STUDY GROUP ON CLASSIFICATION OF ATHEROSCLEROTIC LESIONS

The Director-General has the honour to present to the Executive Board the report of a Study Group on Classification of Atherosclerotic Lesions.¹

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REPORT OF THE STUDY GROUP ON THE CLASSIFICATION OF ATHEROSCLEROTIC LESIONS

held in
Washington D.C., United States, 7-11 October 1957.

CONTENTS

1. Introduction ........................................ 5
2. Adoption of the Agenda ................................ 6
3. Definition of Terms ................................... 6
4. Classification and Grading of Atherosclerotic Lesions .... 7
5. Geographic Pathology of Atherosclerosis ................. 15
6. Co-ordination of Studies ............................ 16
7. Classification of Experimental Degenerative Arterial
   Lesions of Animals ................................. 18
8. Conclusion ........................................... 18

Annex I Diagrams for Comparative Atherosclerosis Studies ..
Annex II Technique of Preservation of Specimens in
   Plastic Bags ........................................
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1. INTRODUCTION

The Study Group on the Classification of Atherosclerotic Lesions met in Washington, D.C., from 7-11 October 1957, to discuss the possibility of developing a classification with uniform terminology for use in the world-wide study of atherosclerosis and to determine the feasibility of establishing acceptable methods of grading of atherosclerotic lesions. This would have all the advantages of a common language, making possible the universal understanding of the qualitative and quantitative implications of the terms applied to characterize these lesions and would allow pathological comparisons of a world-wide scope.

Dr J. S. Peterson, in opening the meeting on behalf of the Director-General, drew attention to the statement made in Section 3.1 of the report of a Study Group of Atherosclerosis and Ischaemic Heart Disease held in 1955; as follows:

"For post-mortem diagnosis, the pathologist may select the number or the surface area of atheromatous plaques, the degree to which the arterial lumen is narrowed, or the presence or absence of calcifications, ulcerations, or thrombi. In assigning grades to any coronary artery disease found, different observers assign a different weight to these several factors. It seems highly desirable that a group of representative pathologists should meet and make suggestions on uniform methods for examining the coronary arteries and for recording the results. Furthermore, in applying such uniform methods to different populations, efforts should be made to reduce observer error to a minimum."

He added that among the recommendations of this Group was one on the need for the standardization of pathological criteria and terminology in respect to atherosclerotic, ischaemic heart disease, and related conditions which appeared so urgent that it was hoped that WHO would give consideration to the organization of a study group for the purpose of agreeing upon methods of examining, assessing, and reporting on necropsies with particular regard to coronary-artery and myocardial lesions. It is on the basis of these recommendations that this present Study Group was convened. A future step would be the convening of an expert committee in 1958 to deal with the question of cardiovascular disease and hypertension from a broad point of view. Thus, the present meeting is an important step in the long-range programme of the World Health Organization in studying, exchanging information, and seeking control measures in the field of atherosclerosis.
Dr Fred L. Soper, Director of the Pan American Sanitary Bureau, Regional Office for the Americas of the World Health Organization, welcomed the members and thanked the officials of the National Research Council for their generosity in providing the meeting place.

Dr J. Watt, Director of the National Heart Institute (USA) which had provided the grant to make the meeting possible, added his welcome to the Group.

Dr J. C. Paterson was then unanimously elected chairman. Dr P. N. Wahi was elected vice-chairman and Drs J. de Brux and J. P. Strong were elected rapporteurs.

2. ADOPTION OF THE AGENDA

After agreeing upon the definition of atherosclerosis given in 3 below, the following agenda was adopted by the Group:

(1) discussion of a classification of atherosclerotic lesions and procedures for processing of specimens;

(2) geographic pathology of atherosclerosis;

(3) establishment of regional centres;

(4) classification of experimental lesions.

3. DEFINITION OF TERMS

An attempt was made to arrive at a definition of atherosclerosis which would be as all-inclusive as possible rather than exclusive.

Atherosclerosis, as defined by this Study Group "is a variable combination of changes of the intima of arteries (as distinguished from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes". A deliberate attempt was made by the Study Group to distinguish this process from the arteriolar changes of hypertension, diffuse intimal thickening, Mönckeberg-type sclerosis, and infectious and allergic arterial changes.

The following definitions were also proposed and accepted:

The term "atheroma" has been used by some workers as synonymous with atherosclerosis. It was not so used by the present Group.

The term "arteriosclerosis" has been avoided by the Group in view of the differing connotation of the term as used by various workers.
(a) the term "fatty streak or spot" is applied to superficial yellow or yellowish-grey intimal lesions which are stained selectively by fat stains. It is not synonymous with atheroma;

(b) the term "fibrous plaque" is applied to a circumscribed, elevated intimal thickening which is firm, grey or pearly-white;

(c) the term "atheroma" is applied to an atherosclerotic plaque in which fatty softening is predominant;

(d) complicated lesions are defined as lesions with additional changes or alterations such as haemorrhage, thrombosis, ulceration, and calcareous deposits.

The Group recognized that there is wide divergence of opinions concerning the pathogenesis of atherosclerosis and no attempt was made at this time to reach an agreement on the progression of various changes from the earliest recognizable lesions to the onset of clinical disease.

4. CLASSIFICATION AND GRADING OF Atherosclerotic lesions

4.1 General Considerations

The Group strongly believed that classification should be based on objective pathological findings rather than on specific schemes of pathogenesis. For immediate purposes atherosclerosis should be considered to be the same process in different arteries.

Grading of atherosclerotic lesions should always be carried out by:

(a) gross examination with the naked eye,

but supplemental use of:

(b) microscopic examination,

(c) other methods, such as chemical analysis, electron microscopy, histochemistry, radiological examination, etc.,

may be very helpful.

There was agreement that the tiny mucoid elevations of the intima, which may also be detected microscopically, are "physiological" in origin and should not be regarded as atherosclerotic lesions.
It was the opinion of the entire Group that relatively simple methods for the quantitation of lesions are needed for a valid determination of the incidence and extent of atherosclerosis in various parts of the world. The desirability of using grossly visible changes was also stressed. Fatty changes should not be unduly emphasized and they should be considered as only one of a number of readily recognizable forms of atherosclerotic change. Simplicity was uppermost in the minds of most of the participants and it was recommended that only the lesions listed under 3a, b, c, and d above be considered. It is to be noted that the listing in paragraph 3 is not necessarily in order of development.

The Study Group considered it of extreme importance that any comparisons be based on the examination of material treated in the same manner in all age-groups. The value of staining of gross specimens with Sudan or other fat-soluble dyes was stressed for the demonstration of fatty streaks and spots in young individuals (below the age of 30 years). It is questionable if Sudan staining is of any value in older age-groups.

Comparability of the classification of atherosclerotic lesions by different investigators can best be achieved by the development and use of standardized and objective methods of making and recording observations. Complete objectivity can rarely be obtained. However, quasi-objective procedures often can be combined with the subjective classification of observations so as to increase the degree of comparability in the reports of different investigators. For example, instead of simply classifying the extent of atherosclerotic lesions as slight, moderate, or severe, without definition of these terms, an index of extent, such as an estimate of the percentage of total surface area involved, may be used.

The usefulness of this index will depend upon the similarity in estimates of the percentage of surface area involved made by different observers on the same lesions, or made by a single observer at different times. For this index there is some evidence that agreement may be acceptably close for lesions that involve either only a small or a very large proportion of the surface. The index would yield reasonably standardized observations on the proportion of very extensive or very minimal lesions in different population groups. This would be worthwhile even though the index is not sufficiently precise to warrant a finer classification of the extent of lesions.
Reversibility of atherosclerotic lesions is a possibility to be considered but there is as yet insufficient evidence to permit a definite conclusion. This factor, however, is unlikely to affect significantly any geographic or other comparisons. There is evidence that malnutrition is compatible with the presence of fatty streaks in the aorta.

4.2 Selection of Material

Every attempt should be made to collect material from comparable sources and detailed attention should be paid to the relative merits of general hospital autopsies and medicolegal autopsies. In some areas there is very little difference in the amount of aortic atherosclerosis from these two sources.

4.3 Macroscopic Grading

The following recommendations are made for classification and registration of atherosclerosis in:

- Coronary arteries
- Cerebral arteries
- Aorta
- Pulmonary arteries
- Renal arteries
- Mesenteric arteries

It is recommended that the changes in the coronary and cerebral arteries and in the aorta be recorded in all cases. It is desirable that the changes in the pulmonary, renal and mesenteric arteries also be noted. All the data should be recorded on charts and plotted on diagrams. (Annex I)

In the interest of later study it is desirable that the arteries listed above be fixed in adequate amounts of 10 per cent. solution of formalin or Kayserling I. A method of preserving specimens in plastic bags has been found useful. (See Annex II) Specimens preserved for histological study, including that of lipid substances, may preferably be fixed in Baker's 10 per cent. formalin which contains 1 per cent. each of calcium chloride and cadmium chloride.
4.3.1 Coronary arteries

Coronary arteries may be examined either by cross-sectioning at intervals of 5 millimetres or by lengthwise opening. If the method of transection is elected, this should be followed by lengthwise opening.

Segments of stenosis should be especially recorded and expressed as, (a) moderate stenosis (more than half the diameter of the lumen preserved) and (b) severe stenosis (less than half the diameter preserved). In view of the small calibre of the vessels involved, no further grading of stenosis appeared to be useful. It should be stated whether stenosis is diffuse or localized and, if so, whether in one or several sites.

The predominant (most numerous) type of lesion, as defined in 3a, b, c, and d, and location of the involvement should be noted (Annex 1, page 2).

When complicated plaques are present, even if not predominant, they should also be noted.

4.3.1.1 Myocardium

The weight of the heart should be given. It is essential to report on the state of the myocardium with regard to the presence of fibrous scars or infarctions or both.

The changes may be determined by the classical method of dissection (Virchow) or by the method in which a cross-section through the heart is made parallel with the atrioventricular ring about halfway between the latter and the apex of the heart. The diagrams in Annex I, page 3, include provision for the plotting of the myocardial changes by either method.

4.3.2 Cerebral arteries

The same standards as for coronaries should be used. The description concerns the arteries at the base of the brain (circle of Willis), including the middle cerebral arteries. The changes should be plotted on the diagram (Annex 1, page 4). Cerebral lesions of vascular origin should be described.

4.3.3 Aorta

As a rule the description should apply to the entire aorta, but for quantitative grading it is suggested that only the descending thoracic and abdominal aorta, in a
segment between the upper margin of the first intercostal arteries and the bifurcation, should be considered. The examination should be performed before fixation. A gross photograph of the aorta may be used to supplement the recording of lesions on a diagram.

For grading of the aorta, two estimations should be made:

(a) total extent of surface involvement - percentage of intimal surface occupied by all types of lesions;
(b) types of lesions - percentage of the total amount of lesions contributed by each constituent type (5a, b, c, d).

These should be recorded in the table in Annex 1, page 5.

The circumference of the aorta at three different levels should be measured and recorded on the diagram (Annex I, page 5):

(a) above the ostia of the coronary arteries;
(b) at the level of the fifth intercostal arteries;
(c) immediately above the origin of the renal arteries.

4.3.4 Pulmonary, mesenteric and renal arteries

Where possible these arteries should also be examined.

4.4 Microscopic Classification

The Group divided the complex histological picture of atherosclerosis into discrete components without taking a stand concerning histogenetical sequence. This permits histological grading of single atherosclerotic plaques, which may be valuable in characterizing special cases. For this study it was thought useful to consider only two kinds of arteries, typified by the aorta and the coronary arteries.

Histological changes found in the aorta include:

(a) fibrin-like film attached to the intimal surface or covered by endothelium;
(b) metachromatically staining material (complex carbohydrates?) increasing the intimal thickness;
(c) lipid deposition, either extracellularly or intracellularly (foam cells) in the form of droplets of variable size;
(d) fibroplasia, largely confined to the subendothelial portion of the intima, in the form of mucopolysaccharides, increased amount of reticulin, collagen fibres and hyalinization;
(e) calcification in fine or coarse granules;
(f) cholesterol crystals, finely granular amorphous glyco-protein material and ulceration;
(g) vascularization, extravasated red corpuscles, hemosiderin and related by-products;
(h) medial changes such as, lipid infiltration, disintegration of smooth muscle fibres, disruption of elastic fibres, cellular infiltration around vasa vasorum, and mucoprotein accumulation or alteration;
(i) secondary changes such as thrombosis with its consequences.

All these lesions can be found in the coronary arteries but additional significance should be attached to the presence of narrowing of the lumen and to large intramural haemorrhages.

4.5 Special Methods of Grading

The Group recommended that further research should be initiated to establish the objectivity and practicability of the following techniques based on physical, chemical or morphological characteristics in relation to the grading of atherosclerotic lesions. Priority should be given to development of methods for those arteries where dysfunction is most liable to result from atherosclerosis, notably the coronary and cerebral arteries.

4.5.1 Measurement of elasticity of arteries

A rough measure of the elasticity of the aorta can be obtained by cutting across the vessel in situ and noting the distance to which the severed ends retract. More complete measures can be obtained by inflating the excised organ with a rubber balloon under various pressures and measuring the range of expansion and recoil by water displacement. Elasticity of the vessel, since it is largely a function of its medial coat, varies with the degree of post-mortem contraction. Measurements obtained are therefore extremely variable and tend to alter as the muscle deteriorates. Nevertheless a rough measure of impairment is obtained from the differences in volume recorded at different pressures and, by standardizing the pressure ranges, a coefficient can be obtained.
The above method is not readily applicable in coronary arteries, but a measure of expansibility can be obtained by injecting radio-opaque fluids of standard viscosity at different pressures and taking skiograms at appropriate intervals. This is a particularly useful method of examining the coronary arteries since it reveals foci of narrowing.

4.5.2 Estimation of consistency and viscosity

Compressibility, flow and viscosity of the elements of the arterial wall can be measured objectively by standard biophysical methods.

4.5.3 Radiography

4.5.3.1 The injection of radio-opaque substances into the coronary artery system at various pressures.

4.5.3.2 Use of image amplifier radiological techniques in the clinical demonstration of vascular lesions. The image-amplifier is a commercially available device to multiply up to one thousandfold the intensity of fluoroscopic or radiographic images. It permits high-speed cinematography and subsequent projection at slow speeds thereby retarding recorded movement. With it, cardio-pulmonary-vascular anomalies have been analysed in living subjects injected intravascularly with safe amounts of inert excretable radio-opaque solutions. The method is already being applied not only to children but adults and should be capable of elucidating disturbances of cardiovascular function.

4.5.3.3 Post-mortem radiological examination of arteries.

4.5.4 Cool-dry methods

The aorta is opened, stripped of its adventitia and outer medial coats, stretched flat on a glass plate and left for 36 to 48 hours in an ordinary electric refrigerator. The effect is drying of the tissues which become semi-transparent revealing vascularization and haemorrhage of the intima, and their relation to atherosclerotic plaques. The vessel can be mounted between glass, readily handled, and transported without deterioration.
4.5.5 Determination of the extent of pathological constituents by electronic scanning and fluorescence

Devices should be developed to measure surface unevenness. Concentration of histochemically demonstrated materials (fat, muco-protein, etc.) should be charted and expressed as percentage of area. Objective measurements could be done most readily with some form of the electronic scanning devices. The electronic scanner for fat might be combined with fluorescence after benzypyrene.

4.5.6 Morphological methods

It can be shown that formalin fixation of the aorta at systolic blood pressure alters the morphology of the atheromatous plaque, thinning the plaque down and decreasing the projection into the lumen to some extent. There is, then, in the use of unfixed specimens, or specimens fixed in the open state (i.e. at atmospheric pressure) a distortion of appearance. This is not a constant distortion in that elasticity of the vessel can vary with age and with non-atherosclerotic disease.

Methods should be devised using fixation at blood (systolic or diastolic) pressure levels. Body temperature fixation might be desirable since internal body fats are probably fluids at body temperature levels. A combination of fixation at blood pressure and at body temperature should be devised.

4.5.7 Simple chemical analysis

4.5.8 Spectrographic methods for the estimation of trace elements

4.5.9 Fluorescent immunochemical methods

4.5.10 Histochemical techniques

Sudan and Oil Red 0 staining methods for fats are by no means the most precise or most modern methods. Sudan Black B, Luxol Fast Blue B, and fluorescence after benzyprene merit study.

Metachromasia with toluidine blue, staining with Hale's colloidal iron or with Alcian Blue offer methods of carbohydrate staining worthy of exploration.

Among the enzyme methods, only lipase and 5-nucleotidase demonstration techniques have been applied to arteries and none have been applied grossly to grading. Oxidative and other enzymes, methods for which have been refined recently, offer potential techniques for gross grading.
5. GEOGRAPHIC PATHOLOGY OF ATHEROSCLEROSIS

The Group noted that there exist marked geographical differences in death rates from cardio-vascular disease in general and from arteriosclerotic and degenerative heart disease in particular, for countries of the world for which data are available from the Epidemiological and Vital Statistics Reports of WHO. Some of the differences are related to lack of medical facilities and incompleteness of registration while some are probably due to differences in terminology used in certifying causes of death and in classification of such causes. The latter is thought to be true in countries such as France and Germany, which have different rates for the common causes but have very similar living conditions. Material presented to the Group indicated wide need for considerable improvement in quality of basic data used for mortality statistics of the cardiovascular diseases.

It was agreed that the value of vital statistics is enhanced in proportion to the deaths confirmed by autopsy findings. Pathologists may thus play a much greater role in certifying causes of death. Death certificates should indicate whether an autopsy has been performed.

To improve vital statistics it is suggested that comparative studies of death certificates of different countries be made to understand the terminology in use and to promote comparable procedures of stating the underlying cause of death and of classifying these causes in accordance with the International Classification of Diseases (WHO, Geneva). In countries where outstanding differences are noted, studies should be extended by use of specific autopsy findings. For these studies use should be made of centres for classification of diseases such as that for Latin America in Caracas and for the world in London.

Studies of pathology related exclusively to prevalence of atherosclerosis give only part of the picture. Relationship between atherosclerotic changes of different vessels and the organs they supply is not a simple one since severe atherosclerosis may occur without lesions of the organ involved and, on the contrary, a slight degree of atherosclerosis may lead to vascular occlusion and fatal infarction. Atherosclerotic lesions are considered to be a necessary prerequisite for coronary thrombosis and

1 Vol. 9, No. 10, 1956 and Vol. 10, No. 5, 1957
myocardial infarction. Because the distinction between atherosclerosis of coronary vessels and myocardial infarction is not always made clear, a clinical and pathological diagnosis of "coronary heart disease" lacks precision. Records of clinically established myocardial infarcts are not always valid as a standard for the incidence of heart disease due to atherosclerosis since some cases of coronary occlusion may die before recognizable infarction occurs. In addition, asymptomatic myocardial infarcts, which are not infrequent, may make difficult the correlation between clinical diagnoses and autopsy findings. In some regions mortality and autopsy figures may be obscured by the fact that unidentified forms of "heart disease" may not be generally recognized as distinct from myocardial infarction. Studies of relationships between atherosclerotic lesions and mortality should be made, and, as much as possible, on unselected material.

6. CO-ORDINATION OF STUDIES

To carry out the programme for the pathologic study of gross and histologic lesions of atherosclerosis and to develop the associated epidemiological studies, the following structural framework might be considered. This framework could be similar in many respects to that recommended to WHO by the recent Oslo Study Group for the planned WHO Study of cancer.

1. An Expert Advisory Panel to WHO
2. An International Centre
3. National and Regional Centres
4. Co-operating Hospitals, Laboratories, and Institutes
5. An International Pathology Group

It was the belief of the Study Group that promotion and co-ordination of world-wide studies by WHO would be desirable. Co-operation of international, non-governmental scientific organizations would be helpful.

6.1 Material to be Collected

Collection of pathologic material of atherosclerotic lesions should be started in a small way. Too ambitious a programme should not be undertaken at the beginning. Detailed methodology will unfold as the programme develops, including details as to records, staff and training facilities.
6.2 Functions and Operations

The international centre for the study of lesions of atherosclerosis will obtain case material to study from its own resources, from the regional and national centres, and from other collaborating pathology laboratories and institutions. This material will consist of well-documented cases sufficient in number to establish gross and microscopic definitions of the different types of lesions and to answer any other questions that are posed by this study. There should be a free exchange of material between the International centre and the regional and national centres. When the results of the work of studying and defining the lesions of atherosclerosis have reached a suitable stage, study sets consisting of gross specimens, moulages, histologic slides, and related descriptive and illustrative material will be prepared and distributed to the various national and regional centres for circulation to the pathologists of the different countries as a guide for classification and grading.

As a result of intercommunication among pathologists in widely scattered parts of the world new opportunities for investigation work will be presented. Possibilities for significant research in atherosclerosis may be present in geographic areas totally lacking in funds for such research, and support may be sought from a variety of agencies.

In addition to the development of a network of centres, co-ordinated individual and team pilot studies might be useful, and such studies should be encouraged. Further, it is highly desirable that individual studies be correlated by promoting general adoption of a standard research protocol.

6.3 Environmental and Epidemiological Studies

The influence of environment on atherosclerosis needs study. In the work of the different pathology centres, therefore, every effort should be made to encourage epidemiological and statistical research. Investigation of other related problems should also be encouraged and facilitated. It may be desirable to associate with one of the national or regional laboratories a special group of scientists to study the relationship between diet and atherosclerosis, or between the use of certain drugs and atherosclerosis.
7. CLASSIFICATION OF EXPERIMENTAL DEGENERATIVE ARTERIAL LESIONS OF ANIMALS

Naturally-occurring arterial lesions in many animal species are far from rare, and it is mandatory that special sampling procedures be carried out to identify their quantity and quality before any claims are made on the experimental induction of arterial disease. Serial sections of large segments of arteries, at fairly wide intervals, are recommended for this purpose. If this is done in exactly the same fashion in controlled and in treated animals, one can readily distinguish between induced lesions and naturally-occurring lesions which are merely accelerated. At the same time a good estimate of the severity of the induced disease, or of the accelerated disease, can be obtained.

In experimentally produced arterial lesions in animals we are presented with a complex of changes which are even more susceptible to misinterpretation than are atherosclerotic lesions in man. Their relationship to atherosclerosis in man has not been clearly established.

8. CONCLUSION

The Study Group believes that a beginning has been made in the assigned project in that atherosclerosis has been defined, standards have been agreed upon for grading, and a suggested international programme has been outlined. The task has been only begun. Continued participation by pathologists is essential for its successful prosecution and completion.
ANNEX I

DIAGRAMS FOR COMPARATIVE ATHEROSCLEROSIS STUDIES

1. Data to be included in tabulating necropsy reports on atherosclerosis

They should include:

A. Identifying information
   - Name and country of hospital or institution
   - Hospital or institutional number or necropsy number, or both
   - Name
   - Age
   - Sex
   - Country of birth
   - Country of residence and number of years there
   - Ethnic origin
   - Occupation

B. General information
   - Immediate cause of death
   - Other important diagnoses
   - State of nutrition (list possibilities)
   - Weight
   - Length
   - Subcutaneous fat thickness over linea alba, midway between xiphoid and umbilicus
a. Areas of stenosis, moderate or severe, and occlusion (show in the diagram below):

SS = Severe stenosis
MS = Moderate
O = Occlusion

b. Predominant type of lesion:

c. Description of complicated plaques, if present:

d. Location of involvement:

Right coronary
Anterior Descending
Left circumflex
Weight: ______ grams

- Scar

- Infarction
CEREBRAL ARTERIES

Annex 1
Page 4

a. Areas of stenosis, moderate or severe, and occlusion (show in diagram):

SS = Severe stenosis
MS = Moderate
O = Occlusion

b. Predominant type of lesion:

c. Description of plaques, if present:
### Estimation of Aorta Lesions (Thoracic and Abdominal Aorta)

<table>
<thead>
<tr>
<th>Total % surface involved</th>
<th>Percent of involved surface composed of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fatty streaks</td>
</tr>
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<td></td>
<td></td>
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</tbody>
</table>
TECHNIQUE OF PRESERVATION OF SPECIMENS IN PLASTIC BAGS

Plastic bags made of three layers of material, impervious to fluids, flexible and transparent, have been found to be highly satisfactory. (Holman, R. L., McGill, H. C. Jr., Stron, J. P. & Geer, J. C., Techniques for studying atherosclerotic lesions: Laboratory Investigations, 1958 (in press)).

For identification autopsy numbers should be written on small strips of light opaque white celluloid dull on both sides, with a soft lead pencil. The writing is rendered permanent by dipping the celluloid in acetone and allowing it to dry. The strip can be inserted into the bag and sealed in with the specimen.

The following technique is used:
(a) the fixed specimen, with the identifying celluloid strip, is inserted into the bag, oriented into the desired position with long forceps, and held in this position by rubber bands stretched around a piece of cardboard;
(b) approximately 20 cc of 10 per cent. formalin is poured into the bag while it is held upright;
(c) the specimen is placed between the two leaves of a press made of two rectangular boards hinged on one end and covered on the inside with foam rubber of one inch (2.5 cm) thickness. The leaves of the press are brought together in a vertical position so that all of the air escapes from the open end of the bag when pressure is applied;
(d) without releasing the pressure, the excess formalin solution is poured off and the press clamped together by two hooks at each side;
(e) the open end of the bag is then sealed by heat.