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# Elastin VII: Aging Effects on Vascular Elastica Staining by Oil Soluble Nigrosin Dyes

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*Summary.* Neutral hydroalcoholic stains with spirit soluble nigrosin (C.I. 50415) and nigrosin base (C.I. 50415B) were applied to a series of human arteries from individuals ranging from newborn to 82 years of age for the demonstration of the selective staining by these dyes of the aging change in their elastica described by Lillie, Pizzolato and Donaldson (1974). The staining is absent in infants and children. It first appears in slight grade in some individuals at age 18. It increases in frequency and intensity with advancing age. It is often seen without obvious other histologic lesion and is regularly present when fibrous and fibroatheromatous plaques appear. In this series it was studied in the aorta of children and in grossly relatively normal areas of the superior mesenteric artery which was selected for the survey because of its usual rather slight involvement in arteriosclerosis. The intensity of the neutral nigrosin staining of the elastica of this artery appeared to be uninfluenced by the extent or severity of aortic lesions in the same individual. This nigrosinophilia appears to be an integral early phase in the development of the arteriosclerotic process and may precede appearance of fibrous or fibroatheromatous plaques by some years. The nigrosinophilia has been assigned (1974) to a lipoprotein alteration of arterial elastica. Prolonged storage in formol in plastic bags induced a strong neutral Solvent black 5 and 7 staining of aortic elastica in the normally negative 10–20 year age group. This reaction is presently considered artifactual, but is being studied further experimentally.

## Introduction

In 1974 we reported that acid alcoholic solutions of nigrosin base C.I. 50415B, Solvent Black 7 (SB-7) served as an excellent genereal stain for elastin, and that neutral hydroalcoholic solutions of alcohol soluble nigrosin C.I. 50415, Solvent Black 5 (SB-5) and of SB-7 electively stained elastica of elderly human arteries but not that of young human and laboratory animal arteries. It was further noted that a neutral hydroalcoholic solution of Sudan Black B (SBB) C.I. 26150, Solvent Black 3 with 24 h exposures also stained the elastica of some but not all elderly human arteries. The suggestion was made that there might be an acid lipoprotein complex appearing in aging arterial elastica.

That study was done on rather limited human and animal material and it was considered necessary to expand the study to determine the age limits at which the SB-5 and SB-7 elastin staining (neutral nigrosinophilia) appeared and its relation to arteriosclerotic lesions appearing at necropsy in the arterial system of each individual. It also seemed desirable to appraise the effect of several

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fixations, routine 4% formaldehyde (F), 0.2 M lead acetate $Pb(OOCCH_3)_2$ (Pb), hot methanol chloroform, 2 d, 60°C, (CM) and prolonged storage in 4% aqueous formaldehyde.

During the course of this study a number of other standard and new elastica stains, such as the Weigert resorcin fuchsin, the Taenzer-Unna 1890 acid alcoholic orcein, the Gomori 1950 aldehyde fuchsin, specific tyrosine and arginine methods, all cited from Lillie and Fullmer (1976), stains with Palatine chrome black 6BN, C.I. 15705, diamond black F C.I. 26695, the Clara hematoxylin reaction, the direct Schiff reaction of Feulgen and Voit (1924) and scattered other procedures as cited by Lillie and Fullmer (1976). These were done on a more limited basis, selecting young and elderly human and laboratory animal arteries and gave rise to more extended tests on other dyes. This material it seems advisable to report separately at a later date.

Most of the specimens were accompanied by adequate identifying data and clinical information. Some cases, however, came from special sources with information only as to age and cause of death, but not sex or race.

The superior mesenteric artery was chosen as the object of study because it seemed to present relatively minor gross arteriosclerotic changes in many necropsies of older individuals. Aorta was often used in the youngest age group (0–19), chiefly because it was more readily available in this age group. A few sclerotic arteries from leg amputations were included as examples of advanced lesions of medium sized arteries. Otherwise, advanced lesions were avoided in the selection of histologic material.

## Material and Methods

Human material (superior mesenteric arteries) was obtained from routine necropsies in the Veterans Administration Hospital (VA), New Orleans, performed by one of us (PP) and fixed routinely in buffered 4% formaldehyde 24–48 h, in 0.2 M lead acetate 5–6 h, followed by 2–5 days in 80% alcohol and in hot chloroform methanol according to Lillie and Fullmer (1976 p. 47). No significant differences were seen in the first 25 cases, therefore the multiple fixation practice was stopped. Since this material was predominantly from older persons, we also used material from arteries of the 18–54 age group of a concurrent study of atherosclerosis in New Orleans in the Pathology Department. Arteries in the 0–19 age group were obtained from the Charity Hospital and from the Coroner's office of Orleans Parish. Finally when only 12 cases were accumulated in the 0–19 age group, 12 additional arteries were selected from the collection of oil red O stained specimens which had been stored in 4% formaldehyde in plastic bags for periods ranging up to 10 years.

Staining Procedures: Blocks from aqueous fixations were routinely hardened and dehydrated in alcohol, dealcoholized in ligroin or naphtha and embedded in paraffin as usual. Chloroform methanol blocks were passed through 2 changes of chloroform into paraffin. Paraffin sections were cut at 5–7 $\mu$, dewaxed in xylene and brought to the appropriate stain solvent as usual. Stains are as in Lillie and Fullmer, 1976, with page no. (LF ascited).

1. For appraisal of the histologic status and presence of local lesions sections were stained in acid alcoholic nigrosin base (SB-7) and van Gieson's picrofuchsin:

Dissolve 100 mg SB-7 in 60 cc 99% isopropanol. Add 1 cc conc (12 N) hydrochloric acid and 39 cc distilled water. Filter before using. Stain 4 h, rinse in 50% isopropanol and in distilled water. Stain 5 min in Lillie Weigert (LF 201) iron hematoxylin, wash in water and counterstain 5 min in the Lillie van Gieson (LF 695) variant: 100 mg acid fuchsin, 100 cc 1.25% aqueous picric acid + 0.25 to 0.3 cc conc HCl: then differentiate in 2 changes of 95% alcohol, dehydrate, clear and mount in synthetic resin (usually xylene-cellulose tricaprate).

2. Sudan black B. Dissolve 100 mg in 60 cc 99% isopropanol. Add 40 cc distilled water. Let stand 24 h. Filter and stain 24 h in the filtrate. Wash in water and mount in Apáthy gum syrup. (LF 119).

3. Nigrosin alcohol soluble SB-5 100 mg/100 cc 60% isopropanol. Stain 30 min, wash and mount in Apáthy gum syrup (LF 704).

4. Nigrosin base SB-7 100 mg/100 cc 60% isopropanol. Stain 18 h, rinse in 50% isopropanol, dehydrate, clear and mount in synthetic resin through alcohols and xylenes as usual (LF 709).

These four were the routine stains applied to all cases.

## Results

Results of the Sudan black, spirit soluble nigrosin and nigrosin base stains are presented in Table 1. Stains were graded in individual sections as black or blue black (4) deep gray blue (3), gray blue (2), pale gray or gray blue (1) and negative (0). The figures in the table represent the number of cases in each age group presenting the several grades of staining.

Inspection of Table 1 shows that only pale grays are given by Solvent blacks 5 and 7 at 18–19 in the second decade. A few examples of moderate and even strong staining appear in the 3rd decade, but weak and negative reactions still prevail. By the 5th decade strong stains now predominate over weak, but occasional negative stains are still seen in the 7th and 8th decades.

If the color grades are expressed by numerals, 4, 3, 2, 1, 0 and the several numerals multiplied by the number of cases in each grade and the sum of these figures is then divided by the total number of arteries for each age group a series of average grades is obtained which for SB-5 progresses from 0 for the first decade, 0.5, 1.1, 1.9, 2.3, 2.6, 2.6 and 2.5 in the last decade. For SB-7 the averages are 0, 0.7, 0.9, 1.5, 2.3, 2.0, 2.4, 2.6. The grades are of course subjective, but they do illustrate the progressive increase in the reaction intensity with age.

There is a similar alteration in the frequency of histologically appreciable lesions and in the proportion of muscular media changing from a yellow to a partly red van Gieson stain. Fibrous and fibroatheromatous intimal plaques also increase in frequency and become combined with medial fibrosis so that the number of lesions noted exceeds the number of arteries reported.

In a number of younger arteries more or less intense elastica staining by neutral Solvent blacks 5 and 7 occurred, in the absence of any lesion demonstrable in paraffin sections. No case showing appreciable medial fibrosis or intimal plaque formation lacked neutral Solvent black 5 or 7 staining of its elastica, except for advanced destructive lesions where no elastica remained. Neutral Sudan black B (SB-3) staining of elastica was much less common and less pronounced. It also attained its greatest frequency and intensity in the 5th to 8th decades. The greatest value of this stain lay in its demonstration of ceroid rims about lipid globules, its demonstration of smaller solid globules, and the presence of ceroid phagocytes, enabling the classification as atheromatous or fibrotheromatous.

*Correlation with Gross Cardiovascular Lesions as Observed at Autopsy.* These were graded according to the usual pattern used in this department for studies in atherosclerosis as previously published by Guzman et al. (1968). Those from the Veterans' Hospital series were graded by one of us (PP). Those for the series used to supplement the younger age groups were graded by JPS.

Table 1. Staining of human arterial elastica by age groups with neutral Solvent

| | | Lesion | | | | TU 5015 B | | | | Solvent black 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Elast | | Media | | | | | | |
| | | FA | FP | MF | — | N | CG+ | Y | Y-R | 4 | 3 | 2 | 1 | 0 |
| 15 | 0– 9 | 0 | 0 | 0 | 15 | 13 | 2 | 15 | 0 | 0 | 0 | 0 | 0 | 1 |
| 6 | 10–19 | 0 | 0 | 0 | 6 | 5 | 1 | 5 | 1 | 0 | 0 | 0 | 3 | 3 |
| 9 | 20–29 | 0 | 3 | 3 | 4 | 9 | 0 | 7 | 2 | 0 | 0 | 0 | 1 | 8 |
| 15 | 30–39 | 4 | 3 | 4 | 4 | 14 | 1 | 10 | 5 | 0 | 0 | 0 | 5 | 10 |
| 15 | 40–49 | 2 | 5 | 5 | 3 | 14 | 1 | 10 | 5 | 0 | 0 | 0 | 6 | 9 |
| 23 | 50–59 | 5 | 15 | 14 | 2 | 22 | 1 | 11 | 12 | 0 | 0 | 12 | 4 | 7 |
| 17 | 60–69 | 9 | 5 | 7 | 1 | 15 | 2 | 6 | 11 | 0 | 1 | 2 | 9 | 5 |
| 10 | 70–82 | 3 | 5 | 6 | 0 | 7 | 3 | 2 | 8 | 0 | 0 | 1 | 5 | 3 |
| 12 | 10–20 | 0 | 67 | 0 | 6 | 8 | 4 | 12 | 0 | 0 | 0 | 0 | 2 | 10 |

Lesions are grouped as fibroatheromatous plaques, containing ceroid rims, globules and areas (FA), fibrous plaques with thin to thick collagenous tissue between lumen and an muscularis (MF). Staining with the 3 neutral Solvent blacks is expressed numerically 4 = unstained. Averages are reached by adding the numerical grades of all stains in the group Nigrosin Base C.I. 50415B

The 0–19 age group comprising 19 cases from the Charity Hospital and the Orleans Parish Coroner's office, in which the oil red O gross staining technic was not used, presented no obvious gross lesions of the aorta. However previous studies have indicated that obvious fatty streaks would be seen in many of them after gross fat staining. In the 15 cases 0–5 years of age no neutral Solvent black 5 or 7 staining of elastica occurred. The remaining 4 cases, aged 15, 16, 19 and 19 and the 2 cases aged 18 from the Strong, oil red treated series, gave an average 0.83 reading for Solvent black 5 and an 0.5 for Solvent black 7.

On the correlation of gross atherosclerotic lesions in the aorta with the staining results in the superior mesenteric artery, only the cases from the Strong series were subjected to the gross oil red O staining technic and the incidence of "fatty streaks" is limited to the 26 cases included in that 18–54 age group.

The 26 cases in which the amount of fatty streak involvement was recorded may be divided into two groups of 13 cases, the one with 0 to 17%, the other with 18 to 54% area.

Table 2. Relation of amount of fatty streak involvement of aorta to neutral solvent black staining of superior mesenteric artery elastica in the same cases

| % Fatty streak | Average age | Average FS area | Total RL average | SB-5 and 7 average |
|---|---|---|---|---|
| 18–53 | 36.5 | 36.5 | 8.4 | 1.5 |
| 0–17 | 41.7 | 11.1 | 25.2 | 1.5 |

blacks 3, 5 and 7, sudan black B, alcohol soluble nigrosin and nigrosin base

| Solvent black 5 | | | | | Solvent black 7 | | | | | SB-3 average | SB-5 average | SB-7 average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | 3 | 2 | 1 | 0 | 4 | 3 | 2 | 1 | 0 | | | |
| 0 | 0 | 0 | 0 | 15 | 0 | 0 | 0 | 0 | 15 | 0.0 | 0.0 | 0.0 |
| 0 | 0 | 0 | 4 | 2 | 0 | 0 | 0 | 3 | 3 | 0.7 | 0.5 | 0.7 |
| 0 | 0 | 2 | 6 | 1 | 0 | 0 | 1 | 6 | 2 | 0.1 | 1.1 | 0.9 |
| 0 | 3 | 7 | 5 | 0 | 2 | 2 | 1 | 7 | 3 | 0.3 | 1.9 | 1.5 |
| 2 | 4 | 6 | 3 | 0 | 2 | 5 | 4 | 3 | 1 | 0.4 | 2.3 | 2.3 |
| 5 | 8 | 6 | 4 | 0 | 1 | 6 | 9 | 7 | 0 | 1.2 | 2.6 | 2.0 |
| 3 | 6 | 7 | 0 | 1 | 2 | 6 | 7 | 1 | 1 | 0.9 | 2.6 | 2.4 |
| 2 | 4 | 2 | 1 | 1 | 2 | 2 | 6 | 0 | 0 | 0.7 | 2.5 | 2.6 |
| 8 | 1 | 0 | 2 | 1 | 2 | 2 | 5 | 2 | 1 | 0.2 | 3.1 | 2.7 |

phagocytes, cholesterol clefts or vacuolated yellow stained material as well as collagenous elastica interna (FP), medial fibrosis with areas of collagenous replacement of the medial black or blue black, 3 = deep gray blue, 2 = moderate gray blue, 1 = pale gray, 0 = a fractile, and of dividing by the number of arteries in the age group. TU Taenzer-Unna Technic with

Table 3. Correlation of percentage area of raised lesion (RL) involvement of the aorta with grade of Solvent blacks 5 and 7 staining of elastica in superior mesenteric artery and ocurrence of local lesions in that artery

| Cases | Age average | PS area | RL area | Elastin stain | | Local lesions | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | SB-5 | SB-7 | MF | FP | FA | Ca | Th |
| 14 | 31.1 | 27.9 | 0 | 1.3 | 1.2 | 0.57 | 0.28 | 0 | 0 | 0 |
| 7 | 40.1 | 24.7 | 14.3 | 2.1 | 2.1 | 1.4 | 1.4 | 0 | 0 | 0 |
| 5 | 46.4 | 10.6 | 50.6 | 2.0 | 1.8 | 1.4 | 1.8 | 0.8 | 0 | 0 |
| Severe cases | | | | | | | | | | |
| 1 | 34 | 14 | 51 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| 1 | 45 | 10 | 49 | 3 | 3 | 2 | 3 | 0 | 0 | 0 |
| 1 | 49 | 9 | 43 | 1 | 1 | 0 | 1 | 0 | 0 | 0 |
| 1 | 50 | 10 | 56 | 3 | 3 | 2 | 2 | 2 | 0 | 0 |
| 1 | 54 | 10 | 54 | 3 | 2 | 1 | 3 | 2 | 0 | 0 |

One concludes that the average area of fatty streak involvement had little influence on the average intensity of Solvent blacks 5 and 7 staining of the elastica.

Segregating the same 26 cases into two groups, the one with no raised lesions 14 cases, the other of 7 cases with 29–40% and 5 cases over 40% area involvement, data are presented in Table 3.

Fatty streak (FS) and total raised lesions (RL) are graded on the percentage of the total area involvement of thoracic and abdominal aorta on oil red O stained specimens. Solvent black 5 and 7 staining is rated on a 4= black or blue black, 3= dark gray blue, 2= gray blue, 1= pale gray blue, 0= unstained. Local lesions are graded on a 0–4 basis in order of severity. MF= medial fibrosis, FP= fibrous plaque, FA= fibroatheromatous plaque, Cal= calcified lesion, Th= thrombosis and sequelae.

Here cases with the higher raised lesion area in the aorta show a significantly greater intensity of neutral Solvent black elastin staining. However this difference correlates also with the increased age of the groups. The 5 individual cases illustrate the variation in local lesion and amount of elastin staining which may occur in cases with comparable extent of aortic atherosclerosis.

In the VA cases an older age group is considered. The oil red O technic was not used and fatty streaks were not recorded. Grading is made on a numerical basis 0= no gross lesions, 1= few scattered fibrous plaques in abdominal and thoracic 2= many fibrous plaques, 3= ulcerative plaques and 4= ulcerated and calcified lesions extensive.

Dividing the 47 cases into two groups, 24 graded 0, 1 or 2 on the above plan and 23 graded at 3 or 4, it is seen that the extent of aortic arteriosclerosis has little or no influence on the intensity of Solvent black 5 and 7 staining of elastica in the superior mesenteric artery (Table 4).

Table 4. Aortic lesion extent and Solvent black staining of mesenteric elastica

| Cases | Range of grade | Average grade | Average age | Average SB-5 | Average SB-7 |
|---|---|---|---|---|---|
| 24 | 0–2 | 1.08 | 55.46 | 2.58 | 2.5 |
| 23 | 3–4 | 3.13 | 63.70 | 2.57 | 2.52 |
| 47 | 0–4 | 2.09 | 59.74 | 2.57 | 2.51 |

Cases were seen with extensive aortic lesions and little or no elastin staining, as well as cases with no aortic lesions and strong mesenteric arterial elastic staining, but in general the two processes appeared to both follow the age distribution pattern: Severe lesions and intense staining in the older individuals, less and weaker in younger.

The VA series included also a series of 10 leg arteries from amputations for diabetic or arteriosclerotic gangrene. These comprised advanced fibroatheromatous lesions, some with organized thrombi and recanalization, some with extensive calcification and even ossification. Where enough of the original intima remained, its elastica stained more or less strongly with SB-5 and 7. Some six of these were included in the general tabulation, the heavily calcified and ossified specimens were not.

## Discussion

It appeared from our previous study (1974) that basic nigrosin staining acted as a solubility reaction of oil soluble dyes, with the extractability in dye and fat solvents and restainability after decoloration which is characteristic of fat stains.

This lipid type alteration of vascular elastica is constantly present in the presence of arteriosclerotic lesions. It appears a decade or so earlier than the histologically evident lesions in paraffin sections, and reaches a high grade a similar period earlier than the fibroatheromatous lesion. It therefore appears to be an integral, and early phase of the arteriosclerotic process. It will be interesting to correlate its appearance with that of the other early phase of the process, the fatty streak of the intima.

The work of Hoff et al. (1975) in localizing lipoprotein to vascular elastin and collagen by immunofluorescence methods probably parallels the present study in its significance. However, the present study appears to indicate the appearance of lipid in elastica not only in the presence of obvious arteriosclerosis, but before the appearance of intimal and medial fibrous lesions.

The Sudan black procedure, by its black outlining of fat droplets and needle shaped cholesterol clefts and the demonstration of ceroid phagocytes enabled the distinction of an intimal plaque as at least partly atheromatous as distinguished from purely fibrous.

Since the 10–19 age group included only six cases a group of 12 aortas were selected from the old oil red stained formaldehyde fixed and stored specimen collection, ranging from 10 to 20 years of age. These 12 cases are tabulated separately at the foot of Table 1. This group, in contrast to the routine 10–19 age group presented the strongest average Solvent black 5 staining of this whole series and the neutral Solvent black 7 staining was comparable with that seen in the 40–82 age groups. This alteration in staining reactions seems definitely assignable to the prolonged exposure to formaldehyde and the protein alterations entailed thereby. It appears from this material that such tissues are not suitable for study of age alterations in elastica staining.

In general the amount and severity of arteriosclerotic involvement of the aorta and the nigrosin staining of the mesenteric arterial elastica both follow the pattern of increasing with age. But these changes in the mesenteric artery in individual cases may differ sharply from the general aortic involvement. The appearance of local lesions in the mesenteric artery varied sharply in individual blocks taken from successive levels of the same artery. But nigrosinophilia of the elastica has been present in more cases than local lesions, and in the presence of such lesions is always present except where in very advanced lesions with organized thrombi, the original intima is completely replaced. Elastic nigrosin staining appears to develop at perhaps the 18th year, before the advent of raised lesions. Material was not adequate between the 5th and 15th year to permit a statement as to whether it precedes or follows the fatty streak phase. In any case it appears to constitute an early phase in lesion evolution in the individual artery rather than being a local expression of a generalized condition of the vascular system.

While the 20–49 age group appeared to show stronger Solvent black elastin staining in the presence of moderate aortic lesions than without them, it is to be noted that the lesion bearing group averaged 43 years of age, the lesion-free group only 31, which could probably account for the staining difference by itself. And in the older group, above 50, the presence of extensive versus minor aortic disease made essentially no difference in intensity of neutral Solvent black 5 or 7 elastin stains in the mesenteric artery.

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