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Microcirculatory Changes Following Early Reperfusion in Experimentel Myocardial Infarction

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Summary. 69 rats underwent temporary or permanent ligation of the left coronary artery and were studied by the injection of colloidal carbon following fixation-perfusion. 10 rats were studied using the same protocol and served as controls. Localized myocardial ischemia was accompanied by microvascular changes which produced capillary obstruction when blood flow was reestablished. This phenomenon of "no-reflow" was characterized by the presence of large non perfused areas seen after brief periods of ischemia (15 min). These areas were increased when the period of ischemia was lengthened. After 30 to 60 min of interruption of blood flow the non perfused area extended over the major portion of the ischemic area.

During reperfusion the "no-reflow" phenomenon displayed during the first hour showing a transitory improvement in capillary perfusion which was soon followed by a progressive reexpansion of the non injected zones. After 24 hours of reperfusion, the latter zones were identical in their extent to those cases showing tissue necrosis following permanent ischemia.

This "no-reflow" phenomenon appears to play a role in the evolution of the reperfused ischemic area by excluding certain areas from the benefits of reperfusion. The most probable factors involved in this process are: increased blood viscosity, endothelial changes peri-capillary edema and the contractile state of the myocardium.

The incidence of these microvascular changes, using various methods of myocardial preservation during open heart surgery operations, as well as the present attempts directed towards metabolic therapy of myocardial anoxia, are under investigation.

Key words: Myocardial ischemia—Reperfusion—Microcirculation—Experimental infarction—No-reflow phenomenon.

Introduction

In spite of recent progress in the Surgery of coronary artery disease, the indications for early revascularization of myocardial infarction remain to be determined (Ross, 1974). Several experimental studies have established that, under certain conditions, early reperfusion may reduce the extent of tissue necrosis (Blumgart *et al.*, 1941; Sommers and Jennings, 1964; Krug and Korb, 1966; Krug, 1970; Maroko *et al.*, 1971, 1972; Ginks *et al.*, 1972, 1974).

In a study using the rat as experimental animal, we have noted that after reperfusion following less than 6 hours of ischemia, the extent of the infarcted myocardium was decreased in 63% of the cases as compared with a group of rats undergoing ischemia for the same period of time without reperfusion (Deloche *et al.*,

Photography: Mr M. Wolfelsperger. The technical assistance of Miss M. Bardin is acknowledged.

1972; Fontaliran *et al.*, 1972). Histoenzymatic studies have shown the heterogeneous nature of the ischemic lesion, notably differentiating a marginal zone which may be revitalized (Cox *et al.*, 1968; Camilleri *et al.*, 1975). Electrophysiological and biochemical data have been reported which supply a scientific basis for these experimental observations (Brachfeld, 1969, 1974).

We have been struck by the wide range of the results obtained in various studies. Certain variations may be ascribed to the different protocols used to the choice of the animal. However, we have noted, in a study using a same protocol, inconstant results with an identical duration of ischemia. The lack of regression of the ischemic lesion or even extension of the lesion following reperfusion in certain cases suggests that other factors may be of importance in addition to the tolerance of the myocardial cell to anoxia.

Several authors have evoked the role of microcirculatory changes. The inability to produce efficient capillary reexpansion may be responsible for the fact that certain areas of myocardium do not benefit from reperfusion. This has been shown to be true for the cerebrum (Majno *et al.*, 1967; Ames, *et al.*, 1968), the dermis (Willms-Kretschmer and Majno, 1969), and the kidney (Summers and Jamison, 1971; Flores *et al.*, 1972). During myocardial ischemia, similar findings have been made by Hausamen and Poche, (1965), Krug *et al.* (1966), Poche *et al.* (1969), Hauschild *et al.* (1970), Willerson *et al.* (1972), and in a recent work in the dog by Kloner *et al.* (1974). The true nature and the importance of these factors during the evolution of ischemic lesions are poorly understood (Doerr, 1973).

Material and Methods

The rat was chosen as experimental animal for the following reasons: the possibility of working with a large series, the ease with which the operation could be performed and the precise localization and reproducibility of the infarction obtained. The present study consists 80 male Wistar rats, aged 5 to 9 months, weighing approximately 250 grams. All animals dying spontaneously, infected or presenting at any stage of the operation technical problems were eliminated from the series. The surgical protocol has been described in previous publications (Deloche *et al.*, 1972; Camilleri *et al.*, 1973). Briefly, the infarction of the left ventricle was created by ligation of the left coronary artery at its origin using the technique of Johns and Olson (1954) as modified by Selye *et al.* (1960).

This original method utilizing a temporary ligature allows reperfusion at variable intervals. Reperfusion of the coronary artery bed was obtained in almost all cases as demonstrated by coronary arteriography using radio opaque dyes and histologic study of the area distal to the ligature. *Electrocardiography* was performed in all animals during operation in order to confirm the presence of infarction (Electronique Appliquée 2.101 type I. G.). Under Ether anesthesia the aorta and vena cave were dissected distal to renal arteries. The aorta was catheterized and the catheter attached to a constant perfusion pump with a flow rate of 50 ml per minute. Following injection into the vena cava of Heparin and 1 ml of Procaine sulfate (1%), the vena cava was divided and ligated. Cardiac arrest occured in diastole and fixation perfusion was performed by the injection of a solution of 2 per cent Glutaraldehyde (buffered with 0.15 M Cacodylate), followed by the injection via the aortic catheter of a suspension of 20 per cent colloidal carbon in gelatin (Pelikan Werke particles of 20–30 nm). The heart was then removed through a thoracotomy and placed in iced normal saline. Transverse sections 0.5 to 0.6 cm from the apex were fixed in a solution of Glutaraldehyde at 4° C. Sections measuring 50 to 100 microns in thickness using a cryostat were examined immediately in aqueous medium or were fixed, dehydrated and embedded in paraffin. Various stains were used routinely: Hematoxylin-Eosin-Safran, Masson Trichrome, Mallory Hematoxylin-phosphotungstic, Hematin-basic-fuchsin.

Table 1. 60 rats with temporary ischemia

Duration of ischemia	Duration of reperfusion							Total
	0 min	15 min	30 min	1 hr	6 hrs	24 hrs	8 days	
15 min	2	2	2	5	4	3	2	20
30 min	2	2	—	3	2	1	3	13
1 hr	1	—	2	4	2	3	5	17
6 hrs	—	1	—	2	—	1	—	4
24 hrs	—	2	—	1	—	—	3	6
Total	5	7	4	15	8	8	13	60

The study of the thick sections previously injected was performed using a binocular loup and the light microscope. In each case, the left coronary artery area and the rest of the myocardium were compared with that the control rats. Finally, for each myocardial section, the surface area of the different myocardial areas were identified and measured by projection on a grid using a simple planimetric method. The results were expressed in percentage of the surface occupied by anterior, lateral and posterior walls as well as the septum of the left ventricle.

The animals were separated into 3 groups:

1) 10 control rats, six of which were not operated upon, and the remaining four underwent simple thoracotomy.

2) 9 rats with permanent ischemia were sacrificed at 15 min and 30 min and 1, 6 and 24 hours, and 8 days.

3) 60 rats with temporary ischemia of variable duration (15 and 30 min, 1, 6 and 24 hours) sacrificed and injected either immediately after reestablishment of blood flow (0 time) or after variable periods of reperfusion (Table 1).

Results

1. Control Animals (10 Rats)

The examination of the epicardial surface and the transverse sections appeared uniformly injected, the myocardium was grey-black in color.

On the sections measuring 50 to 100 microns in thickness, the capillaris showed a rich network which were richly anastomotic and closely applied to the muscular bundles (Fig. 1). They were long, straight or slightly curvilinear, and odnnected to each other by multiple transverse branches which were straight or oblique with various aspects forming Y, T or H configurations. They were fed by transmural arterioles which were few and irregulary spaced.

The draining veinules were more numerous, larger and of irregular calibre. They drained to a rich veinous network which was sub-epicardial whereas the distal branches of the coronary arteries were situated deep in the myocardium. The arteries were further identified by the rounded appearance on cut sections and their walls which were thicker, forming clear halos between the capillary network and the arterial lumen containing the opaque particles (Fig. 2).

In the sub-endocardial areas, the capillary network was as dense as in the rest of the myocardium. In some areas, fine vascular channels opened into the

Fig. 1. Non ischemic myocardium. Colloidal carbon injection. 50 μ thick section. Regular perfusion of the capillary bed with carbon black. $\times 375$

ventricular cavities. On the transverse sections of the muscle bundles, the capillaries formed a regular and areolar network. For the ten control animals, the injection was complete and uniform. We noted however in several subepicardial areas small portions which were not completely injected. These incompletely injected areas never represented more than 1% of the surface area of the various walls of the left ventricle.

Fig. 2. Non ischemic myocardium. Colloidal carbon injection. 50 μ thick section. Complete filling of the vasculature. $\times 105$

II. Early Changes Following Ischemia (First 48 Hours)

a) Controls before Ligature Removal (8 Rats)

Regardless of the lenght of time of ischemia (15 min, 30 min, 1 hr, 6 hr, 24 hr) there was, as soon as the thorax opened, a large pale pink zone which was not injected and occupied major portion of the left ventricle. On the thick sections,

the avascular zone, which was not injected with carbon black was similar to the usual location of the infarctions obtained by this method (Fontaliran *et al.*, 1972). It occupied the anterior and lateral aspects of the left ventricle and all or part of the posterior wall, excluded in some cases a thin sub-endocardial layer, and in most cases, the greater portion of the septum. Sometimes, the latter was avascular in its upper portion. The papillary muscles were most often partially injected. In the avascular zones, there was a partial injection of a few capillary tufts or rare veinules which were recognizable by their irregular course. The transmural perforating arteries were always empty of carbon.

The extent of the non injected surface areas was of the order of 55% of the total surface area.

b) Changes Immediately Following Removal of the Ligature (5 Rats)

In all of the cases injected immediately following removal of the ligature, we observed an incomplete injection of the ischemic area (Fig. 3). There were either large transmural areas in which no evidence of injection was present, or isolated areas which were apparently avascular of variable size located intramurally or sub-endocardially. The injected zones alternated with other zones which were incompletely injected. In none of the ischemic areas, we could find, at this stage, a homogeneous injection similar to that of the normal adjacent zones. The systematic comparison between the ischemic area with the rest of the myocardium enables us to eliminate artefacts due to poor injections technique.

Finally all gradations existed between the total absence of injected material and subnormal injections such as those observed in certain subepicardial and intramural zones. Often the injected material displayed distal perforating arteries and certain arteriolar tufts, but little had reached the capillary level (Fig. 4). In certain areas, a few draining veinules superimposed their shadow over the empty vascular space thus created. In certain areas which were apparently avascular, especially those next to the marginal zones, there were groups of very fine capillaries which were injected in a discontinuous fashion (Fig. 5). This irregular injection of various areas rendered difficult the planimetric measurement of those areas not injected by the carbon suspension. The extent of the areas which were not reperfused were in direct relation to the lenght of time of ischemia. For an ischemic period of 15 min, approximately two thirds of the ischemic area lacked reperfusion.

For ischemic periods lasting 30 min, and even more strikingly for 60 min, the non injected areas were similar to those seen in controls which had been injected prior to the removal of the ligature.

c) Effects of Reperfusion (44 Rats)

The results were analyzed in relation to the duration of ischemia (Fig. 6 and 7).

1. Early Reperfusion (Temporary Ischemia of 15–30 Minutes Duration) (26 Rats). During the first hour following removal of the ligature, the capillary bed

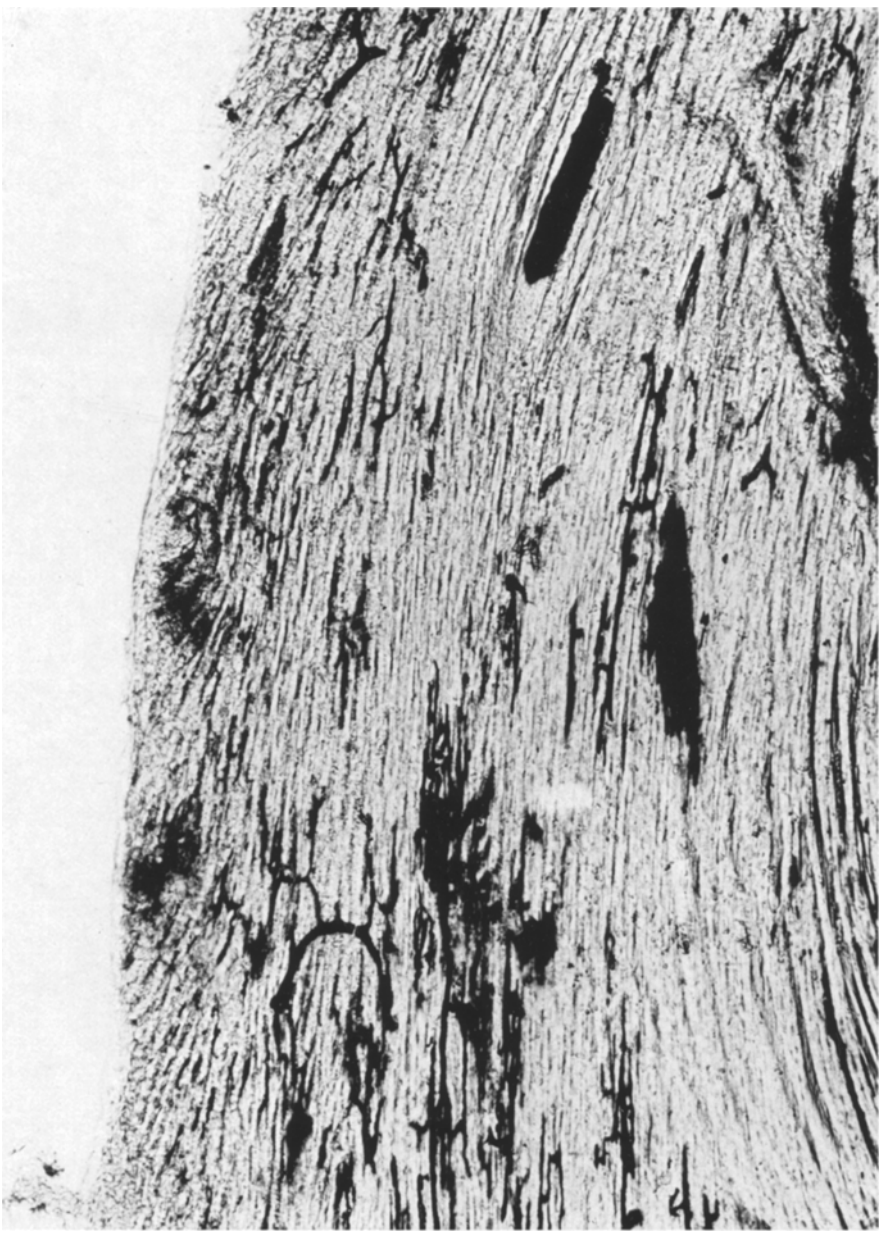

Fig. 3. 15 min temporary ischemia. Immediate carbon injection. Note large areas of impaired perfusion. ×105

was partially reexpanded and the non injected area decreased (Fig. 8 and 9). After the first hour, there was a progressive extension of the non injected areas. After 6 hours, the non perfused area was greater than that present at the start of reperfusion (time 0). After 24 hours of reperfusion, there was a definite decrease in failed perfusion zones, as compared to those seen in controls with permanent

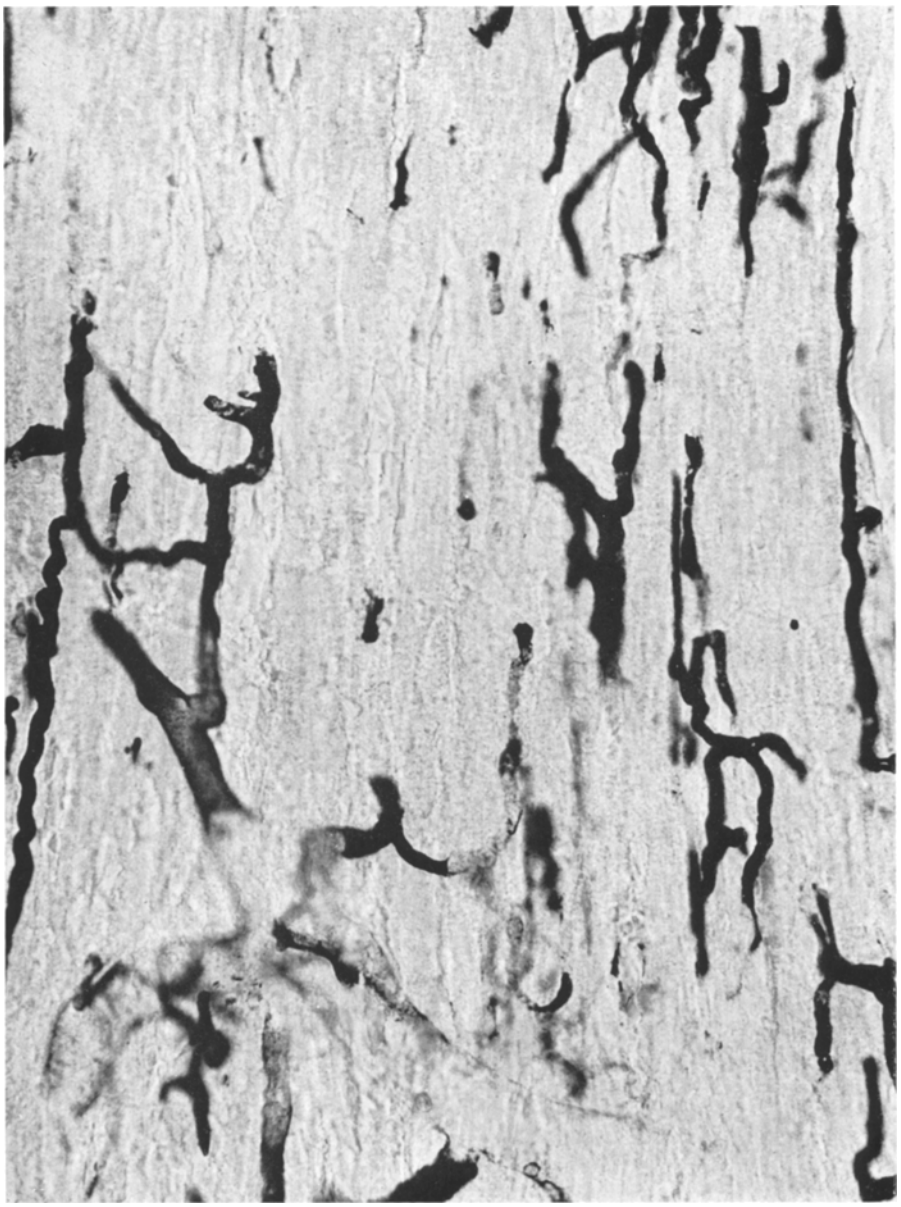

Fig. 4. 30 min temporary ischemia. Immediate carbon perfusion. Carbon black is present in arteriolar tufts but little has reached the capillary level. ×375

ischemia (Fig. 10). The non injected areas at this stage corresponded to tissue necrosis. There were numerous dilated capillaries filled with red blood cells.

2. Late Reperfusion (Temporary Ischemia of 1–6 Hours Duration) (18 Rats). The initial improvement was again present but brief and discrete. It was followed by a slow extension of the non perfusion areas during the first six hours, after which it remained unchanged, similar to that seen in controls.

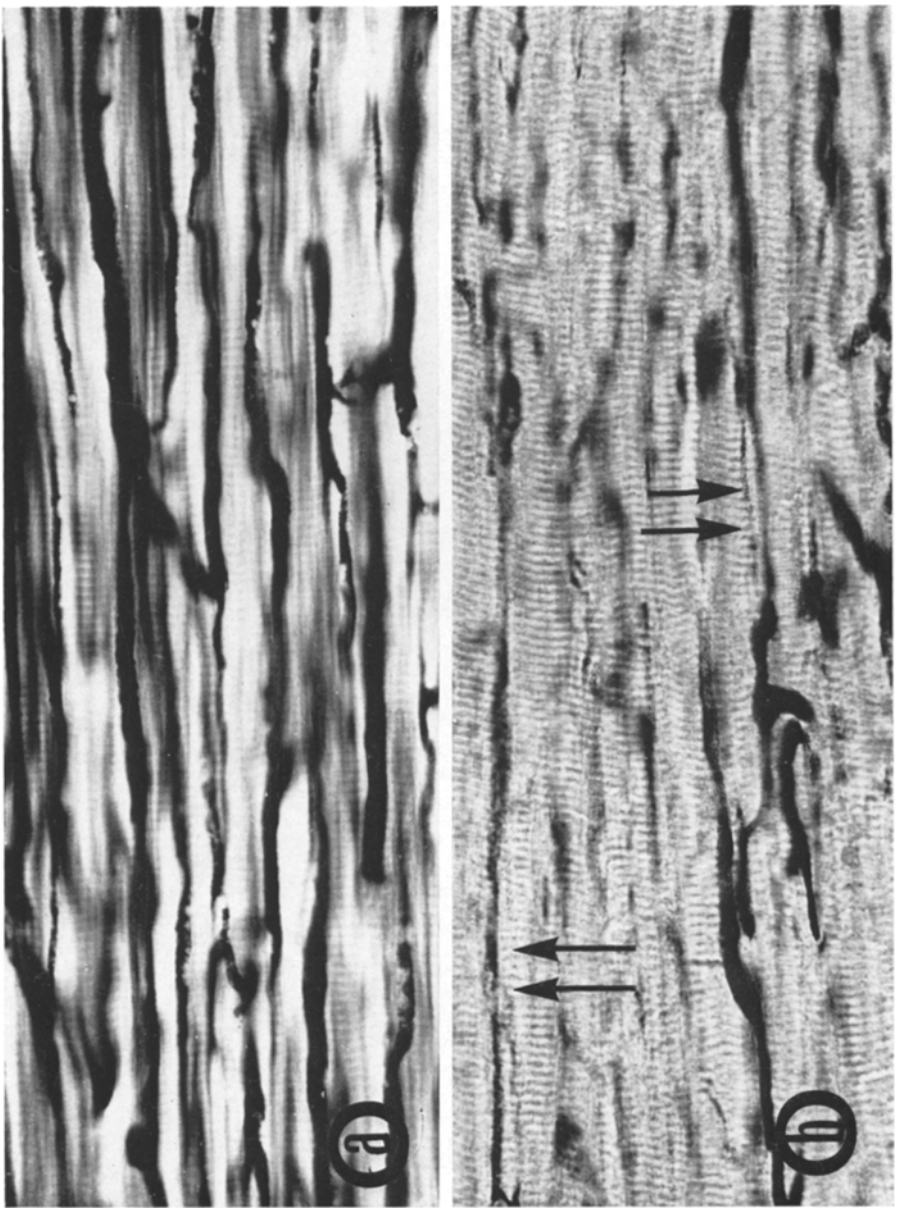

Fig. 5 a and b. Detail of the early changes following ischemia. (a) Control. (b) Incomplete capillary injection following temporary ischemia of 30 min duration. Little carbon is present in fine collapsed capillaries (arrows). ×600

III. Late Changes Following Ischemia (8 Days, 15 Rats)

This was the stage of scar formation. The area of the infarct was characterized by a thinning of the wall and a slight bulging of the ventricular cavity.

Study of the sections following carbon injection showed the presence of rare neo-vessels within scar tissue which were recognizable by their variable sizes and irregular patterns.

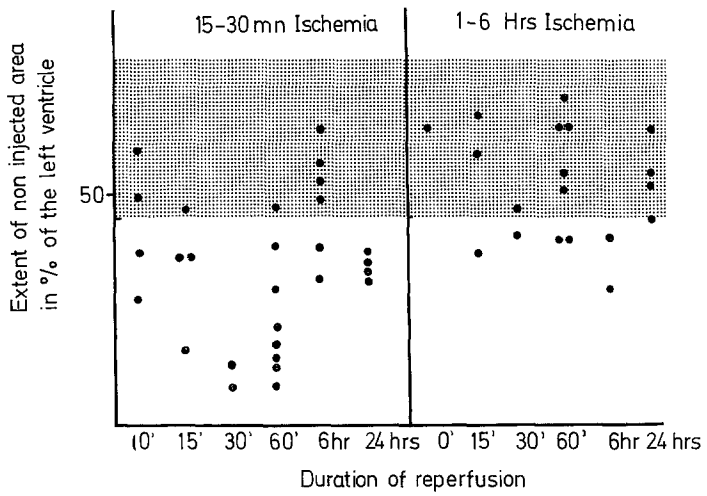

Fig. 6. Extent of areas of impaired perfusion as related to duration of ischemia and reperfusion. The no-reflow area is expressed in percentage of the left ventricle cut surface. ▒ Extent of non injected area in controls before ligature removal. 0 min =time of removal of the ligature

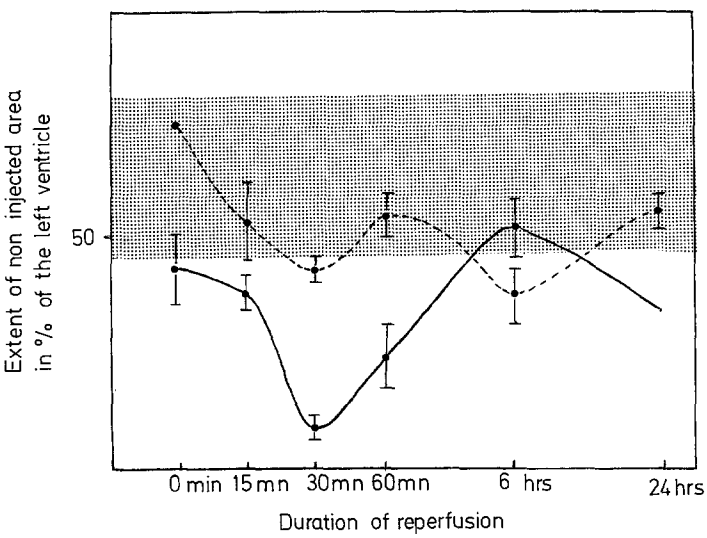

Fig. 7. Diagram illustrating the relationship between the non injected area and the time of reperfusion. Following early reperfusion, note the striking reduction of the no-reflow area within the first hour of reperfusion. After later reperfusion, there was a discrete initial improvement followed by a definite increase of non perfused zones which were similar to those seen in controls. Values are mean±SD. ▒ Extent of non injected area in controls before ligature removal

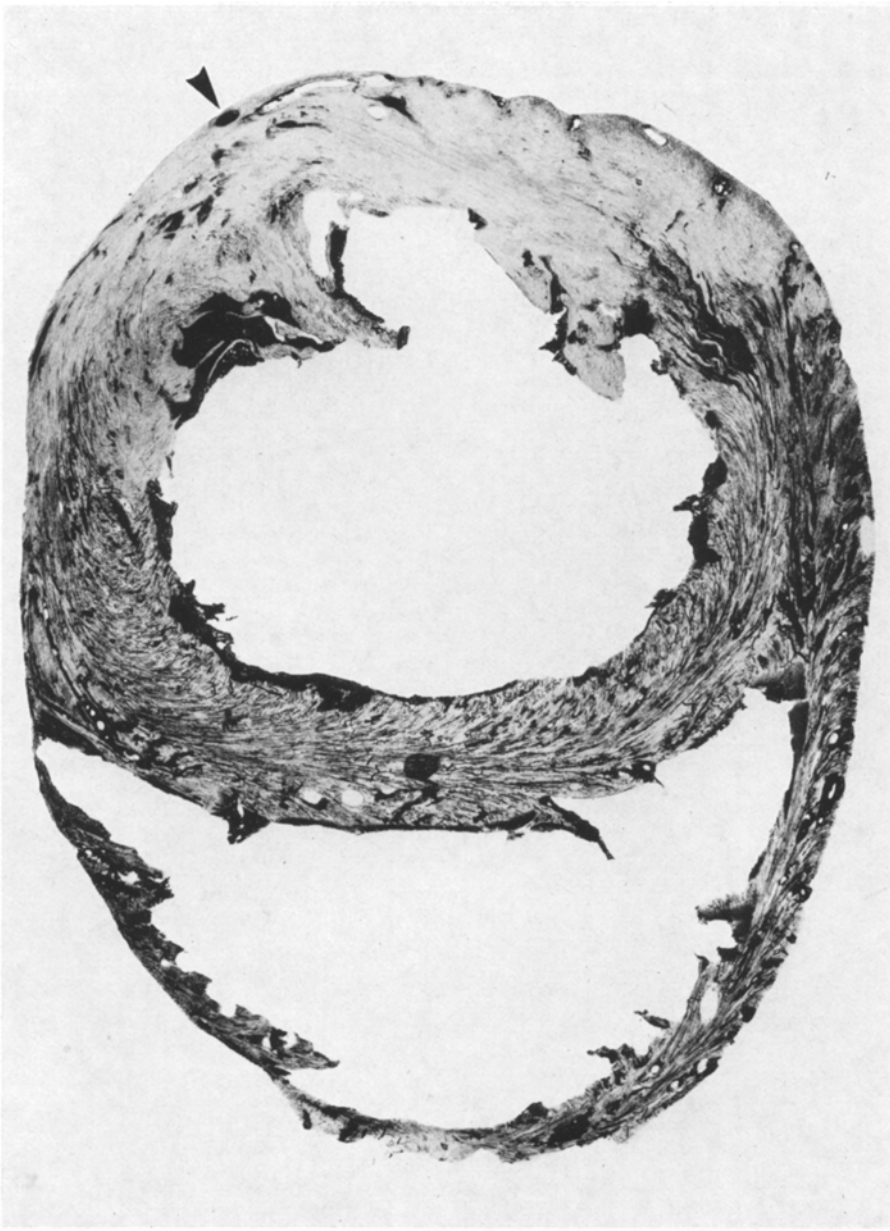

Fig. 8. 15 min ischemia. Carbon injection 15 min later. Note complete lack of capillary perfusion in ischemic area. Some intramyocardial branches of left coronary artery are filled with carbon black (arrow). ×11

Their injection was often incomplete. In the sub-endocardial zones and the papillary muscles which had not indergone necrosis, the vascular pattern was regular and similar to the adjacent areas.

The planimetric measurement of the scar area was difficult due to cicatricial retraction and the hypertrophy of the remaining myocardium. Comparison of the various scars formed showed a definite difference which was related to the lenght

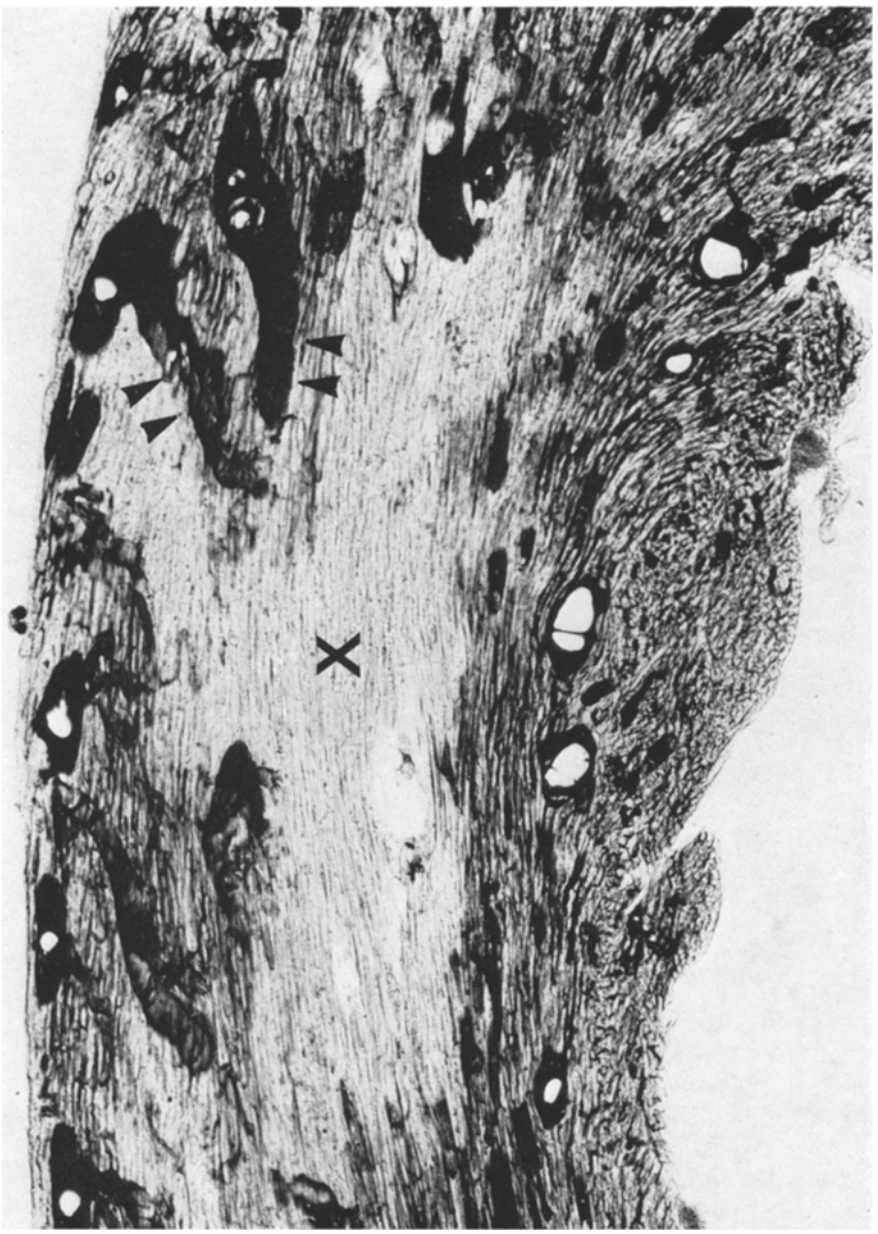

Fig. 9. 15 min ischemia. Carbon injection 1 h later. Note small intramural defect (×) surrounded by dilated veinues (arrows). ×63

of time of ischemia. For temporary ischemias lasting 30 min to 1 hour (10 rats) the scars were one half the size of scars obtained after 24 hr of ischemia (3 rats).

Discussion

The study of the microvasculature by injection of a suspension of colloidal carbon after fixation-perfusion is representative at a given time of the state of

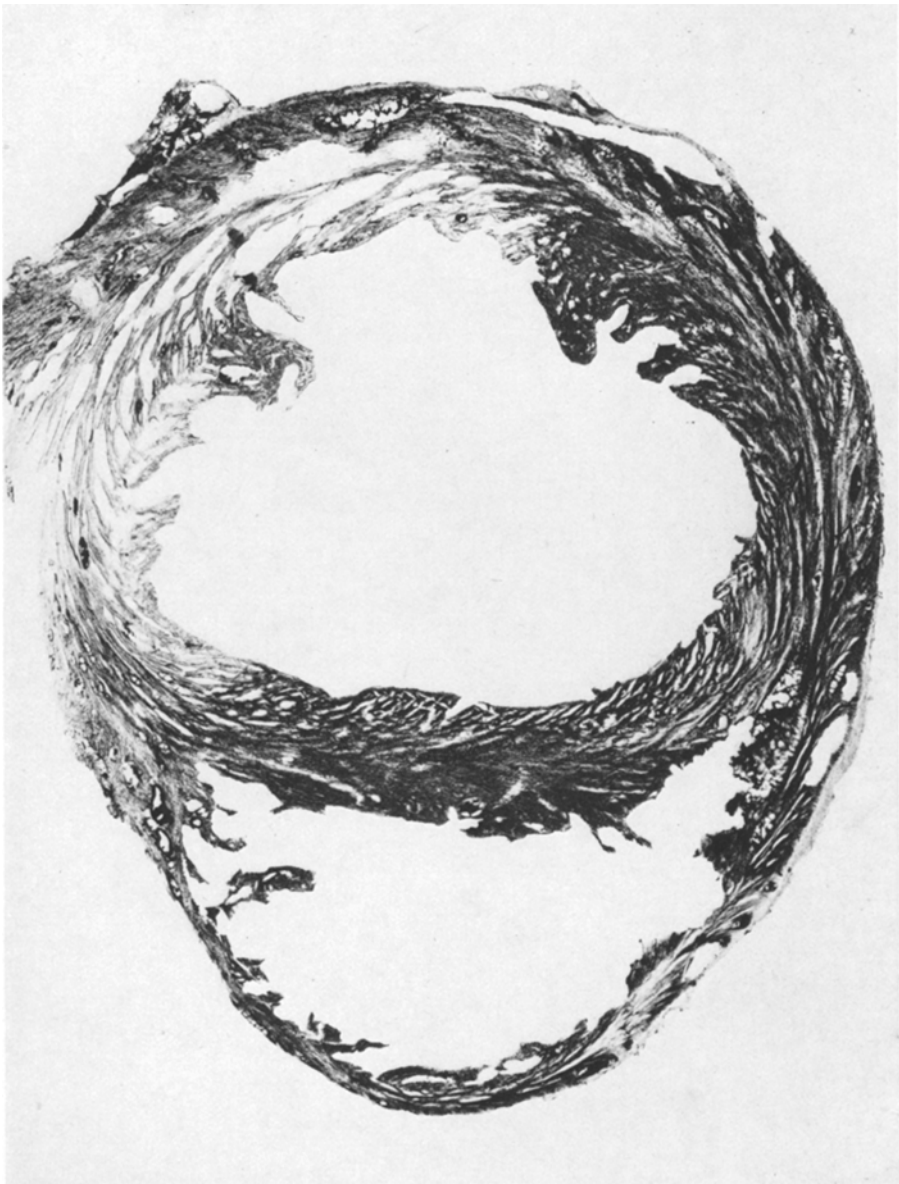

Fig. 10. 15 min ischemia. Carbon injection 24 hrs. Anterior transmural and sub-endocardial defect of failed perfusion, dependent on the necrotic area. ×11

the capillary bed. The use of the entire rat heart allowed us, by comparing the various coronary areas, to eliminate artefacts due to poor injection technique. In light of our results, it appears that localized myocardial ischemia is accompanied at the moment of reperfusion by an abnormal distribution of coronary blood flow at the level of the microcirculation. The immediate reexpansion of the arterial bed is in contrast to the absence of capillary injection in large myocardial areas. These morphological data suggest the existence of a capillary block.

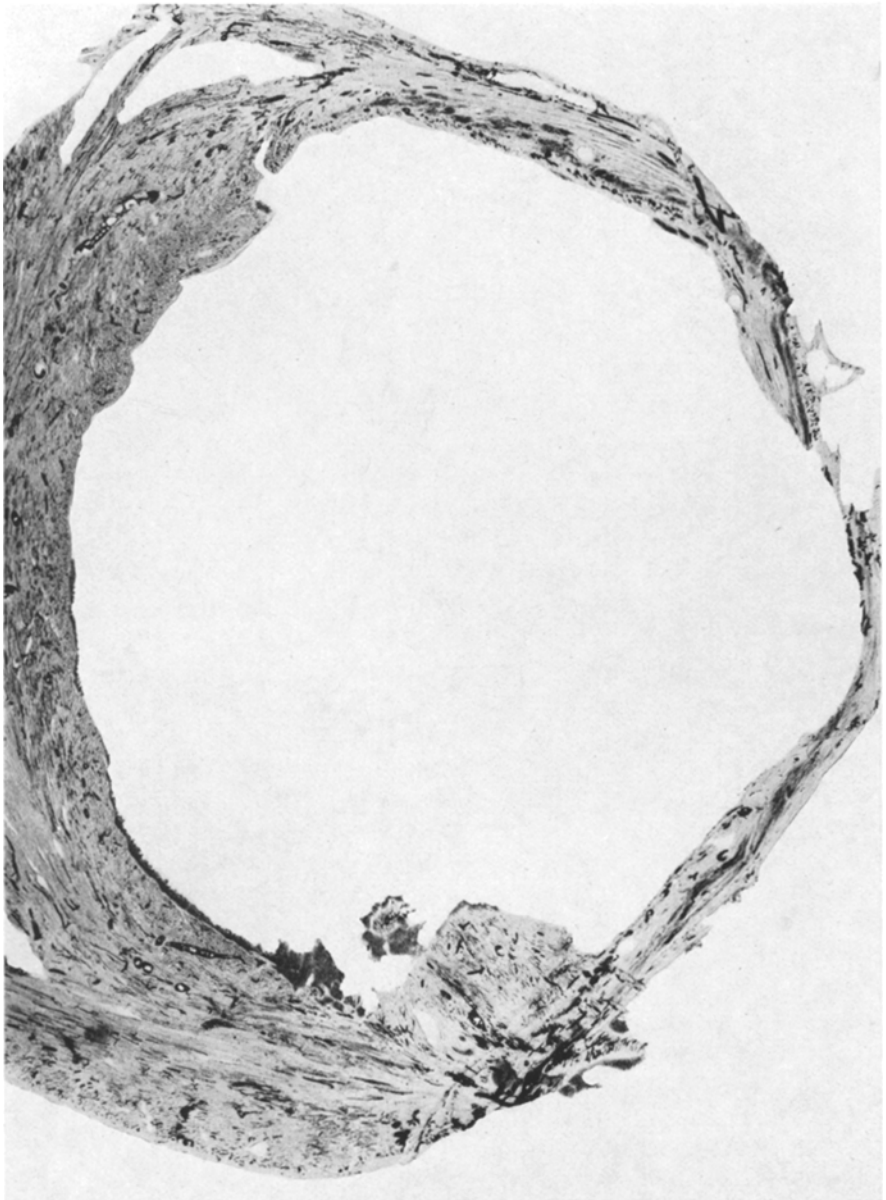

Fig. 11. 48 hrs ischemia. Carbon injection 7 days later. Large scar with thinning of the left ventricular wall. Carbon black filled neo-vessels in the scar. ×14

In 1948, the existence of post-ischemic changes of capillaries was noted by Harman in an original study of striated muscle. It has only been recently that an early objective demonstration was obtained using techniques of injection of fluorescent markers in ischemia of the cerebrum (Ames *et al.*, 1968; Chaing *et al.*, 1968), the dermis (Willms-Kretschmer and Majno, 1969), the kidney (Summers and Jamison, 1971; Franklin *et al.*, 1974). This phenomenon has been called "no reflow". Insofar as the myocardium is concerned, Krug *et al.* (1966) showed, by

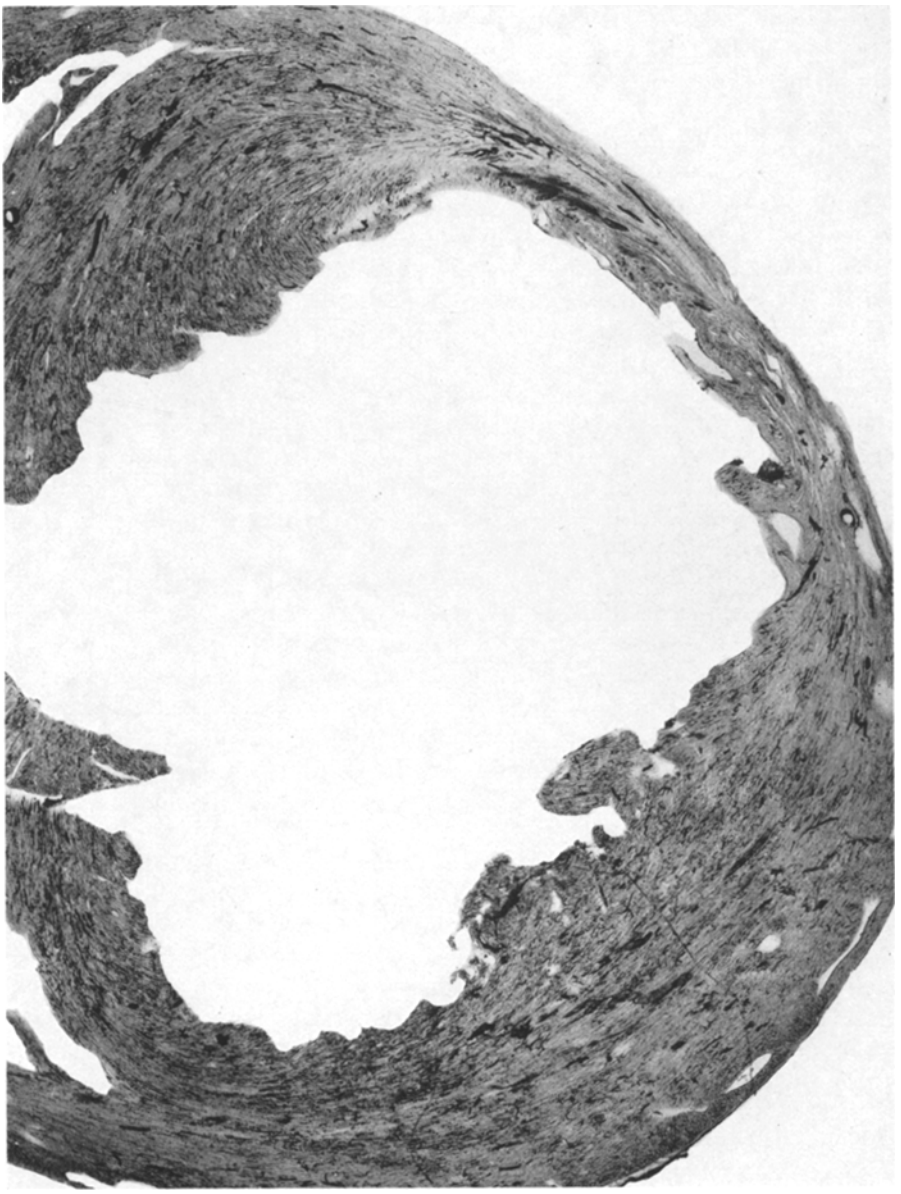

Fig. 12. 15 min ischemia. Carbon injection 7 days later. Reduced anterolateral scar. Note the cicatricial retraction and the hypertrophy of the remaining myocardium. ×14

injection of fluores particles in the cat after temporary ischemia of 30 to 60 min, that the reestablishment of coronary blood flow was followed by hypoperfusion of the deep subendocardial layers. In 1970, Hauschild *et al.*, Baghirzade *et al.*, noted, in the isolated guinea pig heart preparation, an increase in coronary artery resistance following ischemic periods lasting 45, 60 and 75 min. Similar findings were reported in the dog by Willerson *et al.* (1972) and more recently by Kloner *et al.* (1974). The latter, using injections of colloidal carbon and thioflavine,

studied the process of reperfusion in terminal vessels: the posterior papillary muscle of the mitral valve after ligature of the circumflex artery. Using this protocol, the phenomen of "non reflow" was observed for periods of ischemia lasting 90 min.

In the present study, with the protocol which we have adopted, the most important observations we have noted may be summarized as follows:

In contrast to the data of Kloner *et al.* (1974), the phenomen of "no reflow" was observed for periods of ischemia of short duration i. e. : 15 min.

The servity of this phenomen was in direct proportion to the length of ischemia time; after 30 to 60 min of blood flow interruption, it involved the major portion of the area dependent on the ligated coronary artery.

In cases of temporary ischemia followed by reperfusion, the "no reflow" phenomenon was dependent upon the duration of reperfusion. For reperfusion lasting less than one hour, we obtained a partial reopening of the microvasculature. This first wawe of improvement in reperfusion at the capillary level was proportionately more marked if the initial ischemic period was brief. The improvement was minimal in ischemias lasting one hour or more and was completely absent in cases of late reperfusion (24 hrs). Between 2 and 6 hrs, we noted a progressive extension of the non-injected zones, after which (24 hrs of reperfusion), the non injected zones were identical to the necrotic areas.

The nature of this process is poorly understood. Several hypotheses may be advanced. Fibrino-platelet thrombosis appears improbable. Systematic staining of tissues for fibrin (PAS) is negative and Heparin does not prevent it. Post-ischemic arterial spasm does not appear to be present. The microcirculatory changes remain localized and appear only after a certain period if ischemia. We have been able to confirm that after 1 min of coronary blood flow interruption, we obtained an excellent filling of the capillary bed.

Two other factors appear to be more important: an increase in blood viscosity and endothelial lesions. Highly suggestive of an increase in blood viscosity is the aspect of red blood cell sequestration sometimes observed in the ischemic zones. They seem to bear witness to the phenomenon of "sludging" which would be capable of decreasing blood flow to these areas. Various authors have underscored the fact that the *endothelial* lesions appear earlier than the parenchymal lesions, and consist of nuclear alterations, dilation of the endoplasmic reticulum, cellular edema, protrusion into the lumen of cytoplasmic blebs. This has been shown in the brain by Chaing *et al.* (1968), in the dermis by Willms-Kretschmer and Majno (1969), in the kidney by Flores *et al.* (1972), in the myocardium by Hausamen und Poche (1965), Poche *et al.* (1969). Krug *et al.* (1966) pointed out the existence of capillary rupture with interstitial hemorrhages and fibrin deposits. These changes were characteristic of late reperfusions (6 to 24 hrs).

In the early stages of reperfusion (first hour), it appeared in our study that capillary filling was accompanied by microvascular collapse confirmed by the small size of the capillaries which were injected by the tracer. We frequently noted that, next to the normal areas in which the tracer. We frequently noted that, next to the normal areas in which the capillaries were of normal size and widely patent due to fixation-perfusion, there were marginal zones in which the capillaries were collapsed and appeared as narrow slits. These capillaries contained

no red blood cells but only a few paricles of injected carbon. The mechanism causing these changes remains to be elucidated. It does not appear to be a diffuse edema of the endothelial cells. In the brain, it has been postulated that this is due to mechanical pressure on the capillary lumen by the surrounding edematous glial cells. In the myocardium, a similar role could be ascribed to the edema of the myocardial cells secondary to early modifications of fluid transfers during the period of ischemia (Jennings *et al.*, 1965; Leaf, 1970; Whalen *et al.*, 1974). Using isolated guinea pig heart preparations, Baghirzade *et al.* (1970), Hauschild *et al.* (1970) showed that the capillary block occured simultaneously with changes in myocardial tension which occured in the early stages of ischemia. This phenomenon which has been previously noted by Coffman *et al.* (1960) appears to be due to edema and to the contractile state of the ischemic myocardium. Other studies will be necessary to confirm these observations but it is probable that a definite relationship exists between microvascular collapse and the state of the myocardial cell.

Finally, *several factors may be present*: increased blood viscosity, cellular alterations, endothelial lesions, the combined effects of myocardial cell edema and contractile state. More than one mechanism may be at work. In the light of our own results, we would like to suggest the *two* following *theoretical successive phases*:

A first phase during the first 30 min of reperfusion which is characterized by a collapse of the capillary bed due to edema and the contractile state of the myocardium.

A second phase after one hour of reperfusion which appears after an initial improvement in the microcirculation and is characterized by capillary engorgement and is due to endothelial lesions and tissue necrosis.

Thus, microvascular changes appear to play a role in the evolution of the reperfused ischemic foci above and beyond the tolerance of the myocardial cell to anoxia. In a recent study, we stressed the variability in the effects of reestablishment of blood flow, the irregular changes in the marginal areas, the presence of localized infarcts following brief periods of ischemia (Camilleri *et al.*, 1975). It is possible, that this phenomenon of "no reflow" removes from the effects of reperfusion certain areas which then progress to necrosis. In these areas, the microvascular lesions represent a point of "no return" whereas the myocardial cells themselves might still posses the biochemical potential to assure their continued structural and functional integrity. The attempts being made at the present time to improve the energy metabolism in the anoxic areas will not be fully efficacious until the capillary block can be at least be partially removed. The incidence of these changes in the present methods of myocardial preservation during open heart surgery are presently under investigation.

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