Correlation of Intimal Sudanophilia and Cholesterol Analyses of 3 Layers of Human Aorta.*† (24010)

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The purpose of this report is to present the the results of cholesterol analyses of 189 samples from a single human aorta which had been stained *in toto* with a fat stain, Sudan IV. Other investigators(1,2,3) have performed chemical analyses including cholesterol determinations on lesions of atherosclerosis, but there has been no attempt, as far as we can ascertain, to correlate intimal sudanophilia with cholesterol concentration in the manner to be described.

This study was undertaken to determine degree of correlation between sudanophilia and cholesterol concentration in human atherosclerosis and whether or not there is a gradient of cholesterol concentration in any of the aorta's 3 dimensions. Since fat stains such as Sudan IV are used for selective staining of atherosclerotic lesions in humans(4-7), and the lesions resembling atherosclerosis in experimental animals(8,9); and since cholesterol is a constant component of atherosclerotic lesions, a study of this nature seemed indicated.

Materials and methods. A single aorta was stained grossly with Sudan IV and divided into 189 blocks. Cholesterol analyses were performed on these blocks. The aorta was obtained 3 hours post mortem from a 25 year old white male who died an accidental death. This case was selected to minimize any effect of terminal illness on aortic lipids. The vessel was opened anteriorly, stripped of adventitial fat, flattened on chipboard, and fixed 24 hours in 10% neutral formalin. Following fixation the entire aorta was stained with Sudan IV (saturated solution of Sudan IV in equal parts of acetone and 70% ethanol) for 15 minutes, differentiated in 80% ethanol for 20 minutes and washed in tap water for one hour. The staining solution, differentiating solution, and wash waters were retained for cholesterol analysis. Fig. 1A depicts the aorta after staining with Sudan IV. Prior to staining there were noted scattered pale yellow streaks and spots on the intimal surface of the aorta, which were most prominent in the abdominal portion. They stained red with Sudan IV and appear black in the photograph. There were no fibrous plaques or complicated lesions (ulcerated, thrombosed, or calcified) present. Fig. 1A illustrates the 63 pieces into which the vessel was initially divided. Each of these pieces was placed flat on a freezing microtome with the intima up-

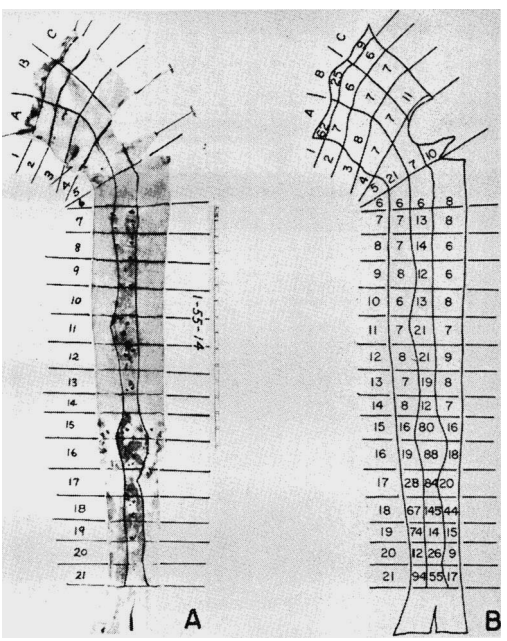

FIG. 1. A. Intimal surface of aorta showing areas of Sudan staining and the 63 pieces into which aorta was initially divided. B. Cholesterol values of intimal layer of aorta expressed to nearest whole number.

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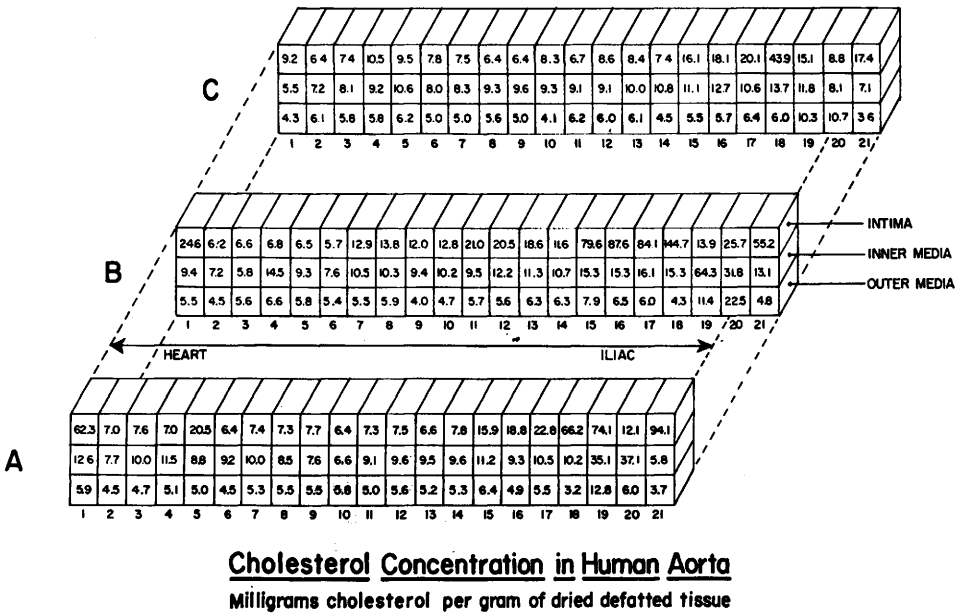

FIG. 2. Exploded block diagram of aorta with cholesterol values expressed as mg/g of dry defatted tissue for intima, inner media and outer media.

permost and quick frozen. A thickness of approximately 160 M was sliced from the intimal surface. This thickness was selected because it was found that this removed the stained portions of the intima from the media in deeply stained areas. The remaining media was divided into 2 approximately equal portions and referred to as inner media and outer media. This yielded 189 specimens for cholesterol analysis. Each sample of tissue was extracted with Delsal's solution(10) with 5 changes of solvent. The first, second, fourth, and fifth changes were decanted after 5 minutes. The third solvent change was allowed to stand 12 hours. The combined solvent extracts were evaporated at 60°C under nitrogen and the lipids re-dissolved in 1:1 acetone-alcohol for a single determination of cholesterol by the Sperry-Webb(11) procedure. The residual tissue was dried at 60°C *in vacuo*, placed under desiccation and weighed repeatedly until a constant weight was obtained. The total amount of cholesterol remaining in the dried defatted tissue was determined by hydrolysing the combined tissues with 30% potassium hydroxide for 16 hours at 100°C, extracting the hydrolysate with ether, and analysing in-replicate for cholesterol according to the Sperry-Webb procedure.

Results. Fig. 2 depicts in an exploded block diagram of the aorta the values for cholesterol expressed as mg cholesterol/g of dried defatted tissue. Fig. 1B shows cholesterol values of the intimal layer for comparison to the photograph of the stained aorta (Fig. 1A). The stained intima has a distinctly higher cholesterol concentration than the unstained intima. The unstained intimal values vary from 6 to 9 mg cholesterol/g of dried defatted tissue, and the stained intimal values vary from 12 to 145 mg with higher values from blocks with more intense and extensive Sudan staining. Values in the inner media and outer media are relatively constant with the inner media averaging 11.7 and the outer media averaging 5.9 mg cholesterol/g of dried defatted tissue. Medial specimens showing widest deviation from usual range of values have penetrating blood vessels in or immediately adjacent to the block.

Analysis of the Sudan IV stain, decolorizing alcohol, and wash waters for cholesterol revealed that 5.6% of total cholesterol recovered from the vessel was removed in the process of staining. Of this 5.6% cholesterol removed in the staining process, 4% was re-

moved in the Sudan IV staining solution, 1.6% in decolorizing alcohols, and none in wash waters. Analysis of the dried defatted tissues by alkali digestion revealed that 0.45 mg or 0.8% of total cholesterol recovered remained in the tissue.

Discussion. Studies of early lesions of atherosclerosis in the human aorta have shown that gross Sudan staining of the entire aorta facilitates detection of early lesions(6). Sudan IV stains all lipid substances except those with high melting points. Cholesterol, excluding low melting point esters, is not stained by this dye(12). Since increased amounts of cholesterol are present in atherosclerotic lesions we were desirous of knowing the relationship between cholesterol concentration and sudanophilia.

This study shows that the extent and intensity of Sudan staining is directly proportional to cholesterol concentration even though cholesterol does not stain with Sudan. This indicates that cholesterol when present in increased amounts is rather constantly associated with other lipids, such as triglycerides, which do stain with Sudan. Areas of the intima, completely free of any staining with Sudan IV, ranged in value from 6 to 10 mg cholesterol/g of dried defatted tissue while those areas that stained with Sudan ranged from 12 to 145 mg cholesterol. Block 19B is the single notable exception to the rule having a cholesterol value of 14 mg and taken from a heavily stained area. This discrepancy is probably due to incomplete removal of the intimal layer since the underlying media has a cholesterol concentration much higher than the mean for that area.

With little exception the cholesterol concentration of the inner and outer layers of the media is quite constant. The values for the inner media lying beneath stained areas are in general higher than that beneath unstained areas which probably reflects incomplete removal of the fatty deposit. Consideration of medial values and unstained intimal values gives no evidence of a gradient of cholesterol concentration along the length of the aorta for man as described in experimental animals (13). In the unstained areas the intimal value is greater than for the outer media, and both of these values are usually less than the value for the inner media.

The staining process removed 5.6% of total cholesterol from the aorta. This is a rather small amount but it must be remembered that this 5.6% probably came first from the surfaces of the vessel in closest contact with the solvents, and principally from those areas containing the largest amounts of cholesterol. It is possible that the generally lower level of cholesterol concentration in the outer media is related to this rather than being a truly lower value.

Summary. A single aorta from a 25-year-old white male was stained grossly with Sudan IV and divided into 189 specimens for cholesterol analysis. The results show that the extent and intensity of Sudan staining is directly proportional to cholesterol concentration in the tissue. Analysis of the data reveals no evidence for a gradient in cholesterol concentration along the length of the aorta. The cholesterol value of the inner media is generally higher than either the intimal or outer media cholesterol concentration in areas where no lipid deposit is contained in the intima. The cholesterol concentration in the outer media is consistently lower than the value for the intima or inner media.

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Immunologic Significance of the Cell Wall of Mycobacteria. (24011)

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Separation of bacterial cells into their major morphological elements (cell wall and internal protoplasm) by a method first described by Salton and Horne(1) offers a new approach to antigenic analysis of bacteria. When aqueous suspensions of bacteria are vibrated with minute glass beads, the rigid cell wall cracks, and protoplasm is liberated in a dispersed state. Cell walls are then separated from protoplasm by differential centrifugation. Examination of a fungus and several bacteria by this technic showed that important antigens are situated in the cell wall(2-6).

Whereas the procedure of Salton and Horne is directly applicable for fractionation of certain bacterial species, it cannot be successfully applied to mycobacteria unless special measures and precautions are taken. To achieve selective disruption of mycobacteria, suspensions of washed cells must be monodispersed when exposed to mechanical vibration, and products of disruption must also be maintained in a dispersed state to allow final separation of cell wall from protoplasm.

Methods. *Mycobacterium butyricum* and 2 strains of *M. tuberculosis* (BCG and H37 Ra) were studied. *M. butyricum* was grown at 37°C for either 3 days on solid Meadow agar medium(7) or for 24 hours in liquid Meadow medium. BCG and H37Ra cultures were grown in stationary liquid Dubos Tween-albumin medium(8) for 8 to 10 days at 37°C. Bacterial cells were collected and washed by centrifugation in cold water containing 0.5% Tween. Washed bacilli could be adequately redispersed in aqueous Tween. *M. tuberculosis* grown on solid or liquid media in which granular growth occurred did not disperse adequately in Tween solution. All procedures described below were performed at 4°C. Suspensions of dispersed washed bacteria were diluted with Tween solution to a concentration having an optical density of 700 mμ in a Klett-Summerson nephelometer with filter No. 420. Five ml of suspension mixed with 5 g Cataphote No. 12 glass beads were shaken in the Mickle tissue disintegrator for 10-second periods for a total shaking time of 4 minutes. Agitation was discontinued between each of these periods for 10 to 30 seconds to avoid raising the temperature of the suspension. About half the cells were broken after this mechanical treatment. It must be emphasized that products of the Mickle process have a marked tendency to clump during vibration and prolonged treatment will produce aggregates to such an extent that cell walls cannot be obtained. Under the experimental conditions employed, a vibration period of 4 minutes was usually suitable. However, the optimum vibration time for different lots of cell suspension must be individually determined by electron microscopy of samples taken from the Mickle tubes. Difficulties regarding aggregation were not encountered during mechanical disintegration of other bacteria.* The mixture of nondispersible aggregates, intact cells, cell walls, and protoplasm was allowed to stand until the glass beads settled. The suspension was then decanted into conical 12-ml tubes, centrifuged, and supernatants containing protoplasm were removed. All cen-

* We recently observed that a commercial pressure cell can be utilized in place of the Mickle apparatus to increase the yield of cell-wall material.