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WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES

No. 441

**THE PATHOLOGICAL DIAGNOSIS
OF ACUTE ISCHAEMIC
HEART DISEASE**

Report of a WHO Scientific Group

WORLD HEALTH ORGANIZATION

GENEVA

1970

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PRINTED IN FRANCE

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OF ACUTE ISCHAEMIC HEART DISEASE

Geneva, 24-29 March 1969

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THE PATHOLOGICAL DIAGNOSIS OF ACUTE ISCHAEMIC HEART DISEASE

Report of a WHO Scientific Group

1. INTRODUCTION

The WHO Scientific Group on the Pathological Diagnosis of Acute Ischaemic Heart Disease met in Geneva from 24 to 29 March 1969. The meeting was opened by Dr J. Karefa-Smart, Assistant Director-General, who welcomed the participants. He stressed that the recognition of early ischaemic changes in the myocardium could well lead to the development of improved methods for the prevention and control of ischaemic heart disease. The Group was invited to review present methods, to assess their value and, if possible, to suggest methods of investigation that could lead to internationally acceptable techniques.

For the purpose of this meeting, the Group defined ischaemic heart disease as "a dysfunction, acute or chronic, arising from a relative or absolute reduction in arterial blood supply to the myocardium". Such dysfunction is most commonly, but not necessarily, associated with disease processes in the coronary arterial system.

2. ACUTE ISCHAEMIC HEART DISEASE AS A PUBLIC HEALTH PROBLEM

Ischaemic heart disease is of world-wide distribution and is a major public health problem in industrial and urbanized countries where it accounts for approximately two-thirds of the total mortality from cardiovascular diseases in the age group 45-64 years.¹ There is evidence that in these countries ischaemic heart disease is increasing, particularly in the younger males (40-49 years). It can be expected that the frequency will also increase in the developing countries as they become more urbanized and industrialized.

In a population-related autopsy study in three countries (Table 1), death was ascribed to ischaemic heart disease in 77% of the men and 50% of

¹ Among males in this age group, about 3 in 10 of all deaths are due to ischaemic heart disease.

TABLE I. PATHOLOGICAL FINDINGS IN SUDDEN DEATH
IN MEN AND WOMEN AGED 40-59 YEARS *

	Men		Women	
	Total No.	%	Total No.	%
All sudden deaths ^a	404	100	145	100
Sudden deaths with fresh myocardial infarction	112	27.7	19	13.1
Sudden deaths with coronary occlusion and fresh myocardial infarction	78	19.3	13	9.0
Sudden deaths with coronary occlusion without fresh myocardial infarction	27	6.7	3	2.1
Sudden deaths with IHD ^b	312	77.2	72	49.6

* Results of WHO population-related autopsy studies in Malmö, Prague 2, and Yalta.

^a In this study, sudden death was defined as non-violent death occurring unexpectedly within 6 hours of the onset of symptoms in an apparently healthy person or in a sick person whose condition was stationary or improving.

^b IHD = Ischaemic heart disease (fresh myocardial infarction, or scars, or occlusions in any coronary artery).

the women in the age group 40-59 years who had died within 6 hours of the onset of symptoms. Fresh myocardial infarction was found in 28% of the men and 13% of the women. The reason why pathological evidence of fresh myocardial infarction was found in so many cases, in spite of the short lapse of time, is not clear; probably the early stage of the attack was silent and had occurred prior to 6 hours before death.

A review of the existing literature showed that mortality from acute ischaemic heart disease is highest during the first 24 hours after the onset of symptoms and that more than one-third of deaths occurred within the first hour. The distribution of deaths during 24 hours in a study carried out in Belfast is shown in Table 2.

The Group did not attempt to develop a definition of sudden death. Before this can be done, it is essential to correlate pathological findings with clinical information obtained during life. It will then also be possible to undertake the classification of the causes of sudden death.

In recognition of the importance of the public health aspects of acute ischaemic heart disease, a separate three-digit category (410) has been assigned to acute myocardial infarction¹ in the 1965 revision of the *International Classification of Diseases*.²

¹ This category includes conditions of up to 8 weeks' duration.

² World Health Organization (1967) *Manual of the International Statistical Classification of Diseases, Injuries and Causes of Death*, 1965 revision, Geneva.

TABLE 2. DISTRIBUTION OF DEATHS FROM CORONARY ARTERY DISEASE IN RELATION TO TIME INTERVAL FROM ONSET OF LAST ATTACK *

Interval from onset of last attack (hours)	Total deaths in stated interval as percentage of all deaths occurring within one year
1/4	22
1/2	28
1	33
2	38
12	53
24	58

* After McNeilly & Pemberton, 1968.

3. PATHOLOGICAL DIAGNOSIS OF ACUTE ISCHAEMIC HEART DISEASE

3.1 Macroscopic diagnosis

The diagnosis of established myocardial infarction is usually easy; it may be difficult, however, to recognize the early lesions of acute ischaemic heart disease when the time interval is too short for the classical changes to have developed.

3.1.1 *Naked-eye findings*

The identification of a fresh thrombus in a branch of the coronary arteries is the most important diagnostic feature. Even in the absence of such a thrombus, it is possible to suspect early infarction by the soft consistency, pallor and oedema of an infarct (measuring, say, 3 cm x 1 cm). These changes may well be detectable even in a patient who has survived for more than 15 hours after the onset of symptoms. They are best seen in transverse slices of the myocardium and are less evident or not recognizable if post-mortem autolysis is marked (see the proposed method for examination of the heart described in Annex 1).

3.1.2 *Coronary artery injection techniques*

Since ischaemic heart disease is very frequently associated with obstructive lesions of the coronary vessels, the postmortem study should include a satisfactory evaluation of the coronary tree.

It is felt that the method usually employed, based only on the dissection of the main sub-epicardial coronary branches, is insufficient for this purpose. A high level of accuracy can be attained, however, if this method is combined with a coronary artery injection technique. Several

injection techniques are available and the choice has to be made in relation to the main purpose of the examination of the coronary system. In a project in which a combined vascular and myocardial evaluation is desired, angiography with a radio-opaque material appears to be preferable.

The injected material should be of such a consistency that it fills the arteries without entering the capillary bed. The injection should be made under controlled conditions at a pressure permitting adequate filling without any artefact.

Two methods of injection have been used. One is coronary cannulation, the other intra-aortic injection after plugging the aortic orifice. The latter is recommended because it demonstrates the coronary ostia and any supernumerary coronary arteries.

Radiographs in multiple different projections of the intact heart permit an adequate evaluation of the coronary arterial tree and do not interfere with the subsequent examination of the organ, to which they may be an important guide.

The injection-corrosion technique using plastic material, combined with myocardial sampling, can be employed when some particular problems (such as three-dimensional evaluation of the intramural vasculature or the anastomotic vessels) have to be investigated.

3.1.3 *Dehydrogenase reactions*

Viable myocardium contains co-enzymes and dehydrogenases that reduce a buffered solution of Nitro Blue Tetrazolium (Nitro-BT)¹ giving a coloured reaction. Dying cells lack these substance and no stain is developed in them. Therefore, the dehydrogenase reaction may be useful for macroscopic identification of areas damaged by ischaemia.

The technique can best be performed on transverse slices of the heart prepared by Šikl's technique (1942) using the methods described by Nachlas & Shnitka (1963), and used also by Ramkinson (1966) and Brody et al. (1967). The incubation solution consists of Sørensen's 0.1 M phosphate buffer, pH 7.4, and 0.5 mg/ml of Nitro-BT. Slices are incubated at 37 °C. Normal human myocardium (within 6 hours after death) stains blue in 15 minutes, while the necrotic areas stain faintly or remain completely unstained. If the post-mortem interval is longer than 6-12 hours, the colour does not develop and sodium succinate should be added to give a final concentration of 0.1 M. The method at least makes it possible to obtain a precise delineation of necrotic areas after 8 hours of ischaemia (according to Nachlas & Shnitka, 1963) and to choose areas for the histological examination.

¹ 2,2'-di-*p*-nitrophenyl-5,5'-phenyl-3,3'-(3,3'-dimethoxy-4,4'-bi-phenylene) ditetrazolium chloride.

3.1.4 *Other methods*

The following methods of investigation appeared to merit further study :

(a) *Potassium determination*

Loss of potassium ions and influx of sodium ions occurs within several minutes after the onset of ischaemia. Based on studies by Iseri et al. (1952), Jennings et al. (1957, 1965), and Herles & Daum (1957), of electrolyte changes in acute myocardial ischaemia, potassium determination was used for the diagnosis of early ischaemic lesions in the human heart by Chait (1964, 1968) and Zugibe et al. (1966). The determination of potassium is performed by flame photometry in homogenates of samples of the left ventricle and septum that are free from old lesions. When no immediate determination is possible, samples should be stored at -20°C or after drying at room temperature. The results are believed to be independent of the autolytic changes that occur between death and autopsy (Chait, 1964, 1968; Zugibe et al., 1966). For a better comparison of the results obtained in various laboratories, the ratio of K/Na should be calculated. Reported values for the K/Na ratio in normal areas of the heart are above 3.0 and in ischaemic areas below 2.5.

(b) *Magnesium determination*

Because magnesium ions rapidly leave hypoxic cells, determination of tissue magnesium levels may be a useful indicator of early myocardial ischaemia. Since calcium ions simultaneously enter the injured cells, alterations in the Mg/Ca ratio of different parts of the heart might permit localization of an ischaemic zone before overt histological changes occur. Tissue samples are chosen similarly as in the method for potassium determination. Analyses are best performed by atomic absorption spectrophotometry. As in the potassium method, samples may be stored in the frozen state and results are thought to be independent of autolysis but may vary with the duration of the agonal period. More data are needed before the method can be recommended.

(c) *Fluorescence methods*

Tetracycline analogues may be used to detect foci of early damage in tissue slices or after injection of tetracycline derivatives into the coronary arteries (Málek et al., 1963). For the evaluation of the usefulness of this technique or of methods using acridine orange (Korb et al., 1965) on human material, more data are needed.

3.2 Histological diagnosis

3.2.1 *Light microscopy*

The value of the histological signs of myocardial infarction is influenced by the interval between death and autopsy and other factors that favour post-mortem autolytic change (e.g., ambient temperature, adiposity). The earliest changes will be seen at the junction of infarcted and non-infarcted tissue, and this zone should be included in the tissue selected for examination by the light microscope, preferably using haematoxylin and eosin, which should be well differentiated.

The histological indications of death of the muscle cells, such as pyknosis, karyolysis, eosinophilia, shrinkage, granularity and loss of striations will not proceed at the same rate in all infarcts. The size of the infarct is clearly the major factor, and these phenomena will be seen earliest and most markedly at the edges.

The evaluation of colour changes in the cytoplasm of the myocardial cells and of very early nuclear changes depends on subjective observation and may be obscured by inadequate eosin differentiation; but the recognition of polymorphonuclear cellular infiltration cannot be misinterpreted or missed, even when autolytic changes are present. At 24 hours these cells are present in considerable numbers at the edges of the infarct; earlier than this it is very difficult to determine the exact time of their appearance. Clearly, they will be seen first around the blood vessels and interstitially as the migrating cells move to the infarcted area. Initial infiltration of leucocytes starting from the blood vessels is a finding which should be very closely looked into by pathologists who are interested in the study of early infarcts. Other inflammatory phenomena, such as oedema and congestion, are less easily recognized. Haemorrhage is not a feature at this stage.

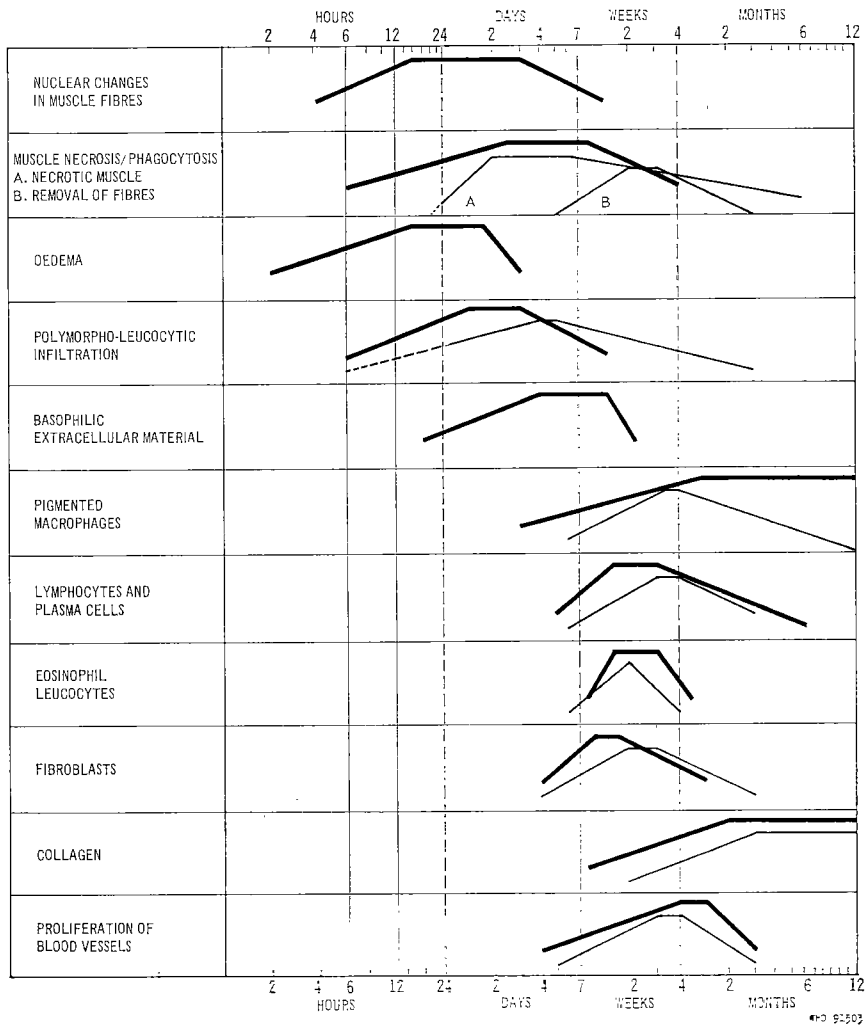
As regards the timing of the changes in myocardial infarction in man (see Fig. 1), the evidence is based virtually on two papers only (Mallory et al., 1939; and Lodge-Patch, 1951). It is clearly essential to obtain more information using routine histological methods, especially on the changes that occur in the first 24 hours after the onset of the lesion.

3.2.2 *Evaluation of refined histological techniques*

(a) "Thick" resin-embedded sections, stained with toluidine blue and examined at higher magnifications of the optical microscope, can give additional information. Current improvements in microtomy make it reasonable to consider the use of this technique whenever possible.

(b) Green fluorescence of the human myocardial cells in cryostat sections of unfixed specimens after the application of acridine orange

FIG. 1. DEVELOPMENT OF MICROSCOPICAL CHANGES IN ACUTE MYOCARDIAL INFARCTION



— Mallory et al. (1939)
 — Lodge-Patch (1951)

(1:10 000 in Michaelis barbital buffer, pH 6.7), as described by Hecht et al. (1961), Korb & Knorr (1962) and Korb & Totovič (1963), must be interpreted with caution.

(c) The demonstration of fuchsinophilia (Selye, 1958; Poley et al., 1964; Lie, 1968) is not recommended because it is a variable and inconsistent technique.

(d) Methyl green/pyronine and Feulgen-oxidized tannin/azo techniques (Malinen et al., 1968) are no more sensitive than haematoxylin and eosin.

3.3 Electron microscopy

Electron microscopic studies of experimental models of myocardial ischaemia have provided much basic information on the pathogenesis of hypoxic heart injury. Correlation of ultra-structural changes with biochemical data confirm the importance of catecholamine release and ionic shifts (loss of magnesium, potassium and phosphate; influx of calcium, sodium and water) in the early evolution of ischaemic injury. An altered cellular metabolism, induced by ischaemia, causes rapid glycogen depletion followed quickly by alterations in the nucleus, mitochondria and sarcotubular system. The myofibril is the organelle most resistant to hypoxia. The severity and speed of development of hypoxic change depend on the mode of induction and vary with the species of animal used.¹

Post-mortem autolysis mimics early ischaemic change very closely, and in fact probably has an initial hypoxic basis. Significant hypoxic-autolytic changes may begin during the agonal state. The timing and techniques of tissue preservation are critical in limiting the amount of artefact. It is currently unrealistic to expect to obtain acutely ischaemic human myocardium soon enough after death to be of value in the estimation of the degree or duration of ischaemia by electron-microscopic techniques. Rapidly progressive autolytic changes preclude meaningful morphological assessment of hypoxic change at the ultra-structural level.

Improved methods of fixation and embedding may be developed that will permit better evaluation of human material in the future. For the present it may be possible to relate sarcomere band patterns to mechanical parameters, such as filling pressure in the post-mortem human heart. In addition, there is a place for further electron-microscopic studies of experimental myocardial ischaemia, particularly in combination with other techniques (e.g., cytochemistry and autoradiography).

3.4 Histochemistry

At least to some extent, histochemistry can fill the gap between morphology (as revealed by the light microscope and electron microscope) and biochemistry. For a proper understanding and interpretation of histochemical data it is necessary to take into account the electron-microscopic and biochemical findings.

¹ For further details, see Heggveit (1969).

The main electron-microscopic findings are a diminution in the number of glycogen particles and a swelling of the mitochondria and sarcoplasmic reticulum which can be observed within a few minutes after onset of ischaemia.

The principal biochemical changes that can be observed in the ischaemic areas after 15 minutes are accelerated anaerobic glycolysis and impaired aerobic metabolism with uncoupling of oxidative phosphorylation. Within a short time there is an efflux of Mg^{2+} and K^+ ions and an influx of Ca^{2+} and Na^+ ions. Later changes include a fall in levels of most dehydrogenases. On the other hand, the activity of glucose-6-phosphate dehydrogenase (G6PD) rises. Many of these functions can be demonstrated histochemically but reliable results are obtained with only some of the methods available.

Experimental studies indicate that these methods are valuable in detecting early myocardial ischaemia in experimental animals. If they are used to trace ischaemic alterations in the human myocardium, two points have to be considered :

(a) There is a lack of knowledge concerning the natural history of lesions in human myocardium and the time of onset of ischaemia is uncertain. Because it is not always possible to take the onset of clinical symptoms as the starting point of ischaemia, many time evaluations of recorded changes found in the literature are probably imprecise.

(b) Post-mortem autolysis affects the nature and timing of the original ischaemic changes.

3.4.1 *Summary of histochemical techniques¹ of proven use in experimental myocardial ischaemias for application to human myocardium*

(1) *Non-enzyme methods*

(a) Demonstration of pseudophosphorylase (dependent on presence of endogenous glycogen).

(b) Demonstration of glycogen.

Both activities are decreased within one hour at 37 °C.²

(2) *Enzyme methods*

(a) Changes in activity of succinate, β -hydroxybutyrate, and isocitrate dehydrogenases (aqueous media, Nitro-BT).

(i) Change from "fine dot" to "large dot" formazan pattern, with or without enhanced intensity of staining (within 3 hours).²

(ii) Decreased activity (within 6 hours).²

¹ All methods applied to unfixed cryostat sections except where otherwise stated; for further details see Pearse (1960, 1968).

² Times quoted refer to animal material.

- (b) Endogenous reaction of Nitro-BT "nothing dehydrogenase" (gel media)¹ (decreased within 2 hours).²
- (3) *Stable components (not substantially decreased within 8 hours at 37 °C)*
- (a) Cytochrome oxidase
 - (b) Succinate dehydrogenase
 - (c) Hydrolases

3.4.2 *Summary of techniques for possible application to human myocardium*

- (1) *Non-enzyme methods*
- (a) Tetraphenyl boron (Collenwijn, 1963) method for potassium (freeze-dried sections).
 - (b) Fluorescence method for lipids (Phosphine 3R).
- (2) *Enzyme methods*
- (a) Inverse relationship between activities of lactate dehydrogenase and glucose-6-phosphate dehydrogenase (gel media; ¹ changes expected in 2 hours).
 - (b) ATPase³ activity (calcium-cobalt method at pH 7.5; increase expected in 2 hours).

The influence of the post-mortem interval on the histochemical pattern is extremely important and must be precisely known.

3.4.3 *Grading of histochemical changes*

Only fresh material taken within 30 minutes of death should be used for the grading of ischaemic changes.

The following 4 grades are proposed :

- Grade I : Only phosphorylase or glycogen depletion present (within 2 hours of onset of ischaemia).
- Grade II : Changes in the formazan pattern (from "fine-dot" to "large-dot") with irregularities in the intensity of the succinate dehydrogenase reaction and decrease in endogenous reduction in the same area where phosphorylase or glycogen depletion is present (within 3 hours of the onset of ischaemia).
- Grade III : Marked differences in the intensity of the β -hydroxybutyrate dehydrogenase reaction or the lactate dehydrogenase

¹ The preparation of a gel medium is described in Annex 3.

² Times quoted refer to animal material.

³ ATPase = adenosine triphosphatase.

reaction in the same area where phosphorylase or glycogen depletion is present (within 8 hours of the onset of ischaemia).

Grade IV : Histological changes in myocardial cells (12 or more hours after the onset of ischaemia).

As soon as sufficient information becomes available, the calcium-cobalt method for determining ATPase activity at pH 7.5, the method based on the inverse relationship between lactate dehydrogenase and G6PD activity, and/or other methods may also be used to assist in grading ischaemic changes.

If no fresh material is available, only certain methods are applicable (see Table 3). The methods for succinate dehydrogenase, cytochrome oxidase and/or $\text{NADH}_2 : \text{TR}$ ¹ (the enzymes most resistant to autolytic changes) can be of help in detecting ischaemic changes, but only large differences in activities should be considered as positive proof of ischaemia.

TABLE 3. SELECTION OF METHODS ACCORDING TO THE POST-MORTEM INTERVAL

Post-mortem interval	Methods applicable
Less than 1/2 hour	All methods
1/2 to 12 hours	G6PD, lactate dehydrogenase, succinate dehydrogenase, cytochrome oxidase, $\text{NADH}_2 : \text{TR}$
More than 12 hours	Lactate dehydrogenase, succinate dehydrogenase, cytochrome oxidase, $\text{NADH}_2 : \text{TR}$

3.4.5 Preparation of myocardial samples for histochemical examination

(a) Large samples up to a maximum of 4 x 2 cm should be taken from as many areas as possible, randomly selected and properly recorded. They should be attached to cryostat tissue holders and frozen immediately at -70°C with dry ice. If necessary, they may be stored for 1-2 months in closed containers or wrappings at -70°C .

(b) Cryostat sections (20-30 μ) should be cut and mounted on slides. These preparations should be used within 48 hours. They can be stored, if necessary, at room temperature.

¹ $\text{NADH}_2 : \text{TR}$ = Nicotinamide-adenine dinucleotide (reduced form) : tetrazolium reductase.

4. FUTURE STUDIES

4.1 Comparative and experimental studies in animal models

4.1.1 *Spontaneous myocardial ischaemia*

Ischaemic heart disease as a consequence of spontaneous occlusion of the extramural coronary artery is extremely rare in animals. In many species, however, some degree of necrosis of the myocardium occurs in association with disease of the small intramural coronary branches. This type of myocardial lesion is often observed in swine (especially the Pietrin breed) and in small ruminants, and these species could be of some value for the study of ischaemic heart disease.

4.1.2 *Experimental myocardial ischaemia*

For studying the physiopathological aspects of the coronary circulation itself and especially the development of collaterals, the dog and the swine are suitable as experimental animals. In contrast, sheep have a very poor potential anastomotic circulation.

For the study of experimental surgery on the atheromatous coronary artery itself, the common laboratory animals (cat, rabbit, dog) are of very limited value. Some species of primates could offer better possibilities.

Experimental models of acute myocardial ischaemia may be produced in the following ways: compromising the coronary circulation in intact animals by extraluminal coronary artery constriction; by generalized hypoxia; or by administering substances such as catecholamines. In such controlled situations the process of acute myocardial ischaemia can be studied in a systematic manner using biochemical, histochemical, morphological, pharmacological, and physiological methods. This approach may shed light on the similar process in humans where the situation is complicated by post-mortem autolysis.

Although no entirely satisfactory animal model for the study of the problem of ischaemic heart disease as a whole can be found, certain species may be useful for particular types of investigation.

4.2 Comparative research

The following areas were identified as being worthy of further comparative study and research:

(1) Analysis of electrolytes, particularly potassium, sodium, magnesium and calcium, by different techniques.

- (2) Fluorescence and isotope scanning of the heart.
- (3) Histochemical methods.
- (4) Examination of "thick" resin-embedded sections.

4.3 Investigations on human hearts

The recognition of the early stages of acute myocardial infarction is essential if this condition is to be separated from other myocardial causes of sudden death. The basic information needed for this purpose is not at present available. One way to obtain adequate data in a usable form would be to study the beating human heart *in vitro*. This would, of course, be preceded by refinement of techniques using larger animals, especially primates.

In principle, the hearts excised from heart transplant patients would be unsuitable for the investigation of the earlier signs of myocardial infarction. The patients have generally been in the terminal stages of their myocardial disease with extensive myocardial damage; those parts of the myocardium that are not fibrosed are not suitable as test material, as they will show the morphological changes associated with severe and prolonged heart failure. The excised heart has lost its sinu-atrial node as well as two-thirds of both atria, and it is doubtful if such a heart could be made to beat in a suitable physiological fashion for the time necessary for the experiment. Nevertheless, the undamaged portions of myocardium may be suitable material for some biochemical, histochemical, morphological, pharmacological and physiological studies if they are maintained in artificial conditions of "survival".

The Group emphasized that the above suggestions were concerned only with the technical feasibility and potential value of experiments with the excised, still beating human heart. The Group did not wish to make any statement concerning the medicolegal and ethical problems involved in such studies.

5. RECOMMENDATIONS

1. Since so little histopathological information is available on early myocardial changes and since the study of such changes by electron microscopy, histochemistry and other refined techniques is hampered by post-mortem autolysis, the Group considered that a prospective pathological study should be carried out. It is therefore recommended that a pilot study should be undertaken as soon as possible, based on the method of studying the heart proposed in Annex 1.

2. Histological slides (stained and unstained) from selected cases provided by centres using the standardized method proposed in Annex 1

should be submitted to a group of independent pathologists for evaluation of the histological findings.

3. Naked-eye and microscopical studies should be co-ordinated, possibly with WHO assistance.

4. In order to permit correlation of clinical and pathological findings, well-trained pathologists familiar with detailed heart studies should be available at the centres where the clinical studies are to be carried out.

5. Since many cases of sudden death from acute ischaemic heart disease become the subject of medicolegal autopsy, it is most important that forensic pathologists should be well acquainted with the most suitable techniques and be able to put them into practice.

ACKNOWLEDGEMENT

The Group acknowledges the special contributions made during its deliberations by the following WHO staff members : Dr A. R. Kagan, Chief, Epidemiology of Non-Communicable Diseases, Division of Research in Epidemiology and Communications Science; Dr R. Masironi, Scientist, Cardiovascular Diseases; Dr Z. Pisa, Medical Officer, Chronic Diseases, WHO Regional Office for Europe, Copenhagen; and Dr R. Vaněček, Head, Second Department of Pathology, Charles University, Prague, Czechoslovakia; Visiting Professor of Pathology at the Institute of Pathology, University of Geneva, Switzerland (*Consultant*).

Annex 1

METHOD FOR EXAMINATION OF THE HEART

The diagnosis of established myocardial infarction is usually easy. However, difficulties often arise in the recognition of acute ischaemic heart disease during the first hours after the accident. For these reasons, the following method of examination of the heart, using the naked eye and, if necessary, injection of the coronary arteries and histological techniques, is now recommended.

1. Removal of the heart and naked-eye examination

The heart should be removed as soon as possible after death : the pulmonary artery and the aorta should be severed 5 cm from the free

margins of the semilunar valves, and the veins connected with the atria should be severed in such a way as to leave the entire atria attached to the heart. After removal, and under proper light conditions, the heart should be examined to identify suspected areas of early myocardial infarction and the following findings should be specially looked for : soft consistency, pallor, oedema, fibrinous pericarditis, epicardial fibrosis, sub-epicardial haemorrhage, aneurysm of ventricle, and rupture. Without opening the coronary vessels, particular attention should be paid to them and any unusual observations noted before they are injected.

2. Injection and study of the coronary arteries

A. PREPARATION OF A RADIO-OPAQUE MASS ¹

Reagents

2-Octanol (secondary *n*-octyl alcohol, secondary caprylic alcohol).

Phenol, liquefied, USP or equivalent grade.

Bacto-Gelatin (Difco Laboratories).

Potassium iodide, granular, USP or equivalent grade.

Buffer salt mixture (sodium and potassium phosphates, pH 6.2).

Barium sulfate (all USP brands labelled as X-ray contrast media are satisfactory).

Stock mixtures

The following amounts are prepared and stocked in bottles or bags :

<i>Mixture A</i>	50.0 ml
2-Octanol	20.0 ml
Phenol	30.0 ml

Mix well and keep in a bottle with calibrated dropper.

<i>Mixture B</i>	61.0 g
Gelatin	23.5 g
Potassium iodide	35.2 g
Buffer salt mix	2.3 g

Contrast medium

Barium sulfate	100.0 g
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¹ This is a modification of Schlesinger's (1967) method.

Preparation of the mass

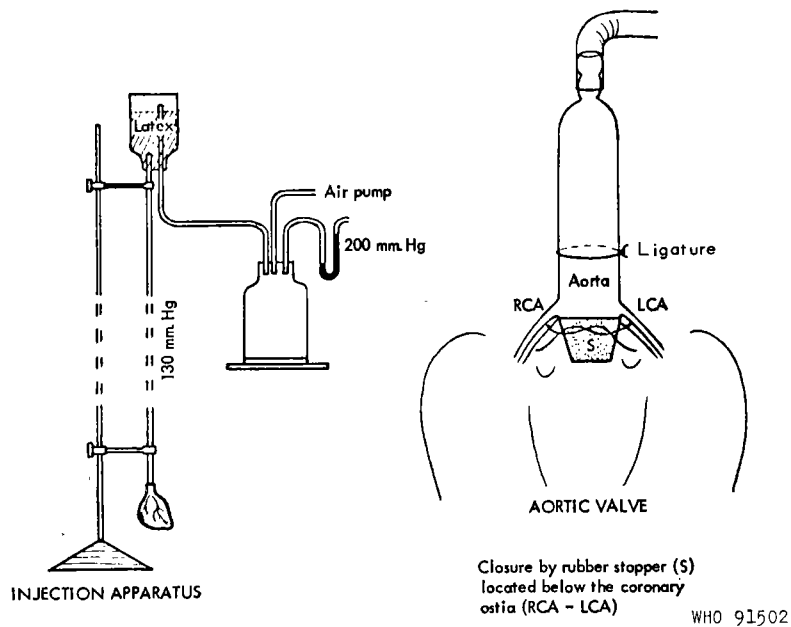
1. Place 163 ml of distilled water in a Waring blender.
2. Add 1 ml of mixture A and spin for 30 seconds.
3. Add 61 g of mixture B and spin for 60 seconds.
4. Add 100 g of barium sulfate and spin for 2 minutes.

If the mass is not to be completely used within two weeks after preparation, store in a refrigerator at 4-6°C. To use, melt the mass by warming to room temperature.

B. INJECTION OF THE CORONARY VESSELS

Under a pressure of about 130 mm of mercury, inject the coronary vessels, utilizing the technique described by Baroldi & Scomazzoni (1967) (see Fig. 2).

FIG. 2. INJECTION EQUIPMENT AND METHOD



C. X-RAY EXAMINATION OF THE CORONARY ARTERIES

Multiple X-rays should be taken of the specimen to demonstrate the entire major coronary arterial tree.

3. Slicing of the heart

(a) The heart should be cut into transverse slices, 1 cm thick, commencing at the apex of the heart and stopping 1 cm short of the mitral valve.

(b) The slices should then be arranged in order, and after washing and removal of post-mortem clots, photographed on a black-and-white plate with a centimetre scale. The slices should be carefully examined for variations in colour and/or consistency. Any changes should be properly documented with colour photographs and/or described. Before the heart is fixed, the coronary arteries should be examined both in the slices and in the remaining basal portion.

(c) The slices and the base of the heart should then be weighed together, after reducing the length of the aorta and pulmonary artery to 2 cm.

(d) The tetrazolium macroscopic method proposed by Nachlas & Shnitka (1963) should be employed for one side of each of the slices and positive findings recorded in colour and black-and-white photographs.

(e) After the tetrazolium reaction has been completed all the slices should be properly fixed in formalin solution.

(f) Sampling for histological examinations should be based on the following principles :

(i) In the presence of infarction, detected either by naked-eye observation or by the Nitro-BT technique, block sections should be taken from the margin of the infarcted area and should cover the full thickness of the wall.

(ii) In the absence of an evident infarction, several block sections should be taken from the myocardium in the neighbourhood of all narrowings or occlusions of the coronary arteries, as revealed by coronary angiography.

(iii) In the absence of changes in the coronary arteries, random sampling of the left ventricle, septum and right ventricle should be carried out, carefully recording the areas from which block sections are taken (as many samples as possible should be taken).

Annex 2

**POSSIBILITIES OF HEART SCANNING
FOR THE DIAGNOSIS OF MYOCARDIAL INFARCTION**

There are two main methods of using radio-isotope scanning for the detection of myocardial infarction :

1. To depict infarcts as "cold areas", using substances rapidly taken up by *normal* myocardium, such as rubidium-86, cesium-131 or iodized (^{131}I) oleic acid.
2. To depict infarcts as "hot areas" by use of substances selectively localized in *damaged* myocardium (neohydrin- ^{203}Hg , iodized (^{131}I) derivatives of tetracyclines, and mercury (^{203}Hg) derivatives of fluorescein.

Neohydrin- ^{203}Hg has been used rather empirically because of its proven usefulness in the diagnosis of malignant tumours. The use of iodized (^{131}I) derivatives of tetracycline analogues and mercury (^{203}Hg) derivatives of fluorescein is based on fluorescence studies, which have demonstrated that both these groups of substances are accumulated and fixed in the damaged myocardium over a prolonged period.

In both methods ("cold" and "hot"), intravenous administration of the compounds is used to detect the ischaemic area and to determine its extent, severity, and localization. The second method seems to be more advantageous; it is easier to obtain a "positive" scan on a "negative" background than a "negative" scan on a "positive" background. Furthermore, in the "cold area" scan method the picture disappears rapidly. On the other hand, the "hot area" method permits long-term retention of the test substance in the damaged tissue, which makes it possible to carry out repeated scanning and to follow the healing process.

Neither method can yet be considered suitable for routine in clinical diagnosis, although the research value of both has been established, particularly that of the "cold area" method. The positive scan method using neohydrin has been found unsuccessful, and clinical trials with mercury derivatives of fluorescein have only recently been started.

Tests with a new mercury derivative of fluorescein, "Mercurascan", have shown considerable promise. This derivative accumulates in the area of ischaemia at a very high rate and is rapidly cleared from the blood and healthy myocardium. The index R I (the ratio damaged muscle/normal muscle) is as high as 80-200 (in comparison with 8 for neohydrin), while the index R II (the ratio damaged muscle/blood) is 90. Because Mercurascan also disappears rapidly from surrounding tissues and organs,

except the liver, all the requirements for a positive scan method are satisfied. Using this test substance it was possible for the first time to obtain a positive scan *in vivo* of experimental ischaemia in the dog heart.

The most important results obtained by this method were :

(1) The proof that it is possible to obtain a positive scan *in vivo* on a breathing animal with a beating heart.

(2) Rapid appearance of the positive scan after intravenous administration of the test substance and after the development of the ischaemic focus. After temporary ligation of the descending branch of the left coronary artery of the dog, a positive scan develops within 1½-2 hours, i.e., before the lesion manifests itself by an increase in the levels of enzymes in the serum (creatinine phosphokinase, GOT,¹ GPT,² and LDH³ isoenzymes). A positive enzyme test, however, can be obtained immediately in the lymph draining the damaged area.

(3) A positive scan appeared in all the animals with experimentally induced infarction. The scan was positive even in animals with permanent ligation of the coronary artery, although the best results were obtained 48 hours or more after the development of the ischaemia.

(4) Mercurascan has been found to be suitable for the dynamic study of myocardial infarction; for example, it was possible to follow the progress of the healing process after one intravenous injection of the substance.

(5) Preliminary results were obtained for the accumulation of the substance in the myocardium after allotransplantation of the heart.

The following further studies on the value of scan methods appear to be justified by the results obtained so far :

(a) The use of scan methods (including the Mercurascan test) in experimental cardiac ischaemia.

(b) Evaluation of the Mercurascan test for the early detection of necrobiotic changes following heart allotransplantation.

(c) Detailed studies on the mechanism of accumulation of substances in damaged tissues. In particular, information is needed on the way in which mercury derivatives of fluorescein are fixed in damaged tissue. This probably involves the formation of a complex. It is known, for example, that tetracycline is fixed in the damaged myocardium primarily through the binding of a tetracycline-calcium complex to hydro-

¹ GOT = glutamic oxaloacetic transaminase.

² GPT = glutamic pyruvic transaminase.

³ LDH = lactate dehydrogenase.

lysed lipids. On the other hand, mercury derivatives of fluorescein seem to be bound directly to the proteins of damaged myocardial cells, possibly by the -SH groups of proteins.

(d) Most of the methods are used for the diagnosis of myocardial infarction *in vivo* after intravenous administration of substances. It would be worth while to correlate such findings with the examination of autopsy material, either after post-mortem perfusion of the heart with test substances or after direct application of such substances to slices of the myocardium.

(e) Clinical trials of Mercurascan in comparison with other clinical diagnostic methods. Preliminary observations indicate that the human myocardium behaves in much the same way as the animal heart, but further careful investigation is required. Coronary care units are particularly suitable for such research.

Annex 3

METHODS FOR THE DEMONSTRATION OF LACTATE DEHYDROGENASE, GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) AND "NOTHING DEHYDROGENASE"¹ IN GELATINE-GEL MEDIA²

I. *Reagents* (the purest grade available is recommended)

Gelatine
 Nitro-BT
 Potassium cyanide
 Magnesium chloride or sulfate
 Disodium phosphate
 Potassium phosphate
 Tris(hydroxymethyl)aminomethane

¹ "Nothing dehydrogenase" is a term first used by Racker (1955) to denote the reduction of NAD and NADP by protein fractions from various tissues in the absence of substrate. The term was subsequently introduced into histochemistry by Zimmermann & Pearse (1959) to indicate reduction of NAD or NADP by tissue sections in the absence of substrate and the analogous reduction of tetrazolium salts to formazans. The reduction was originally ascribed to the protein-bound -SH groups, but is now considered to be due to the -SH groups of alcohol dehydrogenase.

² Lojda (1968).

NAD (nicotinamide adenine dinucleotide)
 NADP (nicotinamide adenine dinucleotide phosphate)
 Phenazine methosulfate
 Sodium lactate
 Glucose-6-phosphate (disodium salt)
 Dimethylformamide

II. Stock solutions

(a) 10% solution of gelatine in 0.1M phosphate or Tris-HCl buffer, pH 7.4 (the pH should be controlled and adjusted by addition of 1N or 0.1N NaOH if necessary)

The solution is prepared in a water bath at 40°C. It can be stored in a stoppered bottle at 4°C and used as long as microbial contamination is not apparent. Before use it should be warmed to 37°C.

(b) Nitro-BT stock mixture :

0.1M phosphate or Tris-HCl buffer, pH 7.4	5 ml
0.2% aqueous solution of Nitro-BT ¹	5 ml
0.1M potassium cyanide (pH adjusted to 7.4 by addition of 1N HCl)	4 ml
0.05 M magnesium chloride or sulfate	4 ml

This mixture can be stored in a refrigerator for several weeks.

(c) 1M sodium lactate

(d) 1M glucose-6-phosphate (disodium salt)

The pH of solutions (c) and (d) should be adjusted to 7.4 and both solutions stored at -10°C or a lower temperature.

III. Demonstration of lactate dehydrogenase

Nitro-BT stock mixture	4.5 ml
1M sodium lactate	1.0 ml
NAD	5-10 mg
(the pH should be adjusted to 7.4 by the addition of 1N or 0.1N NaOH)	
Phenazine methosulfate	0.5-1 mg

The solution is warmed to 37°C, mixed thoroughly with 5.5 ml of gelatine stock solution, poured on clean cover glasses of suitable dimen-

¹ The required amount is dissolved in a few drops of dimethylformamide and distilled water added to the required volume.

sions (about 0.3 ml is required for a cover glass 24 x 32 mm) and allowed to gel at a temperature below 15°C.

Apply the cover glasses, gel side down, to dry cryostat sections mounted on slides. Incubate at room temperature in the dark until the required intensity of staining is obtained (for about 15-30 minutes). Examine and take a photomicrograph. If permanent preparations are required, remove the cover glass in warm water. Fix in 4% formaldehyde for 5 minutes. Mount in glycerine jelly or in a similar medium.

IV. *Demonstration of G6PD*

Nitro-BT stock mixture	4.5 ml
1M glucose phosphate (disodium salt)	1 ml
NADP	5-10 mg
(the pH should be adjusted to 7.4 by the addition of 1N or 0.1N NaOH)	
Phenazine methosulfate	0.5-1 mg

The procedure is similar to that for the demonstration of lactose dehydrogenase (see above) except that the incubation period is about 30-60 minutes.

V. *Demonstration of "nothing dehydrogenase"*

The procedure is the same as for lactose dehydrogenase (see above) except that the IM sodium lactate solution is replaced by water and the incubation period prolonged to about 30-60 minutes.

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