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MACROSCOPIC IDENTIFICATION OF EARLY MYOCARDIAL INFARCTS BY ALTERATIONS IN DEHYDROGENASE ACTIVITY

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The identification of early myocardial infarcts at necropsy is a frequent source of difficulty, even for experienced prosectors. Unequivocal gross changes of infarction do not become apparent for some 24 to 48 hours following the occlusion of a major coronary artery in man [1,2] or for 7 to 24 hours in experimental animals.[3–7] Furthermore, even though minimal microscopic evidence of infarction can be recognized as early as 6 hours in man [1,2] or 2 to 6 hours in experimental animals,[4,5,8] in the absence of gross changes the involved area can be missed when random blocks are taken for histologic examination.

Recent experimental and clinical reports indicate that elevated levels of several enzymes appear in serum shortly after myocardial infarction, and that concurrently there is a demonstrable decrease in the activities of these enzymes in the infarcted area of the heart.[9–15] These biochemical data are supported by the earlier histochemical findings of Wachstein and Meisel [16] that succinic dehydrogenase activity is lost from necrotic heart muscle within 6 to 8 hours of the onset of ischemia. With the foregoing information available, it seemed worth while to investigate the possibility of using an enzyme reaction in the gross identification of early myocardial infarcts. Accordingly, histochemical methods were applied to large transverse slices of heart muscle in attempts to visualize

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sites of activity of cytochrome oxidase, succinic dehydrogenase and certain other dehydrogenases linked either to diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN).

During the course of this study, the recent work of two European groups came to our attention. In 1956, Neoral and associates[6,17] described their experimental results in dogs using sodium tellurite as an oxidation-reduction indicator for the macroscopic diagnosis of infarcts of 5 to 10 hours' duration. Their subsequent experience with a small number of human hearts obtained at necropsy indicated that an infarct of 7 hours' duration could be recognized by this method.[18,19] Reduction of sodium tellurite by malic dehydrogenase activity in normal myocardium resulted in a dark gray stain, while infarcted muscle with its decreased dehydrogenase activity remained unstained. About the same time and for the same purpose, Sandritter and Jestädt employed triphenyltetrazolium chloride (TPT).[7,8] This oxidation-reduction indicator yielded a bright red formazan pigment at sites of normal dehydrogenase activity. Their studies with guinea pigs, and their experience with 112 human hearts [8] suggested that it was often possible to recognize infarcts at necropsy by this histochemical staining technique in patients who died as early as 4 to 24 hours after the onset of clinical signs and symptoms.

The work reported herein confirms and extends the findings of the European investigators. In an initial group of experiments, an attempt was made to identify the biochemical lesions of early muscle necrosis that could be exploited for the macroscopic demonstration of recent myocardial infarcts with oxidation-reduction indicators. It will be shown that with Nitro-BT, a new, sensitive tetrazolium salt with favorable chromogenic properties, experimental myocardial infarcts in dogs can be outlined initially by virtue of loss of endogenous substrates and coenzymes and, at later stages in their evolution, by the added loss of respiratory enzymes. Results with 23 human hearts, suspected of harboring recent and old infarcts will be compared. As will be shown, our technique offers the following distinct advantages over those previously reported: (1) Only a 20 to 40 minute incubation period is required to achieve good differential staining, as opposed to 3 to 8 hours with the tellurite method. (2) The reaction product deposited in the region of healthy heart muscle is a dark blue formazan, which provides for heightened color contrast over the brick red pigment produced in the triphenyltetrazolium (TPT) reaction. (3) It is possible to correlate macroscopic and microscopic changes with certainty, because the formazan of Nitro-BT is not extracted from tissue blocks by the solvents used in the preparation of paraffin sections.

Material and Methods

With Nembutal® as the anesthetic agent (2.6 mg. per kg. of body weight) and the Emerson resuscitator to maintain respiration, the left thoracic cavity was entered. In 40 dogs the anterior descending branch of the left coronary artery (LAD) was ligated within 1.5 cm. of its origin and just distal to the exit of the first major branch. In 6 experiments the ligature was applied loosely according to the method of Le Roy, Fenn and Gilbert,[20] and 2 to 3 weeks later traction was placed on the loose ends, thus tightening the ligature about the LAD at a time when the animals had recovered from the effects of thoracotomy. These animals were sacrificed at 6 hours after coronary artery occlusion and are referred to as the "closed-chest occlusions."

The hearts were examined at intervals of 1 to 120 hours after the coronary artery occlusion. They were sectioned transversely at 5 mm. intervals through both ventricles, from the apex to the site of the ligature, according to the method of Sikl.[21] Twenty-one human hearts were also examined from persons with clinical histories of atherosclerotic heart disease. Necropsies were performed 2 to 70 hours after death. Seven of the patients died suddenly or within minutes of the onset of acute chest pain or dyspnea. Six patients with "acute myocardial infarction" died 2, 7, 8, 30, 48 and 55 hours, respectively, after the onset of typical acute symptoms; in 4 of these cases the diagnosis was supported by electrocardiographic and laboratory findings. Six other patients succumbed during convalescence, 1 to 4 weeks after myocardial infarction. Two cases of severe congestive heart failure without recent infarction were also included in the series. Of the foregoing 21 patients, 8 were known to have had one or more earlier clinical episodes of myocardial infarction followed by recovery. Finally, also studied were 2 hypertrophied hearts which had been arrested by local hypothermia and maintained without coronary perfusion for 60 to 100 minutes. These were instances of open heart surgery with attempted surgical correction of severe calcific aortic stenosis.

Early in the course of this investigation, the heart slices were placed in various substrate media employed for the histochemical demonstration of succinic dehydrogenase, DPN diaphorase, lactic dehydrogenase, malic dehydrogenase, TPN diaphorase, and cytochrome oxidase.[22–25] The tellurite [6] and triphenyltetrazolium [7] methods were also compared with our reagents. It became apparent that of the dehydrogenase reactions, no one method in particular was strikingly superior to the others for the gross demonstration of myocardial infarcts of 24 hours' duration. These preliminary experiments also indicated that greater contrast between normal and injured myocardium could be obtained in early infarcts by making conditions for the histochemical reactions suboptimal. This was done by omitting all exogenous substrates. Thus, the incubation solution which was found to be best suited for the macroscopic identification of myocardial infarction 2 to 24 hours after coronary ligation consisted of Sorensen's phosphate buffer (0.1 M) pH 7.4 and 0.5 mg. per ml. of Nitro-BT [2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride]. For convenience, stock solutions consisting of phosphate buffer at 1 M concentration and Nitro-BT at 5 mg. per ml. were kept. When ready for use, the incubation solution was prepared by mixing 1 part buffer, 1 part Nitro-BT, and 8 parts water. The Nitro-BT obtained from 2 commercial laboratories (Dajac Laboratories and Nutritional Biochemicals Corporation) worked as well in these studies as the tetrazolium salt prepared in our laboratory. The slices of dog hearts were rinsed briefly in running cold water to remove traces of blood and tissue juices from the cut surfaces, and then were placed in the buffered tetrazolium solution for a 30-minute incubation at 37° C. in a water bath. Normal myocardium stained dark blue within 15 minutes, while the infarcts remained unstained or stained only faintly.

In order to determine which anaerobic dehydrogenase of heart muscle was mainly responsible for the production of formazan pigment from Nitro-BT, experiments were carried out in which various substrates (succinate, 0.1 M; DL lactate, 0.1 M; malate, 0.1 M; isocitrate, 0.1 M; DPN, 0.5 mg. per ml.; TPN, 0.5 mg. per ml., and glucose, 1 per cent) and dehydrogenase inhibitors (malonate, 0.01 M; oxaloacetate, 0.01 M; oxalate, 0.01 M; p-chloromercuribenzoate, 0.001 M, iodoacetate, 0.01 M, and N-ethyl maleimide, 0.01 M) were added to the standard buffered Nitro-BT solution described above.

The effect on the differential staining reaction of the loss of endogenous substrates and coenzymes from early infarcts was assessed by adding succinate (0.1 M), DL lactate (0.1 M), and DPN (0.5 mg. per ml.) to the standard buffered Nitro-BT solution. The rate of staining of infarcted and normal muscle in comparison with similar controls incubated without a substrate was then noted. The effects of postmortem autolysis on normal myocardium were assessed by storing portions of canine and human hearts at room temperature and at 4° C. for periods up to 72 hours. Freshly cut slices were then incubated in the standard buffered Nitro-BT solution, with and without the added substrates listed above. It was recognized from the foregoing experiments that it would be advantageous to add 0.1 M succinate to the standard buffered Nitro-BT solution, when dealing with human hearts obtained after a postmortem interval of more than 6 hours, in order to compensate for loss of endogenous substrates from normal heart muscle. This modification in technique was used with 12 human hearts examined during the latter part of the study.

After the histochemical reactions employing Nitro-BT had differentiated normal from necrotic muscle, it was possible to correlate the gross findings with alterations in histologic structure in both the canine and human hearts. This was done by cutting tissue blocks (1 by 1.5 by 0.4 cm.) at a junctional site, fixing for 12 hours in neutral 10 per cent formalin and then processing the blocks for the preparation of paraffin sections. Since the tetrazolium reduction occurred only on the surface, by the proper orientation of the blocks on the object holders of the microtome one could obtain preparations with the tetrazolium marker along one edge of the tissue section. The histologic treatments used for the preparation of tissue sections stained with hematoxylin and eosin did not remove the formazan of Nitro-BT.

Results

When the slices of dog hearts with 24-hour infarcts were placed in suitable incubation media, intense staining was noted within 15 minutes in the normal portions of myocardium, and a much weaker staining reaction appeared in the ischemic myocardium. The pigment deposited by the activity of cytochrome oxidase was reddish-purple and did not provide sharp color contrast between the normal and necrotic muscle fibers. The activities of all the dehydrogenases resulted in the reduction of Nitro-BT to a dark blue formazan, which gave good contrast between active and inactive portions of heart muscle. However, since all enzymatic activity in these, and more particularly in earlier infarcts, was not lost, differential staining could be missed by allowing the reaction to continue for 30 minutes before making observations. This shortcoming was corrected by omitting all exogenous substrates from the incubation solution. The dehydrogenases utilized endogenous substrates in the heart muscle; when these were consumed, tetrazolium reduction

ceased. In this way, differential staining between normal and necrotic heart muscle became apparent, and persisted even when the incubation was allowed to continue for 1 to 1½ hours. The only exceptions to this statement were in the hearts where the coronary artery had been occluded for only 2 to 4 hours before sacrifice.

One of the first questions that arose was whether early myocardial infarcts failed to stain in buffered Nitro-BT solution because endogenous substrates had been depleted or because respiratory enzymes had been lost from the ischemic myocardium. Theoretically, one would expect that most of the effect in hearts examined after a short period of arterial occlusion would be due to substrate rather than to enzyme deficiency. This point was tested by incubating the heart slices in buffered Nitro-BT alone and with succinate, DPN and DL lactate, as sources of exogenous substrate. In hearts with a 2 to 4-hour period of ischemia, the differential staining was not apparent when exogenous succinate, DPN, or DPN and lactate, were present. Lactate alone did not repair the biochemical "lesion." After 24 hours of arterial occlusion, the addition of lactate and DPN had no effect, while succinate still partially restored activity in 3 out of 4 hearts. Thus, the effects noted in hearts with brief periods of ischemia were due primarily to substrate depletion, while at a later time, some of the respiratory enzymes were absent.

The use of suboptimal histochemical conditions to exaggerate the biochemical differences between the normal and ischemic myocardium was employed unknowingly by the two European groups who were studying this problem independently. Neoral and associates [6,17–19] utilized sodium tellurite which is such a poor oxidation-reduction indicator that even after the addition of substrate, sodium malate, the required incubation period was from 3 to 8 hours. These authors also tested neotetrazolium and triphenyltetrazolium, but found them deficient in the color contrast which they produced.[18] Sandritter and Jestädt [7,8] used a tetrazolium salt, which is a better oxidation-reduction indicator than tellurite, but chose one of the least sensitive electron acceptors in this class of reagents. Availability and low cost were their reasons for selecting TPT. In this gross technique, the insensitivity of TPT is not as pronounced as was seen when this redox dye was used for biochemical assay or histochemical visualization of enzymatic activity.[26] In many of our experiments both the tellurium and TPT methods were compared with our Nitro-BT method. Tellurium did outline the area of infarction after a somewhat prolonged reaction time (3 hours or longer). TPT was likewise accurate with fresh tissues although we agree with Neoral and co-workers [6,17–19] that the red color of its formazan did not produce

as striking a color contrast as the black tellurium or the dark blue pigment obtained on reduction of Nitro-BT.

The second question that arose was whether any single dehydrogenase was mainly responsible for pigment production. With the tellurite method, the increased yield of tellurium by the addition of sodium malate was clearly due to malic dehydrogenase activity. Sandritter and Jestädt [7,8] frequently added sodium succinate to their incubation solution, although they did not notice a marked resultant increase in pigment production. To clarify this issue, experiments were carried out in which various substrates (succinate, lactate, malate, isocitrate, DPN, TPN, and glucose) and dehydrogenase inhibitors (malonate, oxaloacetate, oxalate, p-chloromercuribenzoate, iodoacetate and N-ethyl maleimide) were added to the standard Nitro-BT buffer solution. In each instance the addition of substrate increased the rate of pigment production in the normal heart muscle somewhat during the first 10 to 20 minutes, but by 30 minutes the test and control slices of heart muscle were intensely and equally stained. The inhibitors, which were not specific for any one dehydrogenase, had a retarding effect, in that during the early incubation period there was less staining than in controls, but by 40 minutes nearly all heart muscle slices were stained equally. The only exception to this statement was with N-ethyl maleimide (10) which completely inhibited all dehydrogenase activity. Thus it appeared that the blue formazan produced by the method described here resulted from many dehydrogenases oxidizing the endogenous substrates present in the heart muscle and simultaneously transferring the liberated electrons to Nitro-BT.

A matter of practical importance was whether hearts could be tested reliably for their dehydrogenase reaction in instances when an experimental animal had died over a weekend, or in the patient whose necropsy examination was performed 24 or more hours after death. Ten dog hearts were stored in the refrigerator at 4° for 6 to 48 hours, after a slice from each had been tested. Pigment appeared uniformly in the refrigerated heart slices, but at a rate of deposition of about one half that seen with the fresh material. The addition of 0.1 M succinate to the buffered Nitro-BT medium restored the original rate and intensity of staining. When a 6 to 24 hour infarct was present, it could be differentiated as readily in the older refrigerated specimen as in the fresh, although the incubation period in the buffered Nitro-BT solution without substrate had to be increased to 30 or 40 minutes. Storage for 48 hours at 4° C. resulted in patchy and erratic staining, with some areas showing the expected dark blue pigment and other areas being unstained. Three human hearts behaved similarly in that staining was

satisfactory in specimens stored for 24 hours but was patchy after 48 hours in cold storage. In several experiments the tellurite and TPT methods were also tried with aged specimens. The reactions with these less sensitive redox dyes were less dependable. Storage for 6 hours at 37° C. and 12 to 16 hours at room temperature produced effects comparable to those observed after 48 hours in the refrigerator. Specimens aged under these conditions of time and temperature could not be evaluated reliably.

After ligation of the LAD, myocardial infarction occurred in all but 3 of the 40 animals tested. Other investigators have also noted that about 10 per cent of their dogs apparently had sufficient collateral circulation to prevent infarction after ligation of a major coronary vessel.[9,11] Only one animal died before the anticipated time of sacrifice, which varied from 1 hour to 5 days (Table I). In general, the infarcts could be grouped into 3 classes which were related to the extent of myocardial involvement. In 31 of 37 animals this was massive and included the anterior portion of the interventricular septum, the anterior wall of the left ventricle and the anterior papillary muscle. The group designated as "confluent" included those with a uniformly negative reaction and death of all the muscle fibers (Figs. 6 and 9), while the 8 hearts described as "patchy" contained significant islands of enzymatically active muscle fibers within the infarct (Fig. 4). In 6 animals, the infarcts were described as "minimal," because in these, dehydrogenase activity was deficient only in the anterior papillary muscle and in a few small foci of the anterior ventricular wall near the endocardial surface (Figs. 1 and 2). In most of the animals there was a 1 mm. zone of viable muscle beneath the endocardium, which presumably derived its blood supply from the left ventricle. At the time of sacrifice 6 hours after ligation of the coronary artery, no differences were seen in extent of involvement between the open and closed-chest occlusions.

Table I summarizes the results following experimental occlusion of the LAD in 40 dogs. The findings on gross examination of the heart and after the enzyme reaction had been completed are described as "definite," indicating that a myocardial infarction could be recognized without question, and "equivocal," encompassing those hearts in which the presence of an infarct was uncertain or nonexistent. For example, in all 15 dogs with periods of coronary artery occlusion varying from 1 to 4 hours before sacrifice, gross examination of the heart did not furnish any suspicion as to the presence of a myocardial infarction. However, after the enzyme reaction had been performed, the infarct could be recognized in 12 of these 15 hearts. In 2 of the 3 remaining cases described as equivocal, no infarction occurred, while in the other heart

TABLE I

RECOGNITION OF EARLY INFARCTS BEFORE AND AFTER TESTING FOR DEHYDROGENASE ACTIVITY *

No. of dogs	Duration of occlusion (hr.)	Gross findings		Enzyme reaction		Extent of involvement			
		Definite	Equivocal	Definite	Equivocal	Confluent	Patchy	Minimal	No infarct
5	1	0	5	4	1	0	2	2	1
5	2	0	5	5	0	1	2	2	0
5	4	0	5	3	2	2	2	0	1
14	6	8	6	11	3	10	2	1	1
8	24	8	0	8	0	8	0	0	0
3	120	2	1	2	1	2	0	1	0

* Forty dogs were subjected to ligation of the left anterior descending coronary artery. The findings listed as "equivocal" include those in which the presence of an infarct was uncertain or not recognizable. The differences between the 3 types describing the extent of involvement are explained in more detail in the text.

the infarct was minimal and was overlooked. In the 14 animals sacrificed after occlusion of the LAD for 6 hours, there were 13 myocardial infarcts, 8 of which were recognized on inspection of the unstained fresh heart. However, after the dehydrogenase reaction, the presence and location of all 13 infarcts became apparent even though in 2 instances (listed as "equivocal") the contrast was not as striking as in the 11 other specimens of this group. Infarcts of 24 hours or longer were usually readily identifiable by simple gross inspection. However, the exact limits of the necrotic muscle were much more sharply outlined after the dehydrogenase reaction, and as pointed out by Jestädt and Sandritter,[8] the extent of the damage was often as much as 0.5 cm. beyond the apparent border noted by examination of the fresh hearts. The dehydrogenase reaction also assisted in the identification of islands of viable myocardium within patchy infarcts. To illustrate some of these observations, the cross-sectional appearance of opposing cut surfaces are shown without (Figs. 3 and 5) and with (Figs. 1, 2, 4 and 6) the dehydrogenase reaction.

As seen in the table, there were 7 of 40 hearts in which loss of substrates and dehydrogenase activity did not clearly differentiate an area of infarction. In 3 instances all the heart muscle stained uniformly. Whether an area of infarction was being overlooked could not be determined, because the respective durations of coronary artery occlusion in these experiments were only 1, 4 and 6 hours, and even after careful microscopic examination of sections stained with hematoxylin and eosin, no abnormalities of the heart muscle could be recognized. Presumably, these 3 dogs had sufficient collateral circulation to prevent infarction after LAD ligation. In another 3 of the 7 reactions listed as "equivocal," the areas of infarction could be recognized, but were not as sharply outlined as in most of the hearts. These occurred in animals having their arteries occluded for 4 and 6 hours. Perhaps these animals too had a better than average collateral circulation. The seventh reaction listed in the "equivocal" column was in a 5-day-old infarction. The heart muscle appeared to stain equally throughout. However, on microscopic examination there were minute foci of necrotic heart muscle which could not be identified at a macroscopic level even though the enzymatic activities in these areas were shown to be absent by histochemical studies of sections cut at 6 μ.

In the Method section it was pointed out that a precise correlation between gross and microscopic findings in the transitional zone between normal heart muscle and the infarct could be obtained by using the deposition of formazan as a criterion of muscle viability. Thus, when microscopic sections were examined, formazan pigment, dispersed dif-

fusely and in small granules between myofibrils, was noted along the previously incised surface where myocardial fibers were normal, while an absence of formazan pigment nearby focused attention on ischemic muscle fibers. At 4 hours after coronary artery ligation, only minimal histologic changes (i.e., congestion of capillaries and venules, and swelling of mitochondria) were evident in the areas of incipient infarction, and these alterations frequently would have been overlooked without the assistance of the formazan marker (Fig. 7). At 24 hours after coronary artery ligation, the sites which failed to develop surface deposits of formazan coincided exactly with areas which also showed histologic features of early ischemic infarction, (i.e., fragmentation of myofibrils, loss of cross striations, acidophilia of sarcoplasm, pyknosis of nuclei, and infiltration by neutrophils) (Fig. 8). There was no marginal transitional zone between normal and necrotic heart muscle which could be interpreted as showing intermediate biochemical changes. Thus, while a tissue section stained with hematoxylin and eosin might be informative enough when dealing with infarcts of more than 24 hours' duration, with shorter periods of ischemia or under unknown conditions, the simultaneous demonstration of a biochemical "lesion" in a histologic preparation had obvious advantages.

Our results with human hearts obtained at necropsy closely paralleled those encountered in dogs sacrificed at various intervals after LAD occlusion, provided that due allowances were made for differences in the postmortem interval. It was found advantageous when dealing with human hearts obtained after a postmortem interval of more than 6 hours to add 0.1 M succinate to the standard buffered Nitro-BT solution. This was done in order to compensate for nonspecific postmortem depletion of endogenous substrates, and thus to double or triple the rate of staining in normal heart muscle, and to eliminate troublesome staining artifacts. However, for reasons indicated earlier, the addition of exogenous substrate decreased the sensitivity of the method for demonstration of infarcts of less than 12 hours' duration. Nonetheless, hearts with older "recent infarcts," "healing infarcts," postinfarction scars and interstitial myocardial fibrosis, obtained from cadavers refrigerated at 6° C. for as long as 48 hours, gave satisfactory results. Storage of hearts *in situ* at 25 to 37° C. for 8 hours or more precluded valid differential staining reactions because of destruction of respiratory enzymes by autolytic processes.

Of the 21 patients with clinical atherosclerotic heart disease, all showed stenosis or occlusion of major branches of coronary arteries. Fresh thrombotic occlusions were found in 4 hearts. The hearts from patients who died suddenly or within minutes of the onset of symptoms, and from 2 patients with severe congestive heart failure, showed no

decrease in staining with standard buffered Nitro-BT solution (without added substrate), or gross or microscopic evidence of recent myocardial infarction. Similarly, in the heart of the patient who survived for 2 hours, there were no distinct biochemical or structural changes in the myocardium, apart from old interstitial fibrosis. In one case in which the time interval between onset of symptoms and death was 8 hours, areas of congestion and slight discoloration suggested the sites of very recent infarction, but did not precisely define their boundaries. This patient was a 75-year-old male with a clinical history of several previous "heart attacks" and mild aortic stenosis. At necropsy, performed 3 hours after death, the anterior descending branch of the left coronary artery was occluded by atheroma near its origin, and the circumflex branch was markedly stenotic. Transverse slices of the heart were incubated for 75 minutes in standard buffered Nitro-BT solution, without added substrate. The histochemical reaction outlined a recent infarct in the anterior wall of the left ventricle and interventricular septum (Fig. 10). Microscopic sections taken from nonstaining areas showed confirmatory structural changes.

In 3 cases in which myocardial infarction had taken place 30 to 50 hours before death, and in 6 remaining cases with infarcts of 1 to 4 weeks' duration, areas of necrosis of muscle displayed yellowish-gray discoloration, and were frequently bounded by a narrow hemorrhagic zone. Histologic sections of areas giving a negative enzyme staining reaction always showed necrosis of heart muscle, or early replacement fibrosis. Conversely, where the enzyme reaction was positive, there was never histologic evidence of necrosis. Even though the presence or absence of healing infarcts could be established readily by gross inspection at necropsy, the Nitro-BT reaction still was of value because it outlined large and small areas of necrosis precisely with a resolution of 0.5 mm., permitted the identification of tiny islands of viable muscle within apparently homogeneous infarcts, and facilitated the preparation of photographs of specimens by enhancing the color contrast between normal and necrotic muscle. The method provided results that correlated well with recorded clinical observations and electrocardiographic tracings. Figures 10 to 16 illustrate the macroscopic changes in 4 cases in our series. Two hearts that had been arrested by hypothermia, and maintained without coronary perfusion for 60 to 100 minutes during open heart surgery, stained uniformly, and at a normal rate, when incubated in a buffered Nitro-BT solution.

Discussion

The paucity of histologic alterations in heart muscle fibers deprived of their blood supply for 6 hours or less has stimulated investigators to

search for easily recognizable biochemical changes. These have been found to include depletion of glycogen, glucose, pyruvate and other labile substrates, a shift to anaerobic metabolism of pyruvate with accumulation of lactate and a drop in pH, consumption of adenosine triphosphate, adenosine diphosphate, creatine phosphate and phosphorylated intermediates of the glycolytic cycle, disappearance of diphosphopyridine nucleotide, loss of intracellular potassium and an accumulation of sodium, chloride and water.[27–30] Enzymes begin to leave the area of infarction later, and when they appear in the blood, are regarded as evidence of irreversible myocardial damage.[11,13,14] Thus, following ligation of a coronary artery in the dog, there was an initial period of little or no decrease in enzyme activity, lasting 40 to 70 minutes with glutamic oxaloacetic transaminase, 2 hours with lactic dehydrogenase, and 4 to 5 hours with succinic dehydrogenase; the tissue levels of all 3 enzymes fell rapidly thereafter, until values of 30 to 50 per cent of normal were reached 12 to 15 hours after ligation.[12] Most of the foregoing work was carried out either by biochemical assays or histochemical studies after ligation of a coronary artery, the specimen being taken from anticipated areas of infarction. Jennings and Wartman[31] stressed that difficulties might arise in such experimental studies because of lack of homogeneity in the infarct. The absence of recognizable gross changes for 12 to 24 hours after deprivation of arterial blood supply also rendered uncertain the accurate selection of blocks of ischemic muscle for microscopic examination. For these reasons techniques for the gross identification of early myocardial infarction are of great value.

The 3 methods available today differ in certain respects. The tellurite method requires several hours of incubation, but furnishes a satisfactory gray-black reaction product.[6,17–19] Sodium malate is included in the reaction medium, and consequently the activity of malic dehydrogenase is responsible for most of the reduction of tellurite, even though the electron transfer is probably carried out through DPN diaphorase. The TPT method is more rapid, and provided that hemoglobin diffuses out of the muscle during the incubation, a good contrast is noted between the brick-red formazan pigment deposited on normal muscle and the pale pink color of the myocardial infarct. Occasionally, however, in small areas of necrosis, in aged specimens, and when the hemoglobin doesn't leach out, the contrast obtained with the TPT method is not as striking as desired. Sandritter and Jestädt were careful to describe their method as the *triphenyltetrazolium* reaction.[7,8] In the light of recent work showing that electron transfer to TPT occurs after cytochrome oxidase,[26] it seems likely that their method is dependent on the combined loss of substrates, coenzymes, dehydrogenases and cytochrome oxidase

from the heart muscle. The method described in this paper, using Nitro-BT as the hydrogen acceptor, reflects the activities of many dehydrogenases oxidizing endogenous substrates and transferring the liberated electrons either directly or through coenzymes and diaphorases to the tetrazolium salt.[23,24,26] The blue formazan is produced in a 10 to 15 minute incubation period and provides good contrast between normal and ischemic myocardium. Furthermore, the increased sensitivity of this method furnishes more reliable results in small lesions, early infarcts, and aged specimens.

A few precautions should be followed to avoid false negative results. Occasionally the commercial supplier of the tetrazolium salt will unknowingly market an inferior batch of reagent. A fresh piece of muscle with known dehydrogenase activity should be used whenever an experiment is run with new reagents. Secondly, the duration of time from death until the reaction is carried out should be known, because some of the biochemical changes of postmortem autolysis closely parallel those of ischemic necrosis *in vivo,* albeit the former occur at a slower rate. In conformity with findings reported by Kent[32] in another context, our method cannot be expected to give reliable results if applied to specimens which have been stored for more than 8 hours at 25 to 37° C., or for more than 48 hours at 4°. Third, a weaker reaction is sometimes seen normally in the bulbospiral muscle of the left ventricle, the fibers of which run parallel to the transverse cuts of the heart slices. This does not mean that these fibers are ischemic, but rather that better exposure of sarcosomes to the reagent solution is obtained when muscle fibers are cut at right angles, as are the superficial muscle bundles. Finally, if the incubation solution used is not sufficient to cover the heart slices for a depth of at least 2 cm., the staining reaction on the top surface may be less intense or even patchy as compared to that found on the undersurface. Apparently atmospheric oxygen competes somewhat for the electrons transferred from respiratory enzymes, but this can be overcome by an increased volume of incubation solution to provide more anaerobic conditions.

With techniques available for demonstrating infarction of heart muscle before striking histologic alterations occur, one might wonder whether the chemical changes were due to ischemia or necrosis of the muscle fibers. The first fact to be noted was that the findings observed in the early 4 to 6-hour lesions were similar in location to those obtained at 24 hours, when the muscle was known to be necrotic. However, they were apparently not as extensive since 5 of the 6 minimal lesions and all 8 of the patchy lesions were seen in the animals with coronary artery occlusions of 6 hours or less. Furthermore, the addition of substrate led

to a disappearance of differential staining with formazan in the early lesions but not in the later ones, indicating that substrate deficiency was primarily responsible for the lack of tetrazolium reduction in the ischemic muscle, while enzyme loss was the cause in the necrotic muscle.

Biochemical assays of ischemic heart muscle and established myocardial infarcts in dogs indicate a loss of 60 to 87 per cent of glycogen[29] and probably other labile substrates within 30 minutes, a loss of 70 to 83 per cent of diphosphopyridine nucleotide within 2 hours,[27] and a loss of 50 per cent of succinic dehydrogenase activity after 15 hours.[12] Thus, when the Nitro-BT reaction described here demonstrated no formazan deposit in an area of heart muscle, this area represented either ischemic or necrotic muscle, depending upon the duration of arterial occlusion. There appears to be little doubt that the technique demonstrates arterial insufficiency which progresses to myocardial infarction when sufficient time elapses. Since this is not a reversible process in the dog whose LAD is ligated, or in the patient with an obstructed coronary artery branch, it is reasonable to consider the gross staining technique as a macroscopic method for demonstrating myocardial infarcts. The maximum duration of ischemia that can be tolerated by functioning heart muscle has not been critically defined. However, 30 to 60 minutes seems to represent the approximate limit usually compatible with survival of ischemic myocardium in normothermic dogs, judging from experiments involving the temporary occlusion of coronary arteries.[33-35]

The final issue to be considered is whether these techniques which outline experimental myocardial infarcts before gross or microscopic changes become manifest can be used to assist pathologists in the recognition of early or small infarcts in the human subject. Neoral and associates[18,19] applied their tellurite reaction to the hearts of 20 patients with suspected or proven myocardial infarcts. The earliest infarct which they recognized was in a patient who died 7 hours after the onset of clinical symptoms. There were neither gross nor microscopic findings. Jestädt and Sandritter examined 112 human hearts,[8] many of which were negative controls. In 11 cases with clinical symptoms 5 to 15 hours preceding death, the TPT reaction demonstrated infarcts in the absence of both gross and microscopic changes. However, their reported findings are difficult to evaluate because cases, such as the above, of 5 to 15 hours' duration were said to have displayed no histologic changes while hearts from other patients symptomatic for 10 minutes to 4 hours did show microscopic evidence of muscle necrosis.[36] More recently, Cain and Assmann[37] reported 14 human hearts stained at necropsy with neotetrazolium chloride. In 6 cases there was a clinical diagnosis of myocardial infarction of 24 hours to a few days' duration, and the histochemical

reaction served accurately to outline areas of acute and subacute myomalacia, post-infarction scars and interstitial myocardial fibrosis. No early infarcts were included in their series, so that the limits of sensitivity of the neotetrazolium method were not defined.

In our own survey of 23 human hearts treated with buffered Nitro-BT solution, 7 hearts from patients who died suddenly or within minutes of the onset of symptoms, 2 hearts from cases of severe congestive heart failure, and 2 hypertrophied hearts arrested by local hypothermia without coronary perfusion for 60 to 100 minutes, all developed homogeneous staining reactions (aside from post-infarction scars and areas of interstitial myocardial fibrosis), and failed to show gross or microscopic evidence of recent muscle necrosis. The earliest infarct in the human heart recognized by the Nitro-BT method was of 8 hours' duration. Associated gross and microscopic alterations in the myocardium were minimal, and their recognition was facilitated by the histochemical reaction. In 15 hearts with proven recent and healing infarcts, post-infarction scars or patchy interstitial myocardial fibrosis, the Nitro-BT method served accurately to outline large and small areas of necrosis and fibrosis with a resolution of 0.5 mm., and by enhancing color contrast greatly assisted in the photographic documentation of specimens. In 6 hearts with recent and healing infarcts, the areas of muscle necrosis were more widespread after staining than could be appreciated by simple gross inspection. Among these specimens the method also identified tiny islands of viable muscle within apparently homogeneous infarcts. It is anticipated that the Nitro-BT reaction herein reported will have useful applications in studies where detailed correlations are required between the gross topography of a myocardial infarct and its pattern of arterial blood supply, electrocardiographic effects and biochemical make-up.[31,38,39]

Summary

A method for demonstrating myocardial infarcts in gross specimens has been described. The procedure utilizes a general dehydrogenase reaction, and takes advantage of both substrate and enzyme loss from the infarct. In the viable muscle where endogenous substrates, coenzymes and dehydrogenases are present, reduction of Nitro-BT yields a dark blue formazan; necrotic muscle fibers remain unstained or faintly stained.

Our technique is more sensitive and convenient than the tellurite method, because results can be obtained in 30 minutes as opposed to 3 to 8 hours. It furnishes a dark blue pigment rather than a red reaction product as from triphenyltetrazolium. Furthermore, the method em-

ploying Nitro-BT is more reliable for visualizing small or early infarcts, and in evaluating aged necropsy specimens.

Following ligation of the anterior descending branch of the left coronary artery in 40 dogs, the hearts were examined at various intervals grossly, microscopically, and after the dehydrogenase reaction described herein. The region of the infarct could be recognized with the Nitro-BT reaction as early as 2 hours after arterial occlusion, in advance of discernible gross or microscopic alterations. The technique demonstrated the effect of ischemia which led to myocardial infarction when sufficient time elapsed.

Evidence is presented to show that it is the depletion of labile substrates from ischemic heart muscle which is initially responsible for differential staining with the dehydrogenase reaction, while at a later time loss of respiratory enzymes comes to play a predominant role.

A survey of 23 human hearts obtained at necropsy disclosed that there was no alteration in the Nitro-BT reaction following acute coronary insufficiency with sudden death, severe congestive heart failure, or elective cardiac arrest by local hypothermia. The earliest human myocardial infarct to show loss of dehydrogenase activity was of 8 hours' duration. Application of the method to hearts with areas of acute and subacute myomalacia, post-infarction scars and patchy interstitial fibrosis provided very precise information concerning topographic relationships.

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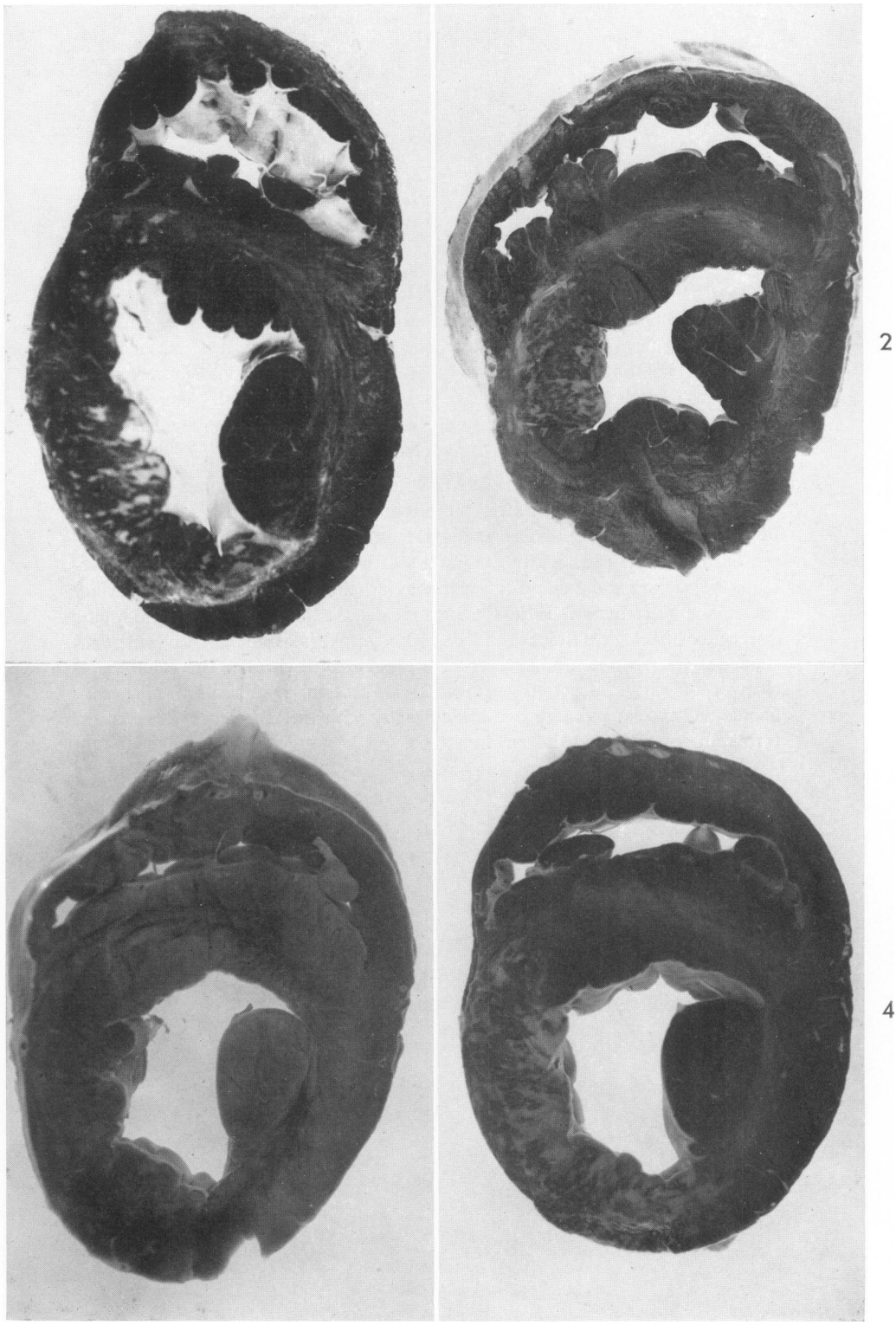
[*Illustrations follow*]

Legends for Figures

Figures 1 to 6 are photographs of 4 different dog hearts. The anterior descending branch of the left coronary artery was ligated 2 hours (Fig. 1), 6 hours (Figs. 2 to 4) and 24 hours (Figs. 5 and 6) before sacrifice. The cross sectional cuts are oriented with the anterior wall of the left ventricle on the left, and the right ventricle in the upper part of the photograph.

Figs. 1 and 2. Two examples of the type of lesion designated in the text as "minimal." It is characterized by small areas of unstained (ischemic) heart muscle with an irregular distribution throughout the anterior wall, which is stained a dark blue color. The light-staining areas in the septum and inner portion of the posterior ventricular myocardium do not represent infarction of muscle, but rather normal muscle fibers less intensely stained because of the orientation of their myofibrils and mitochondria in a parallel rather than cross-sectional plane (bulbospiral muscle). In the specimens there was no difficulty in differentiating these areas from the infarct.

Figs. 3 and 4. Opposing surfaces of a dog heart, 6 hours after LAD occlusion. On the left is the unstained heart which appears normal. After the enzyme reaction (Fig. 4) the areas of infarction become apparent. This type of result is designated as "patchy" (see text) because there are many areas of viable muscle in the infarct of the anterior ventricular wall.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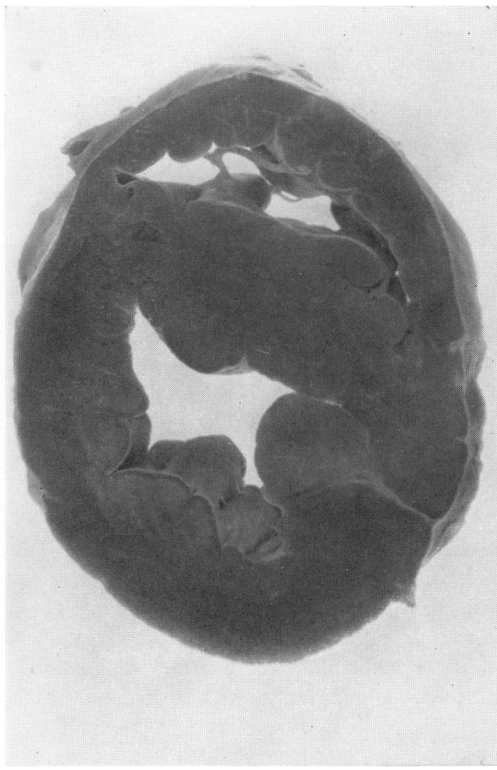
FIGS. 5 and 6. Opposing surfaces of a dog heart sectioned 24 hours after LAD occlusion. The unstained photograph on the left shows a broad pale area on the endocardial half of the anterior ventricular wall which was more easily recognized in the fresh specimen, but even there was not well demarcated. Figure 6 shows the adjoining surface of heart muscle which has been stained by enzymatic activity. The infarct is classified as "confluent" (see text).

FIGS. 7 and 8. Sections of heart muscle processed in the usual manner and stained with hematoxylin and eosin after the enzymatic reaction had been performed. Fig. 7. Four hours of ischemia. Although the muscle fibers look uniform, formazan deposition may be seen in the lower left border of the section. This represents the surface staining indicating viable muscle, while the upper left surface (unstained by formazan) is the ischemic muscle. At this early stage there are no discernible histologic differences between the normal (stained) and ischemic (unstained) zones. × 100. Fig. 8. After 24 hours of ischemia, the microscopic changes of early infarction (neutrophil infiltration, hyalinization of muscle fibers, and nuclear pyknosis) coincide exactly with the muscle which lacks the formazan deposition on the surface. × 60.



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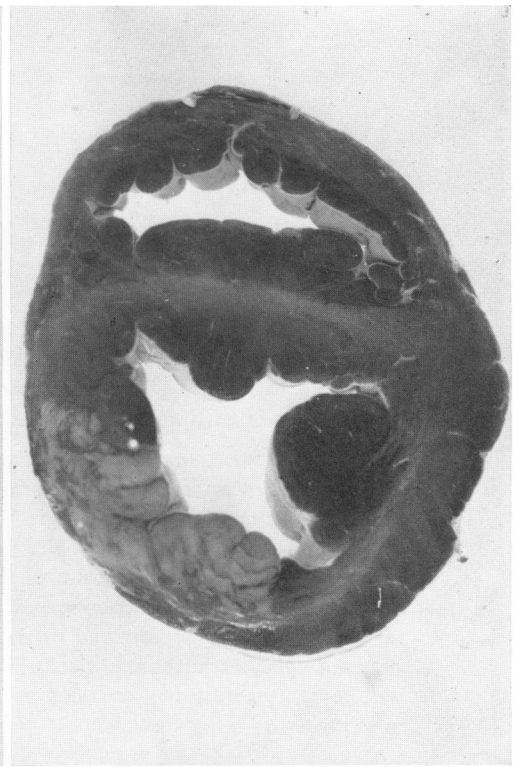

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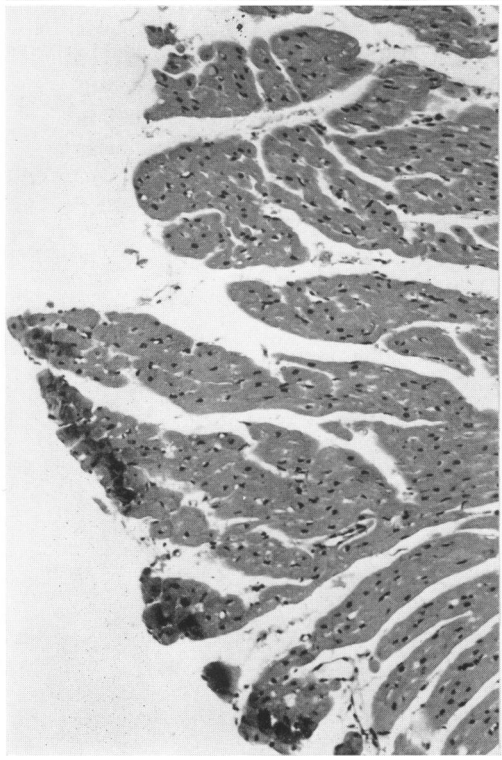

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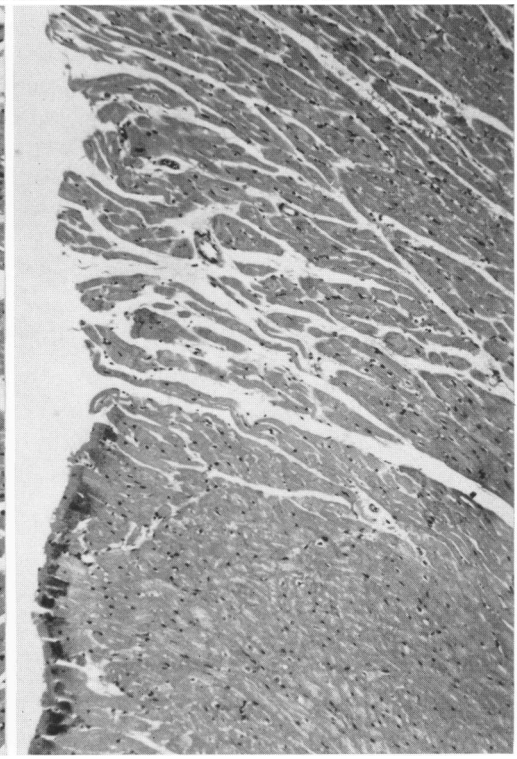

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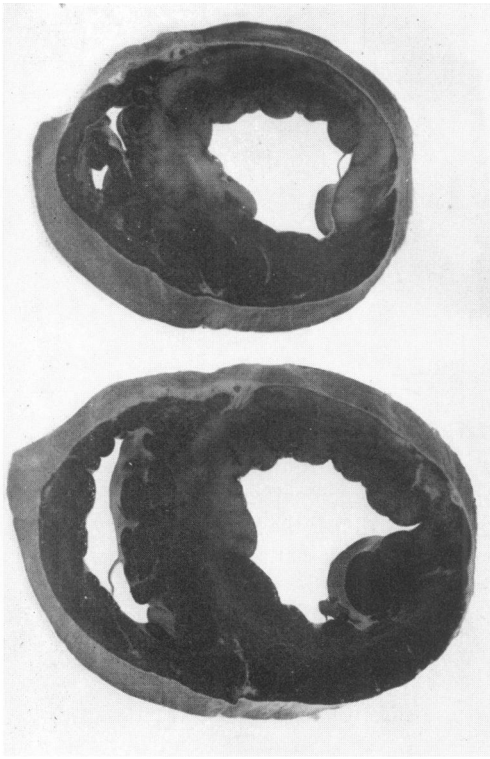
FIG. 9. Adjacent transverse slices of dog heart after 6 hours of ischemia. The upper specimen was stained with buffered Nitro-BT medium. The lower specimen was incubated with Nitro-BT medium plus DPN, under otherwise identical conditions. There is nearly complete correction of the biochemical lesion.

FIG. 10. Stained specimen from a heart with an 8-hour infarct as substantiated by history, electrocardiogram, and microscopic lesions. Past history indicated several previous myocardial infarctions. The reaction was run 3 hours post mortem without added substrate. The older post-infarction scars are visualized as the whiter nonstaining areas distributed through the septum and remainder of the left ventricle. There is a superimposed extensive fresh infarction which is shown as gray areas in the septum and anterior wall. The extent of the fresh infarction was not apparent in the unstained slice. The anterior descending branch of the left coronary artery was occluded by atheroma and the circumflex branch was stenotic.

FIGS. 11 and 12. Figure 11 is the unstained opposing surface to Figure 12. The clinical history indicated an infarction of 2 hours' duration, but the histologic findings were more consistent with a process of 4 to 7 days. Note the marked increase in clarity of the stained specimen. The recent thrombotic occlusion of the left circumflex artery produced the very patchy subendocardial infarct in the posterior wall and septum. Interstitial fibrosis in the anterior wall was also delineated by the enzymatic reaction.



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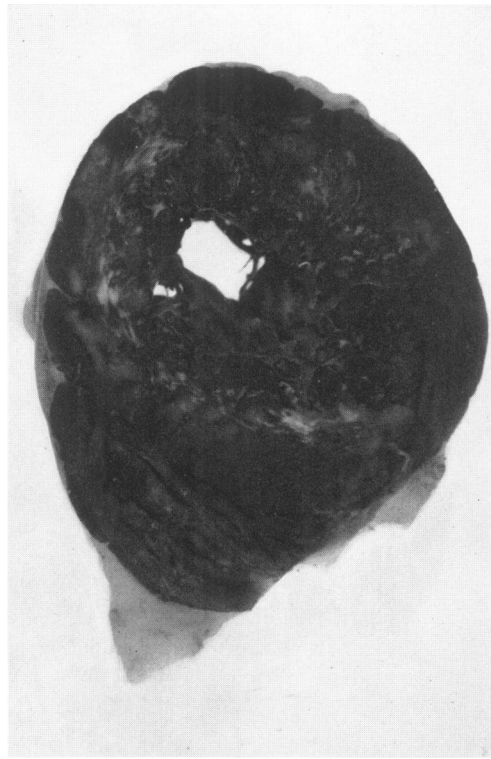

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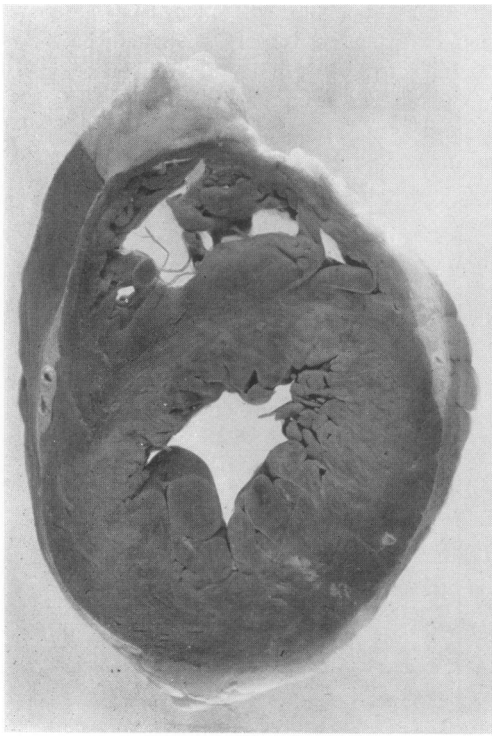

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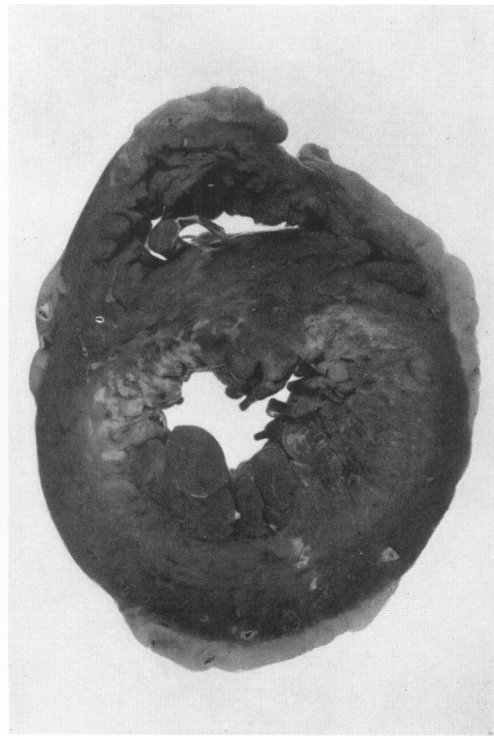

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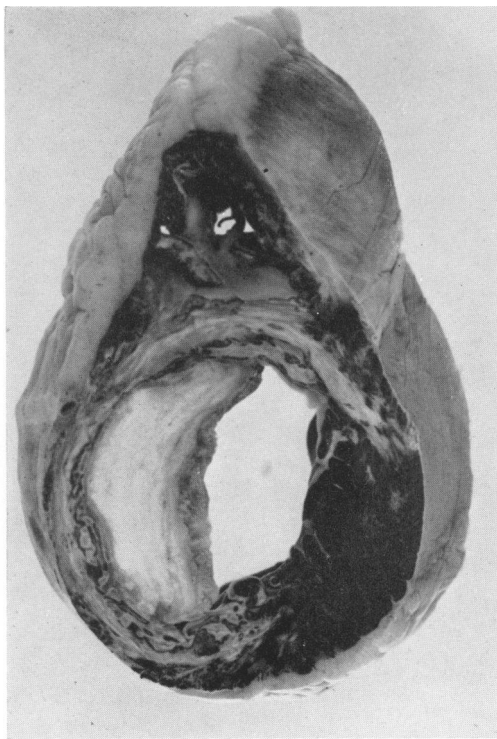
FIG. 13. There is a massive post-infarction scar involving the entire anterior wall and septum with an attached organized mural thrombus. The staining procedure demarcated numerous bundles of viable muscle within the scar.

FIG. 14. In this patient there was a history of posterior myocardial infarction 7 years ago, anterior infarction 30 days previously and a terminal readmission *in extremis*. The post-infarction scar in the posterior wall and the large area of myomalacia in the anterior wall were recognized on gross examination. However, a more recent extension of the month-old infarct into the anterior septum and lateral wall of the left ventricle was not obvious until completion of the staining reaction. Microscopic examination of the 3 sites confirmed the different ages of these infarcts.

FIGS. 15 and 16. A fresh and stained transverse slice of heart muscle from a 40-year-old man with a 30-hour history of chest pain, hypotension and electrocardiographic changes indicative of massive anterior wall infarction. The necropsy examination was performed 20 hours after death. Inspection showed the lateral wall involvement (Fig. 15), but the full extent of the lesion was recognized only after the staining procedure. It may be seen from Figure 16 that the process extends into the anterior wall and septum. The addition of exogenous substrate was necessitated by the long postmortem interval.



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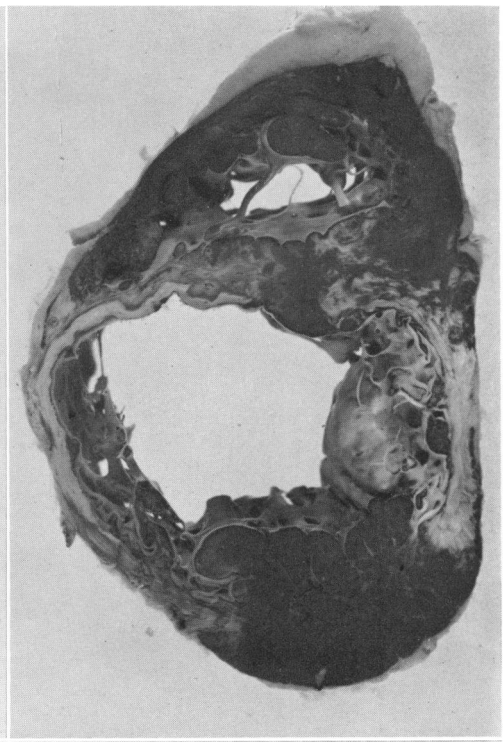

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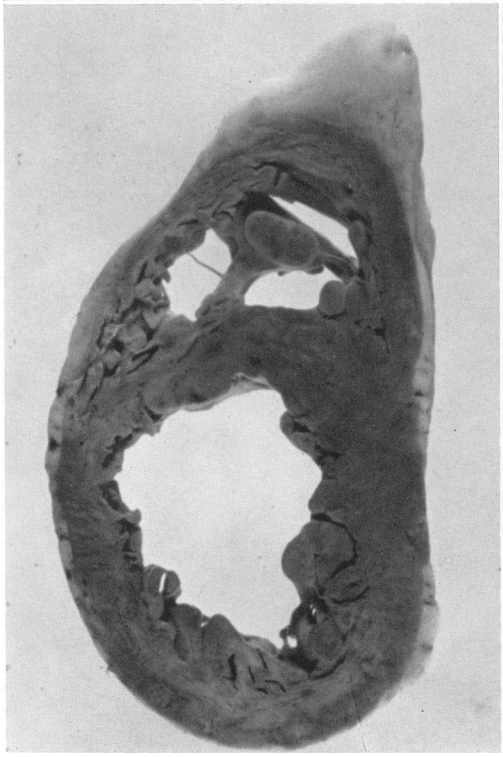

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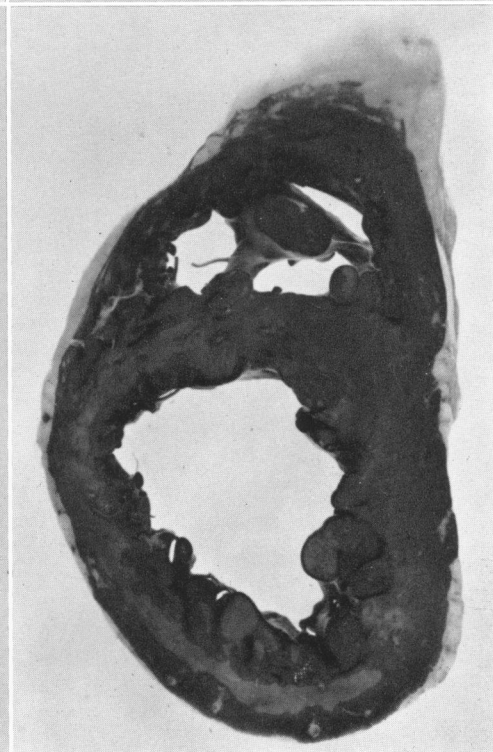

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