempt to influence body weight (Siegel, 1971).

Based on gross observation, all animals appeared normal with no physical defects apparent in any of the groups. Coccidiostat was administered for the first 16 weeks of life with no drugs being utilized thereafter.

Animals were subjected to hot air brooding followed by rearing in floor pens supplied with wood shaving litter. Natural lighting conditions were employed during the first 24 weeks of life after which all birds were subjected to a timed cycle of fourteen hours light followed by a ten hour period of darkness. Excessive environmental pressures such as crowding or temperature extremes were non existent.

All animals were provided with rations, administered as indicated in Table I, with water ad-libitum. Starter ration was fed until birds were 8 weeks old, at which time they were switched to grower ration until 24 weeks of age (Table I). Antibiotic feeding ceased at 16 weeks and the grower diet was replaced with breeder ration on which the birds were continually maintained (Siegel, 1971). At no time were hormones, arsenicals, supplements or other additives of any kind employed in the diets of these animals.

Hens were killed by cervical dislocation using the "English kill" style. Both legs were then disjointed and a frontal plane incision was made along the longitudinal axis (Storer and Usinger, 1959). Gross examination of the viscera was carried out for signs of atherosclerotic lesions, blood clots or other abnormalities.

Both iliac complexes were dissected with samples being fixed for 48 hours in neutral buffered formalin, according to Lillie (1954). After 48 hours, tissues were washed in running distilled water, dehydrated in a graded series of alcohols (2 hours in each step), and cleared in 2 changes of Xylene for 1 hour each. All tissues were vacuum

Table I. Diets

Nutrients		Chicken Starter A2.1	Chicken Grower B2.1	Chicken Breeder C2.0
Crude protein	%	20.0	16.0	16.0
Crude fat	%	3.0	3.0	3.5
Crude fiber	%	6.0	7.0	4.5
Calcium (min.)	%	0.95	0.95	3.5
Calcium (max.)	%	1.2	1.2	4.0
Inorganic phosphorus	%	0.3	0.25	0.25
Manganese	mg/lb	25.0	20.0	25.0
Preformed vitamin A	IU/lb	1800.0	1800.0	1800.0
Vitamin D3	ICU/lb	350.0	350.0	700.0
Riboflavin	mg/lb	2.5	2.5	3.0
D-pantothenic acid	mg/lb	6.0	5.0	6.0
Niacin	mg/lb	25.0	25.0	25.0
Choline	mg/lb	500.0	425.0	425.0
Vitamin B_{12}	mcg/lb	6.0	4.0	6.0
Methionine	%	0.35	0.28	0.28
Antibiotic	g/ton	10.0	---	---

Ration analysis data, Siegel (1971)

embedded at 58°C in 3 changes of paraffin for 2 hours each. Four to six micron sections were cut at intervals on a model "820" AO Spencer microtome for histologic and histochemical examination.

Histological and Histochemical Procedures

Tissues were examined utilizing the following techniques: (1) Routine histologic examinations were conducted using neutral buffered formalin fixed tissue stained with Delafield's hematoxylin for 12 minutes and counterstained with Eosin-Y for 2 minutes (McManus and Mowry, 1965). (2) Carbohydrates were stained following the PAS technique of Barka and Anderson (1965). Control slides were treated for 1 hour at 37.5°C in 0.1% malt diastase[1] in 0.02M phosphate buffer (pH 6.0) followed by 3 minutes washing in distilled water prior to staining. (3) Further carbohydrate studies were carried out utilizing 0.1% toluidin blue-0 in veronal acetate buffer at pH 2.6, 3.5, and 4.9 for 25 minutes in each. Prior to staining, control slides were digested for 2 hours in testicular hyaluronidase[2] in 0.2M NaH_2PO_4 buffer

[1]Fisher Scientific, Silver Spring, Maryland.

[2]Nutritional Biochemical Corporation, Cleveland, Ohio.

(pH 6.0) at 37.5°C (Pearse and Everson, 1961). (4) Neutral buffered formalin fixed sections stained with 0.1% Alcian blue in 2% acetic acid (pH 2.6) were utilized as an ancillary technique for the characterization of acid protein polysaccharides. All tissues were stained 25 minutes in Alcian blue following treatment in 2% acetic acid to aid maintenance of tissue pH. (5) Elastic fibers were selectively stained using the McManus and Mowry (1965) modification of Verhoeff's procedure. Sections treated for 22 minutes in Verhoeff's stain and differentiated for 3 minutes in 2% ferric chloride gave optimum results. No counter stain was employed with this technique. (6) Extensive lipid histochemistry of these plaques was conducted previously by Gerity and Steeves (1970) and the results of that investigation preclude the necessity to repeat this work herein.

Histochemical Quantitation

Serum cholesterol quantitation was conducted using the colorimetric method of Zlatkis et al. (1953). Four replicates of serum from each animal were run and absorbancy readings at 560 u were obtained on a Bausch and Lomb Spectronic 20. From these readings mean values were

obtained for each animal by plotting against a standard curve prepared from chemically pure, ash free, dry cholesterol.[1]

Arterial elastin determinations were conducted utilizing a modification of the technique of Naughton and Sanger (1961). Five micron paraffin sections were mounted, 6 per slide. Tissues were deparaffinized and carried through a graded series of alcohols to distilled water followed by drying for 48 hours at 38°C. Slides were then weighed on a Mettler balance. Tissue sections were removed with a specially made scraper followed by thorough cleaning and drying of all slides. After this treatment all slides were re-weighed and mean net tissue weights were calculated. Alternate tissue sections were prepared and stained in 0.75% Orcein in 95% alcohol for 25 minutes (Gray, 1954). Tissue sections were deparaffinized, brought to distilled water and washed for 20 minutes in running distilled water to remove all residual stain. All tissue sections were then digested in a 50 ug/ml concentration of electrophoretically pure elastase[2] in Na_2CO_3 buffer (pH 8.8) for 5 hours. After 5 hours this

[1] Baker Chemical, Phillipsburg, New Jersey.

[2] Nutritional Biochemicals Corporation, Freehold, New Jersey.

solution was recovered by aspiration, and absorbancy read in a Bausch and Lomb Spectronic 20 at 525 u. The amount of solubolized elastin was then determined by comparison with a standard curve prepared from Orcein impregnated elastin.[1] All mean values were ultimately converted to a ugm elastin per ugm dry tissue weight basis to give a more easily recognizable value. A high degree of reproducibility and accuracy is apparent based on the extremely small standard errors obtained from the three replicates.

Arterial collagen determinations were conducted using a composite modification of the photometric methods of Moore and Stein (1948) and Mandl et al. (1953). Mean net tissue weights were obtained by the same technique previously employed for elastin quantitation. Following the normal hydration procedure, tissues were washed for 20 minutes in running distilled water. Tissues were then subjected to digestion for 18 hours in a 0.5 mg/ml electrophoretically pure collagenase[2] solution prepared in phosphate buffer (pH 7.4). This solution was aspirated off at the end

[1] Worthington Biochemical Corporation, Freehold, New Jersey.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

of the incubation period and rendered pink with Schiff's reagent. Following a 15 minute color development period, absorbancy was read in a Bausch and Lomb Spectronic 20. These readings were then plotted against those obtained from a standard curve prepared from purified collagen[1], treated identically to tissues with Schiff's reagent. Mean values were then converted to a ugm collagen/ugm dry tissue weight basis.

Following staining, all tissues were examined using a Zeiss model RA microscope. Selected representative tissues were photographed using a Zeiss automatic camera unit and adox KB-14 film. Plates were then reproduced using a polaroid MP-3 copy stand and Kodak Panatomic-X film.

Statistical Analysis

Based on histochemical staining response to toluidin blue-O (pH 2.6, 3.5, and 4.9), all tissues were scored on the staining properties of each of three regions: (1) tunica adventitia; (2) tunica media; and, (3) tunica intima. Arbitrary scores ranging from negative staining (0), to a

[1]Worthington Biochemical Corporation, Freehold, New Jersey.

maximum of plus 4 (indicating extremely positive staining response) were assigned accordingly. Alcian blue stain (pH 2.6) was used as an ancillary method for verification of acid protein polysaccharide response, but is not included in the statistical analysis due to extremely negative response.

Dimensional analysis of arteries was conducted by making twelve measurements of representative sections from each animal, and calculating means and standard errors for each of five parameters: (1) overall diameter; (2) bore diameter; (3) tunica adventitia; (4) tunica media; and, (5) tunica intima thickness. All measurements of vessel regions were treated using the "Hierarchical" classification for nested factors (Li, 1964). The model used for analysis of variance: $\hat{y}=\bar{y}+(\bar{y}_a-\bar{y})+(\bar{y}-\bar{y}_a)=(y-\bar{y})$ with nests consisting of plaque, non-plaque animals within each age group.

Plaque-like formations found present in the arteries were scored according to a modification of the method of Tennent et al. (1957). These scores range from 0, indicationg no presence of plaque, to 5.00 indicating virtually total lumen occlusion (50%-90%).

Serum cholesterol mean values, and standard errors calculated from these means, were obtained from 4 replicate

samples. All values were then subjected to analysis using the "Hierarchical" model of Li (1964) outlined above. This analysis model was also applied to the replicate data obtained from elastin and collagen histochemical digestion techniques.

RESULTS

General Findings

Examination of iliac complexes revealed these vessels to be extremely tough and resilient, similar to findings reported by Steeves and Siegel (1968). Upon gross examination, no apparent signs of atherosclerotic lesions were observed. Similarly, there were no external symptoms indicative of cardiovascular pathology. One exception to this was an extremely severe anemia accompanied by overall weakness and muscle flacidity found in a 1.0 year old pit fowl hen # GH-06. Very little peripheral blood was present, and cardiac muscle exhibited signs of degeneration, with all body tissues generally lacking tone.

Histology

Histologic examination of vessels indicated that the severity of spontaneous lesions, based on the modified plaque scoring system of Tennent et al. (1957), increased from a mean value of 2.83 in animals 1.0 year of age to a mean of 4.91 (indicating nearly total tunica intima participation in plaque formation) for animals 2.5 years of age. A

mean value of 3.50 was obtained for animals having a mean age of 4.4 years (Table II). While plaque severity suggests a normal curve with its mid-point at 2.5 years of age, plaque frequency data yield a linear increase from 37.5% to 75%, and 87.5% for birds having mean ages of 1.0, 2.5, and 4.4 years, respectively. A summary of plaque severity is presented in Plate 1 and may be readily compared with data contained in Table II. One extreme case of iliac arterial occlusion was apparent in a 2.0 year old hen, animal # GH-76 (Table II, and Plate 5). This animal represents the most severe example of spontaneous atherosclerotic plaque formation (approximating 95% occlusion) identified in this study.

Spontaneous atherosclerotic plaques were characterized by (1) numerous vacuolar formations in the tunica intima; (2) a marked localization and fragmentation of elastic tissue fibers within the intima and underlying media region (Plates 2, 3, 5) (Gerity and Steeves, 1970); and (3) alterations to tunica media collagen, similar to findings reported by Krista et al. (1969a) and McCully (1970). The tunica intima frequently exhibited a group of vacuolar cells which failed to respond to lipid histochemical techniques as reported by

Table II. Tennent Plaque Scores of Atherosclerotic Lesions

Animal Number	Non-Plaque or Plaque	Age (years)	Game Hen Iliac Arterial Complex
GH-01	N	1.0	0.00
GH-02	N	1.0	0.00
GH-03	N	1.0	0.00
GH-04	N	1.0	0.00
GH-08	N	1.0	0.00
GH-05	P	1.0	1.00
GH-06	P	1.0	4.50
GH-07	P	1.0	3.00
Pooled		1.0	2.83[b] or 1.06[c]
GH-1	N	2.0	0.00
GH-2	N	3.0	0.00
GH-3	P	3.0	5.00
GH-4	P	2.0	5.00
GH-5	P	2.0	5.00
GH-6	P	2.0	4.50
GH-7	P	3.0	5.00[a]
GH-8	P	3.0	5.00
Pooled		2.5	4.91[b] or 3.68[c]
GH-18	N	5.0	0.00
GH-11	P	4.0	1.50
GH-12	P	4.0	4.50
GH-13	P	4.0	2.50
GH-14	P	4.0	5.00
GH-15	P	4.0	1.50
GH-16	P	5.0	4.50
GH-17	P	5.0	5.00
Pooled		4.4	3.50[b] or 3.06[c]

[a]Lumen totally occluded.
[b]Mean based only on animals containing plaques.
[c]Mean based on all animals in age group.

Gerity and Steeves (1970). It is evident that these cells correspond to the cells described as "foam cells" by DiLuzio (1967), Levy (1967), and confirmed in pigeons (Simpson et al., 1969), and in rats (Trillo et al., 1970) (see Plates 4, 6, and 7 for a detailed view of "foam cell" formations).

Measurement data for all regions of iliac arteries are given in Table III. Many significant ($P \leq .05$) differences were found. A significant reduction in tunica adventitia thickness between age groups was observed to regress from a mean of 78.70 u for 1.0 year old animals, to 69.78 u for 2.5 year olds, and 42.88 u for animals having a mean age of 4.4 years. A general reduction in adventitia thickness of plaque containing animals was apparent within each age group, with only the reduction in the 2.5 year old group being significant. Tunica media dimensional changes for the three age groups peaked at 2.5 years of age with 79.10 u, 159.52 u, and 98.83 u mean thickness for 1.0, 2.5, and 4.4 year old groups, respectively. While all three age groups exhibited an increased medial thickness in plaque containing animals, only the 2.5 and 4.4 year old groups closely approached the ($P \leq .05$) level of significance. Tunica intima measurement

data also yield a peak at 2.5 years. Intima mean thickness data indicated significant differences for the three age groups with 27.37 u, 50.29 u, and 36.80 u for animals 1.0, 2.5, and 4.4 years of age, respectively. All plaque containing animals observed exhibited significant tunica intimal thickening ranging from 6.0 u-8.0 u for non-plaque animals to 40.99 u-65.96 u for plaque containing animals. Mean values obtained for overall diameters of vessels, once again produce a peak at 2.5 years, with significant differences of 666.00 u, 998.64 u, and 662.55 u observed for animals 1.0, 2.5, and 4.4 years of age, respectively. A slight increase (not significant) in plaque bearing animals 1.0 year of age was observed. Overall diameters were observed to decrease significantly only in the 2.5 year old plaque animals. The 4.4 year old plaque group, however, was observed to parallel this finding, but the difference in this case when compared with non-plaque animals was not significant. Mean bore diameters for the three age groups also yield a peak at 2.5 years with 254.52 u, 326.03 u, and 251.61 u being significantly different for animals 1.0, 2.5, and 4.4 years of age respectively. As evident, based on

Table III. Weighted Means and Standard Errors for Arterial Dimension Analysis (u)[1]

Group	Age (years)	Region	Non-Plaque	Plaque	Pooled
01-08	1.0	AD	80.00±4.15	76.53±4.41	78.70
		MED	72.76±5.24	89.66±8.72	79.10
		INT	8.08±0.39	59.51±2.63	27.37
		OD	657.38±19.92	680.36±21.09	666.00
		BORE	293.34±23.58	189.83±28.38	254.52
1-8	2.5	AD	179.79±4.29	33.12±2.46	69.78
		MED	140.20±6.61	165.96±12.18	159.52
		INT	6.00±0.29	65.96±2.22	50.29
		OD	1330.20±24.91	888.12±32.48	998.64
		BORE	517.91±26.54	262.07±27.02	326.03
11-18	4.4	AD	31.80±0.82	44.47±1.60	42.88
		MED	83.90±1.42	100.97±9.77	98.83
		INT	6.00±0	40.99±1.17	36.80
		OD	699.10±2.03	657.32±29.01	662.55
		BORE	328.40±3.09	240.64±32.44	251.61

[1] See text (p. 19) for those means that are significantly different.

AD - Adventitia
MED - Media
INT - Intima
OD - Outside Diameter

plaque severity, mean bore diameters for plaque-containing animals were significantly reduced in each of the three age groups.

Histochemistry

Carbohydrate. Various iliac complex histochemical differences were evident in the three age groups of pit fowl examined and are summarized in Table IV. Tunica adventitia staining response with toluidin blue-O and alcian blue stains was extremely negative. Differences in tunica adventitia were not observed between the three age groups or between plaque and non-plaque containing animals.

The staining response of the tunica media with toluidin blue-● at pH 4.9, for the three age groups, follows a curve similar to plaque severity data. Histochemical scores of 2.41, 2.50, and 2.46 (based upon a scoring system with 4.0 being the most positive staining response) were registered for 1.0, 2.5, and 4.4 year old animals, respectively. Response of the media to toluidin blue-O pH 3.4 for these age groups remained equal (0.25) for 1.0 and 2.5 year olds, with 4.4 year olds exhibiting an increased response to 0.41. Media scores for all ages stained with toluidin blue-O pH 2.6

were virtually identical (either 0.09 or 0.10) (Table IV).

Toluidin blue-O at pH 4.9 produced a marked increase in staining response of the tunica media in all animals containing plaques, accompanied by an extremely intense gamma metachromasia (Table IV). Response to staining in toluidin blue-O pH 3.4 demonstrated no change between plaque and non-plaque animals 1.0 year of age, an increase from 0 to 0.33 in plaque animals 2.5 years of age, and a decrease from 0.50 to 0.39 in animals 4.4 years of age. An extreme decrease in response to toluidin blue-O pH 2.6 was noted in plaque containing animals of all three age groups (Table IV).

Tunica intima alterations noted with toluidin blue-O pH 4.9 for the three age groups once again simulate a curve with a peak at 2.5 years of age, with scores of 1.37, 1.87, and 1.56 for animals 1.0, 2.5, and 4.4 years of age, respectively. Response to toluidin blue-O at pH 3.4 produced a similar pattern for tunica intimas of the three age groups with scores of 0.31, 0.41, and 0.06 for animals 1.0, 2.5, and 4.4 years of age, respectively. Tunica intimal response to toluidin blue-O pH 2.6 was relatively constant for all three ages, ranging from 0.09 to 0.12 (Table IV).

Table IV. Weighted Means for Iliac Complex Histochemical Scores (Toluidin Blue-O)

Group	Age (years)	pH	AN	AP	AG	MN	MP	MG	IN	IP	IG
01-08	1.0	4.9	--	--	--	1.91	3.33*	2.41	1.00	2.00	1.37
		3.4	--	--	--	0.25	0.25	0.25	--	0.83	0.31
		2.6	--	--	--	0.16	0.05	0.09	0.08	0.15	0.10
1-8	2.5	4.9	--	--	--	2.00	2.66*	2.50	2.00	1.83	1.87
		3.4	--	--	--	--	0.33	0.25	0.25	0.46	0.41
		2.6	--	--	--	0.25	0.04	0.09	0.12	0.12	0.12
11-18	4.4	4.9	--	--	--	2.00	2.53*	2.46	1.00	1.64	1.56
		3.4	--	--	--	0.50	0.39	0.41	--	0.07	0.06
		2.6	--	--	--	0.50	0.03	0.10	0.25	0.10	0.09

A - Adventitia
M - Media
I - Intima
G - Group mean
N - Non-plaque mean

-- - Negative response
* - Gamma metachromasia
4.0 - (Plus 4) Extremely positive response
P - Plaque mean

Tunica intimas of plaque containing animals 1.0 and 4.4 years of age exhibited an increase from 1.00 to 2.00 and 1.00 to 1.64, respectively, with toluidin blue-O at pH 4.9. Contrariwise, plaque containing animals 2.5 years of age showed a decrease in response from 2.0 to 1.83. Tunica intimal response with toluidin blue-O at pH 3.4 was found to be substantially higher in all cases of animals containing plaques (Table IV). Tunica intimal response to toluidin blue-O pH 2.6 increased from 0.08 to 0.15 in plaque animals 1.0 years of age, remained stable in the 2.5 year old group, and exhibited a decrease from 0.25 to 0.10 in plaque animals 4.4 years of age (Table IV).

Elastin. Extremely severe fragmentation in elastic lamina and elastica interna was apparent based upon examination with Verhoeff's, Alcian blue, and PAS stained tissue sections (Plates 2, 4, 5, and 7). The elastica interna was fragmented in every case where intimal plaques appeared to be developing, regardless of the severity of plaque stage (Plates 1 and 4). In more severe cases, the elastica interna had degenerated completely. Fibers of elastin were frequently arranged in a columnar pattern of radiating bundles

(Plates 3 and 5) resulting in a perpendicular configuration similar to that noted previously in pit fowl by Gerity and Steeves (1970) and reported in turkeys by Krista et al. (1969a).

Iliac arterial complexes of the three age groups exhibited significant ($P \leq .05$) differences in elastin content, with mean values of 26.2%, 37.1%, and 14.5% for animals 1.0, 2.5, and 4.4 years of age, respectively (Table V). An apparent increase in elastin accompanies aging if one examines mean values given in Table V for non-plaque groups from all three ages (from 36.7% in 1.0 year old animals, to 50.3% in 2.5 year olds, and then to 58.8% for animals 4.4 years of age). A significant ($P \leq .05$) decrease in arterial elastin from 36.7% to 8.8%, 50.3% to 32.7%, and 58.8% to 8.2% was found in plaque animals 1.0, 2.5, and 4.4 years of age, respectively. The overall decrease from 42.8% for non-plaque animals to 19.9% for plaque animals is significant ($P \leq .05$) (Table V).

Collagen. Modification in the tunica media region with respect to collagen were observed in PAS stained tissues from the three age groups. These differences are

Table V. Weighted Means and Standard Errors for Elastin Determination of Iliac Complex (ugm/ugm expressed as %)

Animal No.	Non-plaque or Plaque	Age (yrs)	% Elastin (based on dry wt.)	Non-Plaque	Plaque
GH-01	N		33.0±0.7		
GH-02	N		67.4±0.6		
GH-03	N		11.0±0.1		
GH-04	N		21.7±0.8		
GH-08	N		50.5±0.1		
GH-05	P		10.6±0.8		
GH-06	P		10.5±0.1		
GH-07	P		5.4±0.7		
Pooled		1.0	26.2±0.2*	36.7±0.7**	8.8±0.7**
GH-1	N		50.0±0.6		
GH-2	N		50.5±0.1		
GH-3	P		55.4±0.1		
GH-4	P		10.9±0.1		
GH-5	P		16.3±0.2		
GH-6	P		17.2±0.1		
GH-7	P		69.2±0.9		
GH-8	P		27.2±0.2		
Pooled		2.5	37.1±0.8*	50.3±0.2**	32.7±0.7**
GH-18	N		55.8±0.8		
GH-11	P		27.7±0.1		
GH-12	P		9.4±0.5		
GH-13	P		2.1±0.7		
GH-14	P		11.6±0.2		
GH-15	P		2.4±0.8		
GH-16	P		3.0±0.1		
GH-17	P		1.2±0.1		
Pooled		4.4	14.5±0.5*	58.8±0.8**	8.2±0.2**
			All Animals	Non-plaque	Plaque
Overall Means			25.8±0.5	42.8±0.1**	19.9±0.4**

N - Non-plaque (animals not containing plaques).
P - Animals containing atherosclerotic plaques.
* - Those means significantly different between age groups ($P \leq .05$).
** - Those means significantly different within age groups -- plaque, non-plaque ($P \leq .05$).

Table VI. Weighted Means and Standard Errors for Collagen Determination of Iliac Complex (ugm/ugm expressed as %)

Animal No.	Non-plaque or Plaque	Age (yrs)	% Collagen (based on dry wt.)	Non-Plaque	Plaque
GH-01	N		7.3 ±0.5		
GH-02	N		20.2 ±0.1		
GH-03	N		35.0 ±0.9		
GH-04	N		5.8 ±0.5		
GH-08	N		10.9 ±0.3		
GH-05	P		30.9 ±0.4		
GH-06	P		11.5 ±0.3		
GH-07	P		0.9 ±0.2		
Pooled		1.0	15.3 ±0.1*	15.8± 0.1	14.4± 0.2
GH-1	N		10.2 ±0.4		
GH-2	N		4.5 ±0.4		
GH-3	P		4.3 ±0.9		
GH-4	P		0.6 ±0.2		
GH-5	P		7.6 ±0.9		
GH-6	P		43.6 ±0.9		
GH-7	P		11.2 ±0.4		
GH-8	P		35.7 ±0.4		
Pooled		2.5	14.8 ±0.1*	7.3± 0.6**	17.2± 0.2**
GH-18	N		20.8 ±0.2		
GH-11	P		16.6 ±0.4		
GH-12	P		7.3 ±0.1		
GH-13	P		2.6 ±0.2		
GH-14	P		7.9 ±0.2		
GH-15	P		1.6 ±0.4		
GH-16	P		1.5 ±0.3		
GH-17	P		1.5 ±0.4		
Pooled		4.4	7.5 ±0.2*	20.8± 0.2**	5.6± 0.2**
			All Animals	Non-plaque	Plaque
Overall Means			12.5 ±0.7	14.3± 0.3	10.7± 0.8

N - Non-plaque (animals not containing plaques).
P - Animals containing atherosclerotic plaques.
* - Those means significantly different between age groups ($P \leq .05$).
** - Those means significantly different within age groups plaque, non-plaque ($P \leq .05$).

more specifically identified in Table VI. A significant ($P \leq .05$) decrease with age in iliac artery collagen content was observed in the three age groups. This decrease consisted of a reduction from 15.3%, 14.8%, to 7.5% for animals 1.0, 2.5, and 4.4 years of age, respectively. The differences between plaque and non-plaque animals of the three age groups consist of a decrease from 15.8% to 14.4% for 1 year olds, and an increase from 7.3% to 17.2% for 2.5 year olds, and a decrease from 20.8% to 5.6% in animals 4.4 years of age, with only the latter 2 being significantly different ($P \leq .05$). The overall decrease from a mean of 14.3% for non-plaque animals to a mean of 10.7% for animals containing plaques was significantly different (Table VI).

Serum Cholesterol. Significant ($P \leq .05$) differences between age groups were noted. Mean values of 213 mg%, 167 mg%, and 215 mg% were obtained for animals 1.0, 2.5, and 4.4 years of age, respectively (Table VII). The comparison of plaque and non-plaque vessels demonstrated a significant difference extant only in the 1.0 year old group (a decrease from 251 mg% to 150 mg%). Serum cholesterol

Table VII. Weighted Means and Standard Errors for Serum Cholesterol Determination (mg %)

Animal No.	Non-plaque or Plaque	Age (yrs)	Serum Cholesterol	Non-Plaque	Plaque
GH-01	N		350 ±1.08		
GH-02	N		338 ±0.62		
GH-03	N		150 ±0.53		
GH-04	N		262 ±0.64		
GH-08	N		155 ±0.62		
GH-05	P		188 ±0.79		
GH-06	P		105 ±0.52		
GH-07	P		157 ±0.74		
Pooled		1.0	213 ±7.07*	251± 3.80**	150± 6.06**
GH-1	N		206 ±1.27		
GH-2	N		125 ±0.67		
GH-3	P		191 ±0.65		
GH-4	P		171 ±0.79		
GH-5	P		129 ±0.64		
GH-6	P		174 ±0.66		
GH-7	P		183 ±0.71		
GH-8	P		156 ±1.17		
Pooled		2.5	167 ±10.53*	165± 19.25	167± 5.91
GH-18	N		197 ±0.63		
GH-11	P		218 ±0.59		
GH-12	P		228 ±0.64		
GH-13	P		240 ±0.62		
GH-14	P		186 ±0.92		
GH-15	P		188 ±1.27		
GH-16	P		216 ±1.17		
GH-17	P		248 ±0.53		
Pooled		4.4	215 ±7.66*	197± 0.63	218± 8.37

N - Non-plaque (animals not containing plaques).
P - Animals containing atherosclerotic plaques.
* - Those means significantly different between age groups ($P \leq .05$).
** - Those means significantly different within age groups plaque, non-plaque ($P \leq .05$).

levels (Table VII), while increasing slightly (165 mg% to 167 mg% and 197 mg% to 218 mg% for 2.5 and 4.4 year old animals, respectively) are not significantly different ($P \leq .05$).

Abbreviations

EE - Elastica Externa
FP - Fibrosed Plaque
FC - "Foam Cell" Formation
FI - Fragmented Elastica Interna
FL - Fragmented Elastic Lamina
L - Lumen
TA - Tunica Adventitia
TI - Tunica Intima
TM - Tunica Media
VI - Vacuolated Intima

PLATE 1

EXPLANATION OF FIGURES

1 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-01) illustrating a non-plaque tunica intima, media, and adventitia. Delafield's hematoxylin and eosin Y (X 600).

2 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating representative plaque severity for animals having a mean age of 1.0 year. Verhoeff's stain for elastic tissue fibers (X 375).

3 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-1) illustrating representative plaque severity for animals having a mean age of 2.5 years. Delafield's hematoxylin and eosin Y (X 375).

4 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-7) illustrating the most severe degree of arterial occlusion. Verhoeff's stain for elastic tissue fibers (X 375).

5 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-12) illustrating typical plaque severity, vacuolated tunica intima, and fragmented elastica interna. Verhoeff's stain for elastic tissue fibers (X 375).

6 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-14) illustrating typical plaque severity and "foam cell" formation of the tunica intima. Delafield's hematoxylin and eosin Y (X 375).

PLATE 1

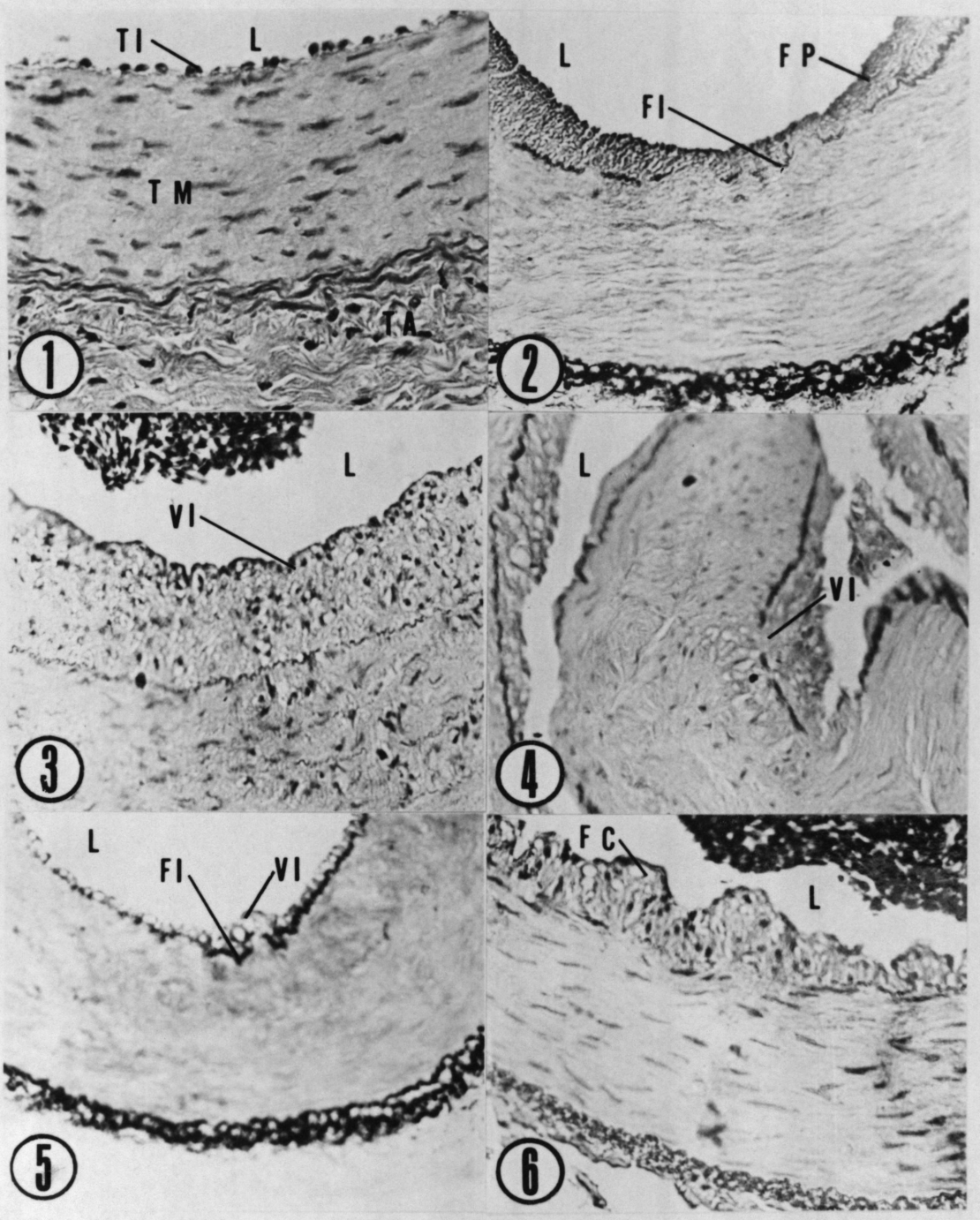


PLATE 2

EXPLANATION OF FIGURES

7 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating typical plaque development, and tunica media staining response. Toluidin blue-O pH 4.9 (X 500).

8 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating the extent of fibrotic plaque development. PAS (X 500).

9 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating extent of plaque development, and advanced fragmentation of the elastica interna. Verhoeff's stain for elastic tissue fibers (X 200).

10 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating degree of elastic tissue fragmentation and organization into intimal plaque. Verhoeff's stain for elastic tissue fibers (X 500).

PLATE 2

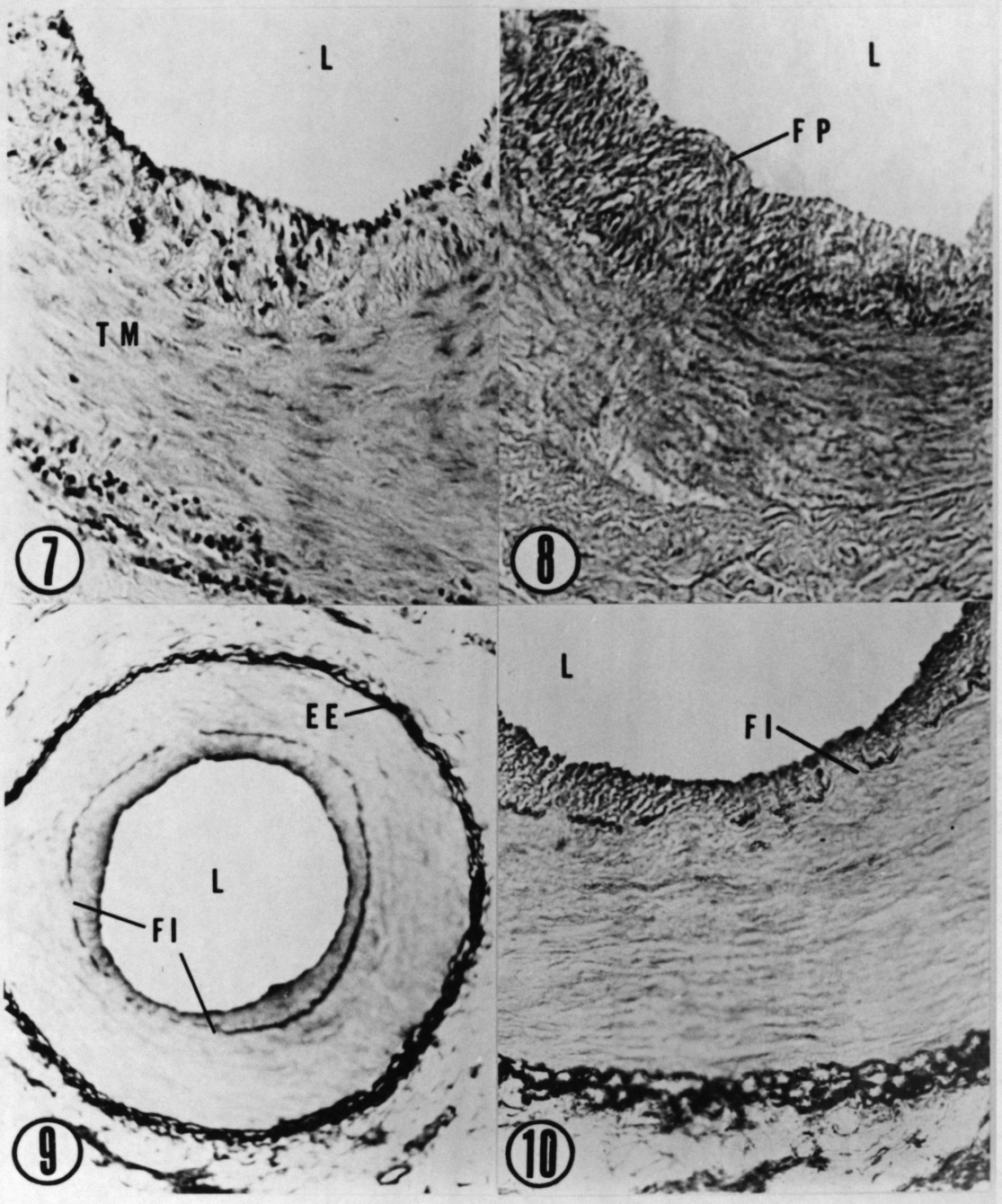


PLATE 3

EXPLANATION OF FIGURES

11 Tangential section of the iliac artery of a 1.0 year old pit fowl hen (GH-05) illustrating early plaque development and relative extent of protein-polysaccharide anomalies in tunica media. Toluidin blue-O pH 4.9 (X 200).

12 Tangential section of the iliac artery of a 1.0 year old pit fowl hen (GH-05) illustrating early plaque development, and degree of protein-polysaccharide involvement in plaque development. Toluidin blue-O pH 4.9 (X 500).

13 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating an overall view of early plaque development. Delafield's hematoxylin and eosin Y (X 200).

14 Cross section of the iliac artery of a 1.0 year old pit fowl hen (GH-06) illustrating intimal thickening in plaque development representative of this age group. Delafield's hematoxylin and eosin Y (X 500).

PLATE 3

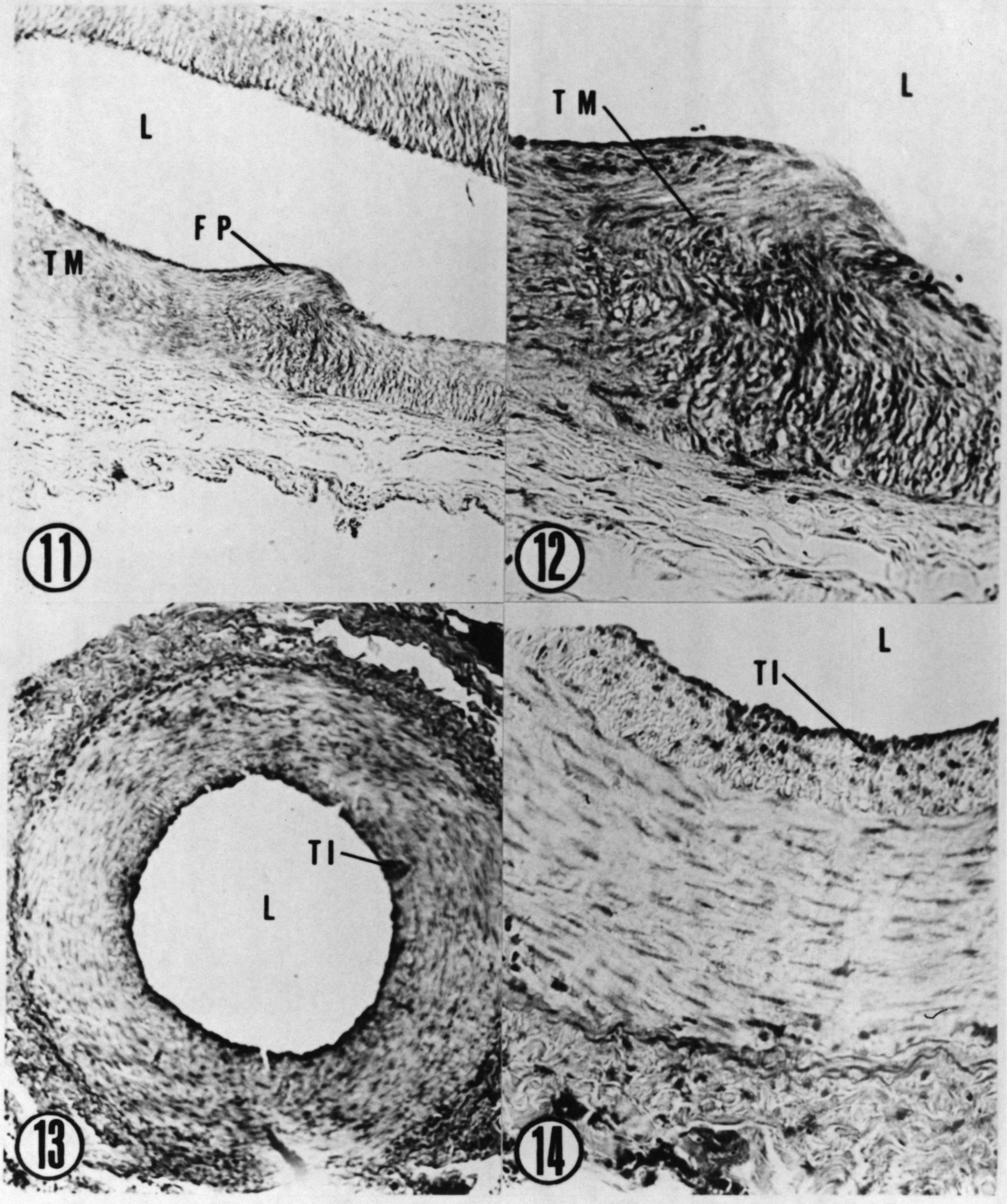


PLATE 4

EXPLANATION OF FIGURES

15 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-2) illustrating fragmentation of the elastica interna, and "foam cell" formation in the tunica intima. Delafield's hematoxylin and eosin Y (X 800).

16 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-3) illustrating advanced vacuolar plaque development, and elastic fiber disorientation. Verhoeff's stain for elastic tissue fibers (X 500).

17 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-4) illustrating fibrosed intimal plaque involving the entire lumen lining, and fragmented elastica interna. Verhoeff's stain for elastic tissue fibers (X 200).

18 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-4) illustrating severe fragmentation of the elastica interna and the elastic lamina, and early intimal vacuolation. Verhoeff's stain for elastic tissue fibers (X 500).

PLATE 4

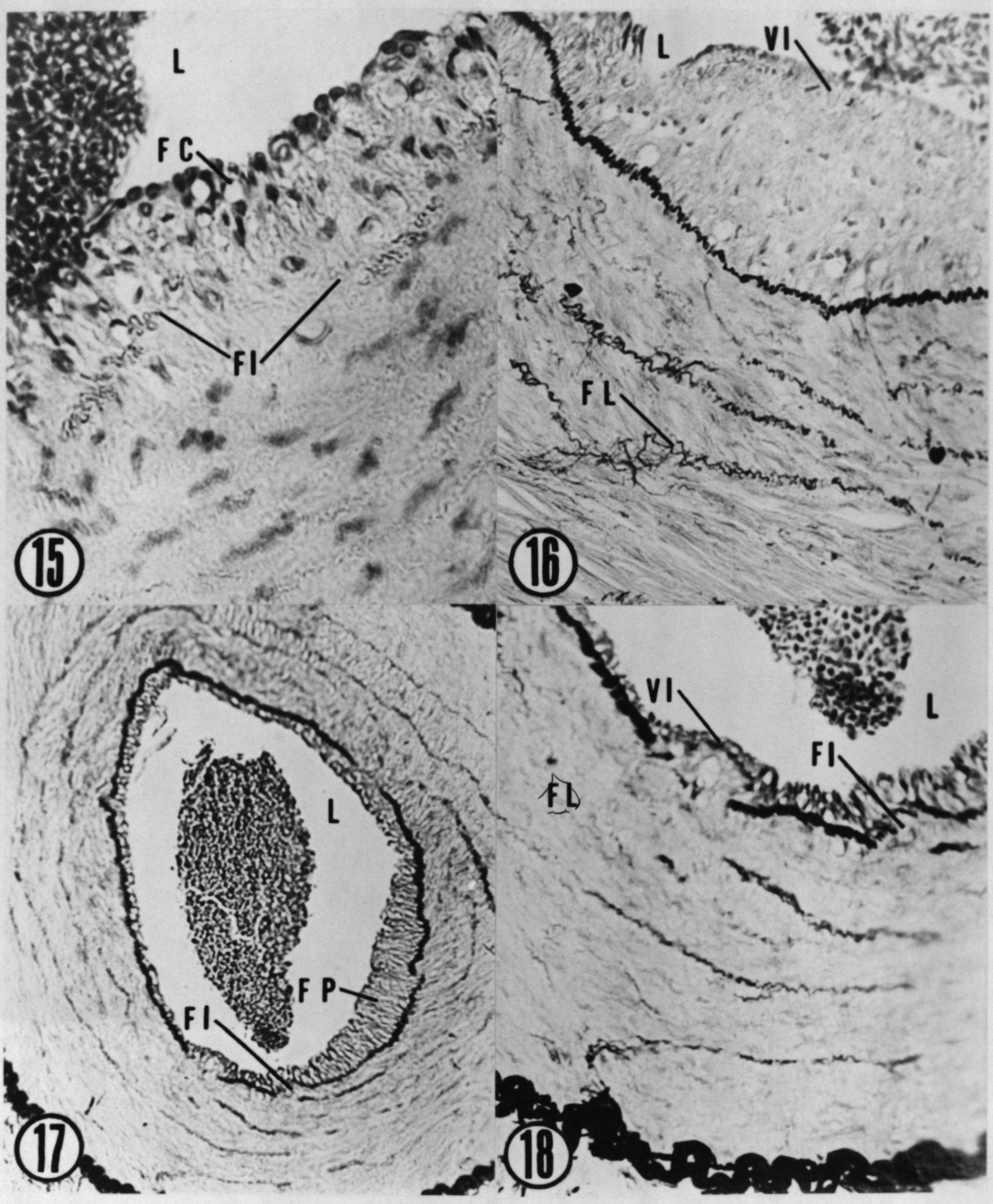


PLATE 5

EXPLANATION OF FIGURES

19 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-7) illustrating nearly total occlusion of the lumen. Verhoeff's stain for elastic tissue fibers (X 500).

20 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-7) illustrating fragmentation of the elastica interna, and vacuolar infiltration of the tunica media. Verhoeff's stain for elastic tissue fibers (X 800).

21 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-7) illustrating occluded lumen and extent of plaque development. Delafield's hematoxylin and eosin Y (X 500). (Note: elastica externa is not inverted, indicating the lack of vessel involution).

22 Cross section of the iliac artery of a 2.5 year old pit fowl hen (GH-7) illustrating totality of fibrous plaque development, and infiltration of fibers into tunica media. Alcian blue stain (X 500).

PLATE 5

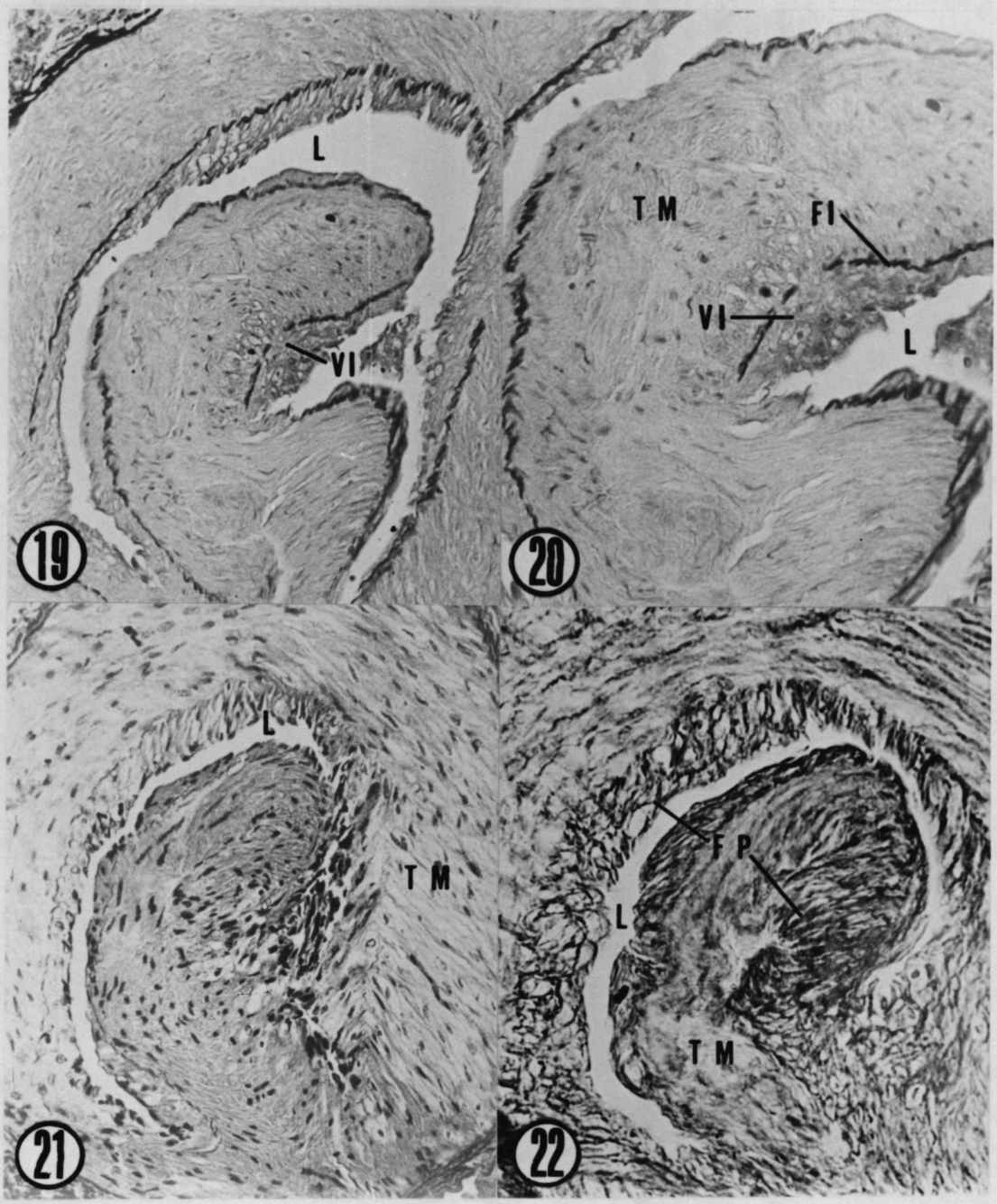


PLATE 6

EXPLANATION OF FIGURES

23 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-12) illustrating early "foam cell" formation in the tunica intima, and typical staining properties of the tunica media with toluidin blue-O pH 4.9 (X 500).

24 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-12) illustrating early vacuolation of the tunica intima. Delafield's hematoxylin and eosin Y (X 800).

25 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-12) illustrating overall severity of plaque development. Verhoeff's stain for elastic tissue fibers (X 500).

26 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-12) illustrating vacuolation of the tunica intima, and fragmentation of the elastica interna. Verhoeff's stain for elastic tissue fibers (X 800).

PLATE 6

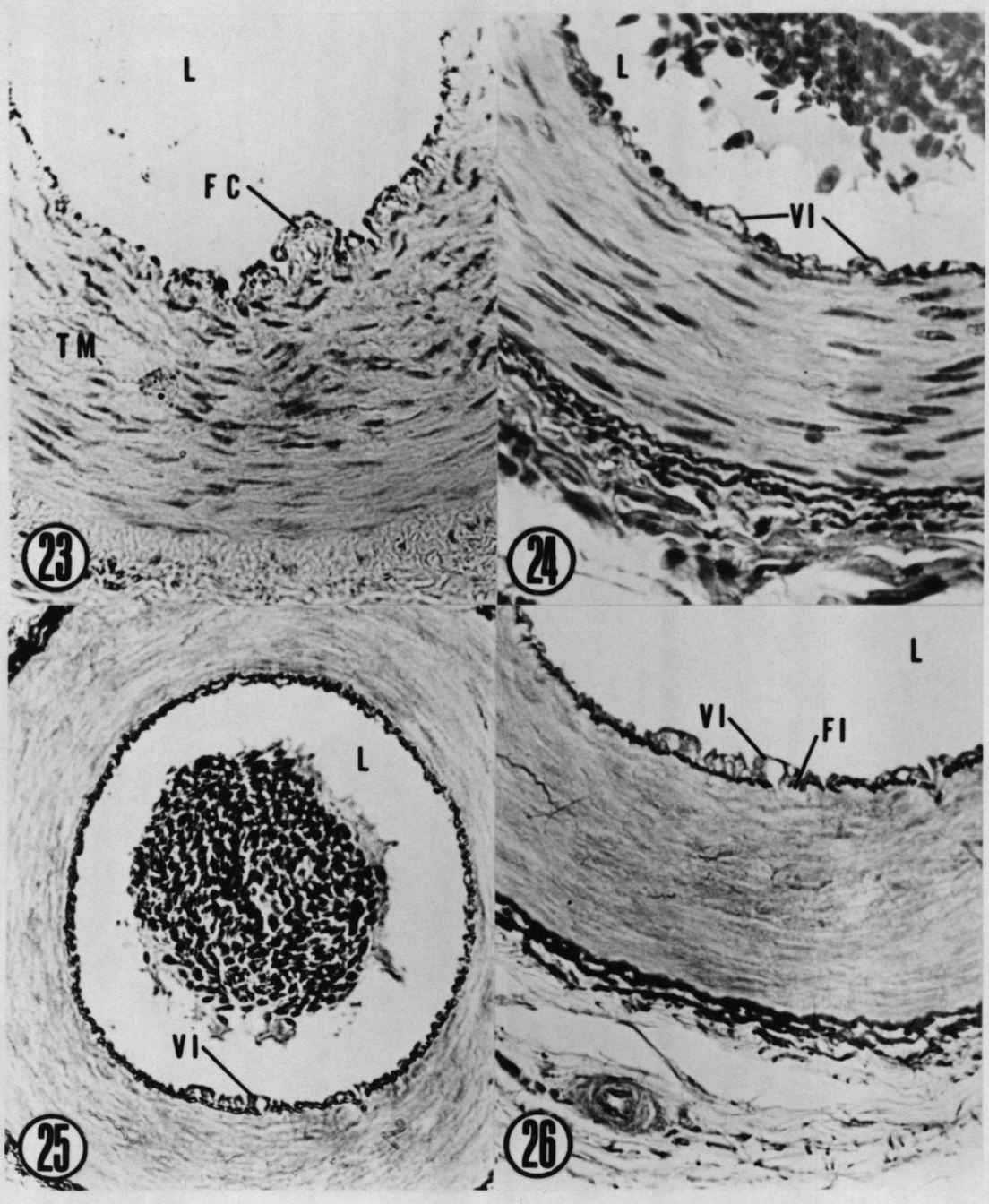


PLATE 7

EXPLANATION OF FIGURES

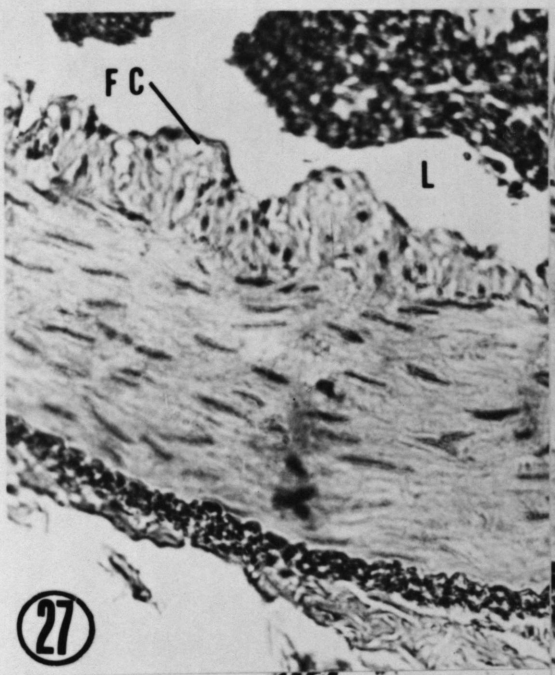
27 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-14) illustrating extensive "foam cell" formation in the tunica intima. Delafield's hematoxylin and eosin Y (X 500).

28 Cross section of the iliac artery of a 4.4 year old pit fowl hen (GH-14) illustrating extensive "foam cell" formation, and plaque severity typical for this age group. Delafield's hematoxylin and eosin Y (X 800).

29 Cross section of artery from the iliac complex of a 4.4 year old pit fowl hen (GH-12) illustrating overall elastica interna fragmentation, and extensive vacuolation of the tunica intima. Verhoeff's stain for elastic tissue fibers (X 200).

30 Cross section of artery from the iliac complex of a 4.4 year old pit fowl hen (GH-12) illustrating extreme elastica interna fragmentation and vacuolar development. Verhoeff's stain for elastic tissue fibers (X 500).

PLATE 7



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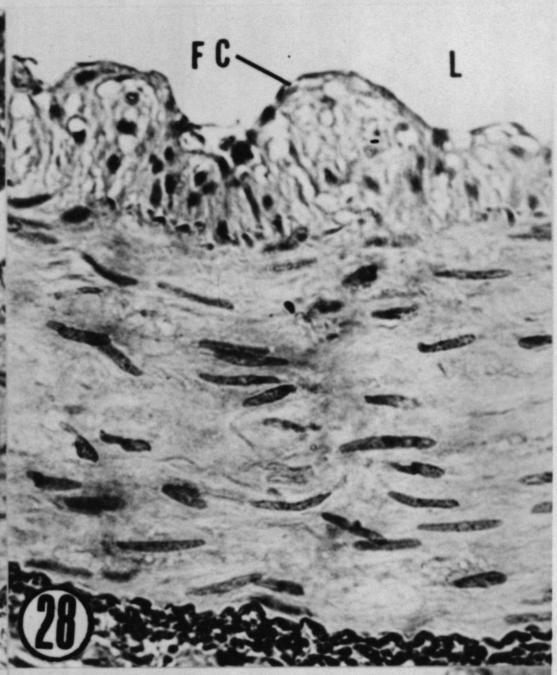

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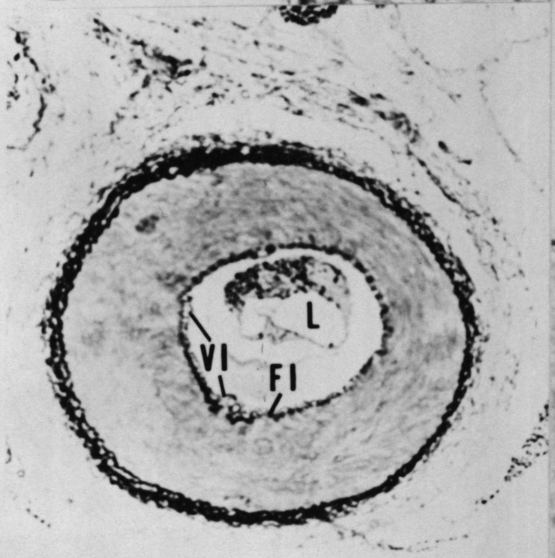

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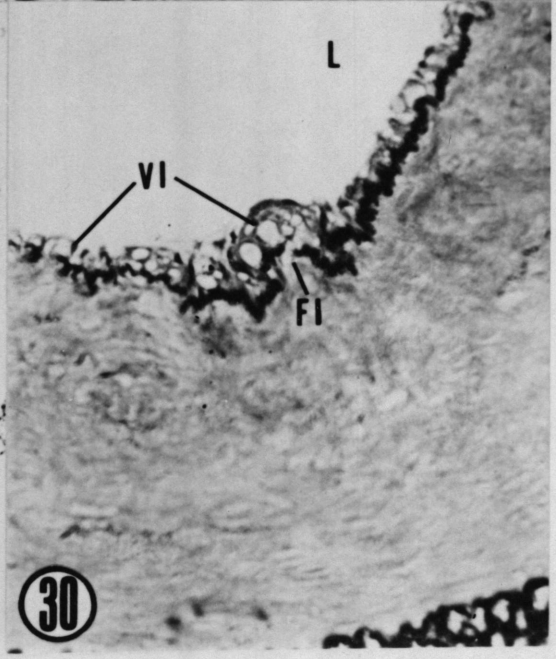

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DISCUSSION

Atherosclerotic plaque frequency data yielded a linear pattern increasing from 37.5% to 75% and 87.5% for birds with mean ages of 1.0, 2.5, and 4.4 years, respectively. Plaque severity data, however, contain a peak with the most advanced plaques within the 2.5 year old group (Table II). The high incidence of spontaneous atherosclerotic plaques was expected based upon earlier findings of Gerity and Steeves (1970). Plaque severity data seem to justify the conclusion that the more severe plaques are effective in eliminating more pre-disposed animals from the flock sometime prior to 4.4 years of age. Since animals have not been observed to die from causes directly attributable to cardiovascular pathology, specifically aneurysm, it would seem that flock deaths have been previously attributed to other causes. It would not be unjustified to suggest that the increased cardiovascular stress imposed by spontaneous atherosclerotic plaques may pre-dispose these animals to increased vulnerability to a multiplicity of additional factors.

Several additional reasons may be cited with respect to the failure to observe aneurysm in these birds. As previously

noted, pit fowl iliac arteries exhibit a 50% increase in thickness when compared with White Rocks (Gerity and Steeves, 1970). A great degree of structural integrity is imparted to the iliac arterial wall by high concentrations of elastin present within the tunica media, effectively increasing the strength of this vessel. Non-plaque iliac arteries exhibited a mean elastin content of 42.8% with a decrease to 19.9% for plaque containing vessels (Table V). This decrease in elastin content corroborates the histologic findings of Krista et al. (1969a) and Gerity and Steeves (1970), which showed a fragmentation of elastic tissue fibers in plaque containing animals (Table V, Plates 2, 4, 5, and 7). This significant reduction in elastin content unquestionably alters the structural integrity of these vessels severely, and suggests a ready pathway open to massive cardiovascular failure and secondary invasion of the system by a variety of pathogens. The net result of this would be high mortality following 2.5 years of age.

Further alterations in the tunica media noted histochemically were verified using biochemical quantitation. Collagen present in the tunica media was demonstrated to decrease with age from 15.3%, 14.8%, and 7.5% for animals 1.0, 2.5,

and 4.4 years of age, respectively (Table VI).

Both primary structural components, elastin and collagen, apparently undergo extreme reduction accompanying aging and the onset of atheromatous plaques. These two factors coupled with an increase in chondroitin sulfate matrices, observed in toluidin blue-O stained sections, combine to effect the ultimate degeneration of the artery wall. Working with swine aortic complexes containing cholesterol induced plaques, Lee et al. (1970) described an increase in lysosomal products. In these spontaneous atherosclerotic plaques, acid protein polysaccharides were not observed to increase (Table IV). Proline hydroxylase activity, however, has been observed to increase in experimentally induced plaques (Langner, 1970). Based upon the alterations to collagen which accompany aging and plaque formation in pit fowl, it would seem logical to suggest that increased proline hydroxylase activity may be involved in the production of spontaneous lesions.

Based upon dimensional data the increased thickness of the tunica media, found in all three plaque-containing age groups, probably further reduces the incidence of, or

predisposition to aneurysm (Table III). Increases in chondroitin sulfate complexes are apparently responsible for the bulk of this increased thickness.

The lack of significant differences between plaque and non-plaque animals of 2.5 and 4.4 years of age may seem somewhat astonishing at first. The observation, however, that plaque formation with its accompanying alterations in chondroitin sulfate, elastin, collagen, and protein polysaccharides would indicate that carbohydrate complexes rather than lipoidal compounds are primarily involved in spontaneous lesions. This fact is further evidenced in the fibrotic rather than lipoidal nature of the plaques themselves (Gerity and Steeves, 1970). It is however, suggested that cholesterol may contribute to spontaneous plaque development through normal metabolic conversion to methionine, amino acids, and proteinaceous compounds (Kleiner and Orten, 1966). The observation that serum cholesterol levels exhibit a peak depression phenomenon ranging from 213 to 167 to 215 mg % for animals 1.0, 2.5, and 4.4 years of age (Table VII) may further reflect a demand for, or increased rate of cholesterol utilization in the production of various steroid hormones (Kleiner and Orten, 1966).

The possible significance of this research is manifold: (1) clarification of the role of specific arterial components in the elaboration of atherosclerotic lesions; (2) the demonstration of the involvement of protein polysaccharides in the etiology of spontaneous atherosclerotic lesions; and (3) the characterization and quantitation of these substances are necessary to the understanding of possible regulatory mechanisms and should have significant impact upon the search for clinical solutions to the problems associated with the atherosclerotic condition.

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HISTOLOGY AND HISTOCHEMISTRY OF ATHEROSCLEROTIC LESIONS IN THE PIT FOWL HEN VASCULAR SYSTEM

by

Peter F. Gerity

ABSTRACT

Iliac complexes from pit fowl hens, in 3 groups of 8 animals each having mean ages of 1.0, 2.5, and 4.4 years, were examined histologically and histochemically for spontaneous atherosclerotic plaques. The purpose being (1) clarification of the role of specific arterial components in elaboration of plaques; (2) demonstration of specific protein polysaccharide involvement in the etiology of these lesions; and (3) quantitation of these compounds.

Examination of vessels indicated plaque severity ranged from 2.83 to 4.91 (total intimal involvement) to 3.50 for animals 1.0, 2.5, and 4.4 years of age. Plaque severity data suggest a normal curve with its mid-point at 2.5 years, while plaque frequency data increase linearly from 37.5% to 87.5%.

Spontaneous plaques were characterized by (1) vacuolization of the intima; (2) marked elastic fiber fragmentation in the intimal and medial regions; and (3) alterations to medial collagen and protein-polysaccharides.

Significant ($P \leq .05$) adventitial reduction accompanied aging and plaque development. Media dimensions simulate a normal curve accompanying age, with mean thickness of 79.10 u, 159.52 u, and 98.83 u for birds of 1.0, 2.5, and 4.4 years. All ages exhibited increased medial thickness in plaque-containing vessels. Intima dimensions of the three age groups simulate a normal curve with mean thickness of 27.37 u, 50.29 u, and 36.80 u for animals 1.0, 2.5, and 4.4 years, respectively. Plaque-vessels exhibited significant intimal thickening from 6.0 u, non-plaque, to 65.96 u for plaque-vessels. Mean bore diameters for the three ages approximate a normal curve with 254.52 u, 326.03 u, and 251.61 u being significantly different for animals of 1.0, 2.5, and 4.4 years, respectively. Bore diameters of plaque-vessels were reduced significantly. Iliac O.D. for all ages differed significantly with 666.00 u, 998.64 u, and 662.55 u observed for animals 1.0, 2.5, and 4.4 years, respectively.

Toluidin blue-O, pH 4.9 produced marked staining response, and intense gamma metachromasia in the media of plaque-vessels, indicating carbohydrate alterations. Decreased response to toluidin blue-O, pH 2.6 was observed in plaque-vessels. Intimal response to toluidin blue-O, pH 4.9, and 3.4 exhibited peak intensity at age 2.5 years.

Iliac complexes of animals 1.0, 2.5, and 4.4 years contained 26.2%, 37.1%, and 14.5% elastin, respectively. While non-plaque vessels exhibited a linear increase from 36.7% to 50.3% and 58.8%, plaque-vessels exhibited severe fragmentation and significant decrease in elastin to 8.8%, 32.7%, and 8.2% in birds 1.0, 2.5, and 4.4 years.

Arterial collagen decreased with age from 15.3%, 14.8%, to 7.5% for birds 1.0, 2.5, and 4.4 years. Plaque-vessels of birds 1.0 and 4.4 years of age showed decreased collagen content, while those of 2.5 years increased. The pooled mean, however, indicates significant collagen reduction in plaque-vessels.

Animals 2.5 and 4.4 years of age exhibited no significant difference for serum cholesterols between plaque and non-plaque animals. Significant differences between age groups, however, were noted.