SELECTIVE NECROSIS OF CARDIAC AND SKELETAL MUSCLE INDUCED EXPERIMENTALLY BY MEANS OF PROTEOLYTIC ENZYME SOLUTIONS GIVEN INTRAVENOUSLY*

By AARON KELLNER, M.D., and THEODORE ROBERTSON, M.D.

(From the Department of Pathology, The New York Hospital—Cornell Medical Center, New York)

Plates 11 and 12

(Received for publication, September 18, 1953)

The action of proteolytic enzymes on susceptible tissues has been implicated in the pathogenesis of the anatomical lesions of rheumatic fever and certain hypersensitivity states (1–4). There has, however, been little evidence to show that proteolytic enzymes are in fact capable of producing morphological changes in specific tissues, particularly when the enzyme is disseminated *via* the blood stream. During the course of experiments undertaken to clarify this point, it was found that solutions of the plant proteolytic enzyme papain when injected intravenously into rabbits and other animals resulted with striking regularity in the production of widespread focal necrosis of cardiac muscle and also, but to a lesser extent, of skeletal muscle.[1] Further investigation revealed that the intravenous injection into rabbits of solutions of ficin, trypsin, and streptokinase also resulted in the development of comparable though somewhat less extensive lesions of the heart and skeletal muscle. It is the purpose of this paper to detail the method of production of these lesions and to describe their histological characteristics.

Materials and Methods

Enzyme Solutions.—Papain is a proteolytic enzyme contained in the dried latex of *Carica papaya*. Crude papain powder obtained from two different commercial sources (Merck and Co., Inc., New York, and Fisher Scientific Co., New York) was used in these studies and identical effects were observed with each. To make solutions of this material for injection, 5 gm. of the papain powder was added to 100 ml. of 0.9 per cent saline; this turbid mixture was shaken for several minutes and then filtered through a Seitz pad. The clear, amber colored filtrate was arbitrarily referred to as a 5 per cent solution. The enzymatic activity of the papain solutions was measured by the ability to clot milk according to the method of Balls and Hoover (5) and the ability to digest hemoglobin according to the method of Anson (6).

* This investigation was supported by research grants from the United States Public Health Service, Federal Security Agency, and the New York Heart Association.

[1] A preliminary report of these studies appeared in *Proc. Am. Assn. Path. and Bact., Am. J. Path.*, 1951, **27**, 753.

The solutions maintained their potency for at least 3 weeks when stored at 4°C. A purified papain solution prepared by the method of Balls and Lineweaver (7) was used in several experiments. None of the papain solutions employed in these studies was specifically activated *in vitro* prior to use.

A 2 per cent solution of ficin, a proteolytic enzyme present in the dried latex of the fig tree, was prepared in a similar manner by adding 2 gm. of dried crude ficin powder (Delta Chemical Works, New York) to 100 ml. of 0.9 per cent saline.

Two preparations of trypsin were employed in these studies. One was a lyophilized pancreatic extract (enzar, Armour Laboratories, Chicago) distributed by the manufacturer in vials containing 200,000 units of tryptic activity, 1 unit being defined as the amount of enzar which upon incubation at 25°C. with a preparation of pure hemoglobin will liberate the equivalent of 0.001 mg. of tyrosine as measured with the Folin-Ciocalteu phenol reagent. Enzar, according to the manufacturer, contains other pancreatic enzymes in addition to trypsin. In a number of experiments crystalline trypsin (two times crystallized, Worthington Biochemical Co., Freehold, New Jersey) was used. This preparation contained approximately 50 per cent magnesium sulfate, and the amount of trypsin injected is stated in each case as the approximate weight of pure trypsin exclusive of the magnesium sulfate. For purposes of injection, the trypsin preparations were dissolved in 150 ml. of 0.9 per cent saline and infused slowly at a rate of 10 to 15 drops per minute into an ear vein.

The streptokinase preparation used was a dried, partially purified concentrate of a streptococcal filtrate which contained, among other things, varying amounts of streptococcal desoxyribonuclease.[2] The streptokinase titers are reported in units according to the method of Christensen (8). The streptokinase powder was dissolved in 15 ml. of saline and injected rapidly into the marginal ear vein of rabbits.

Animals.—Market-bought adult rabbits of both sexes and of mixed breeds weighing from 2000 to 4000 gm. were used. A small number of chickens, guinea pigs, adult male and female rats of the Wistar strain, and adult white male Swiss mice were also employed. All animals were maintained on the usual laboratory diet.

Anatomical Studies.—Rabbits were sacrificed by means of air embolism at varying intervals of time following injection. Rats, mice, and guinea pigs were killed with chloroform. Complete postmortem examinations were made on all animals that were sacrificed and also on those animals that died following injection. In each case the entire heart and blocks from a number of skeletal muscles were fixed in Zenker-formol solution. Additional blocks from smooth muscles and from the thoracic and abdominal viscera were taken in a number of animals. Following fixation, a block was cut from the heart in the coronal plane to include portions of both auricles and ventricles and portions of several valves. The blocks of tissue were embedded in paraffin and sections were stained with hematoxylin and eosin. In selected cases sections were also stained with phosphotungstic acid-hematoxylin and with Masson's trichome stain. With very few exceptions only a single section was examined from each heart, and no section was considered to be positive unless it contained at least two areas of characteristic necrosis, to be described in detail further on.

Potassium Studies.—Serum potassium levels were determined in duplicate, using an internal standard flame photometer.

Necrosis of Cardiac and Skeletal Muscle in Animals Given Papain Solution Intravenously

Focal necrosis of cardiac and skeletal muscle was produced readily in rabbits by means of a single intravenous injection of 5 per cent papain solution. The

[2] The streptokinase was generously supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

optimal amount for this purpose was found to be 0.7 to 1.2 ml. per kilo of body weight; the development of lesions was irregular when doses lower than this were employed, and when larger doses than this were given, a consider-

TABLE I

Incidence of Necrosis of Cardiac and Skeletal Muscle Induced in Rabbits by Intravenously Injected Enzyme Solutions

Enzyme	Amount injected	No. of rabbits*	No. with lesions in	
			Cardiac muscle	Skeletal muscle
Papain	5 per cent solution, 0.7–1.2 ml./kg.	24	22	9
Ficin	2 per cent solution, 0.25–0.35 ml./kg.	2	0	—
	2 per cent solution, 0.5–2.0 ml./kg.	5	4	—
Trypsin	Enzar, 25,000–50,000 units	4	3	1
	Enzar, 100,000 units	7	5	0
	Enzar, 200,000 units	2	2	1
	Enzar, 200,000 units plus CSBTI, 100 mg.	5	0	5
	Crystalline, 15 mg.	2	0	0
	Crystalline, 25–30 mg.	3	0	0
	Crystalline, 50 mg.	4	3	1
	Crystalline, 50 mg. plus CSBTI, 100 mg.	4	0	0
Streptokinase	250,000 units	3	0	0
	500,000 units	4	2	0
	1,000,000 units	9	5	2

—, skeletal muscles not examined.

CSBTI, crystalline soy bean trypsin inhibitor.

* Animals that died during injection or within 6 hours following injection have not been included in these totals.

able number of the animals died within a few minutes to 24 hours following injection. Within this dosage range, however, the injections were well tolerated.

Each of 24 rabbits was given a single intravenous injection of a 5 per cent papain solution in amounts of 0.7 to 1.2 ml. per kilo into the marginal ear vein. The animals were then sacrificed at intervals from 6 hours to 7 days following injection. Focal necrosis of the myocardium was present in random sections of the heart in 22 of the 24 rabbits in this series (see Table I). Numerous control animals studied at the same time failed to reveal similar lesions. Focal necrosis of skeletal muscle was observed in nine cases, usually in sections taken from the diaphragm and the masseter muscle; the skeletal muscle lesions were fewer in number and smaller than the myocardial lesions in the same animal. Rabbits injected with papain solu-

tion developed, in addition, an unusual blood coagulation defect which will be described in detail in a separate paper.[3]

Morphological Characteristics of the Papain-Induced Lesions.—The lesions observed in rabbits following the intravenous injection of papain solution were in all cases limited to the cardiac and skeletal musculature. No lesions attributable to the enzyme solution were found in smooth muscles or in any of the viscera, though these were sought for repeatedly, nor were lesions present in the heart valves, the epicardium, or the endocardium. The lesions in the heart appeared to have a random distribution; they were observed in the muscle fibers of both the right and left ventricles, and bore no constant relationship to blood vessels (Fig. 1). There were no thrombi of any type found in the blood vessels near the lesions, nor was there evidence of agglutination of platelets or leukocytes. Despite the clotting defect induced in the animals by the injection of papain solution, there were no hemorrhages associated with the lesions. Careful search for viral inclusion bodies, bacteria, and parasites was made of the involved areas in the heart and skeletal muscle of a number of animals; these were uniformly negative.

The changes in the myocardium consisted essentially of focal areas of necrosis of muscle fibers. The necrotic areas were small and with very few exceptions could not be seen by the naked eye upon examination of the heart in the gross. The lesions in any one heart all appeared to be in the same stage of development, suggesting that the causative agent affected widely scattered muscle fibers simultaneously and for but a brief period of time. The earliest myocardial changes were apparent in animals that died or were killed 6 hours after injection and consisted of swelling, fragmentation, and marked eosinophilia of the muscle fibers with obliteration of the individual myofibrils and pyknosis of the muscle nuclei. These changes were more advanced and more striking in animals killed 12 hours after injection (Fig. 2). At this time, too, a few polymorphonuclear leukocytes were seen in and about the larger lesions; the inflammatory reaction in ensuing stages in the progression of the lesions, however, consisted for the most part of mononuclear cells. 1 to 3 days following injection the changes within the muscle fibers had progressed to complete necrosis of the affected fibers with disappearance of the sarcoplasm, in some cases leaving only the sarcolemmic sheath (Figs. 3 and 4). There was an associated active proliferation of mononuclear cells both within and about the necrotic fibers. These mononuclear cells had poorly defined cytoplasm and large oval vesicular nuclei and resembled in some respects Anitschkow myocytes (Figs. 4 and 5). On the 3rd day the reactive mononuclear cells showed abundant mitoses and multinucleated giant cells were evident in places. From the 3rd to the 7th day following injection there was considerable variation in the appearance of

[3] An abstract of these observations has appeared in *Fed. Proc.*, 1951, **10**, 361.

the lesions from animal to animal. The larger lesions were gradually replaced by fibrous scars. In a few instances the damaged areas were partially calcified at 7 days, and in some instances multinucleated giant cells were abundant in the involved areas (Fig. 6). Calcification of the necrotic fibers was noted on occasion as early as 2 days after injection of papain (Fig. 1). It is of interest that in a few animals killed 2 weeks after injection, apart from an occasional very small fibrotic area, virtually no evidence of the antecedent lesions was apparent. A few of the muscle fibers, however, appeared somewhat basophilic and had indistinct myofibrils and an increased number of nuclei, suggesting that a reparative process had taken place within the damaged muscle fibers.

The lesions in skeletal muscle were similar in most respects to those found in cardiac muscle. They were small and focal in character and, in contradistinction to the myocardial lesions which were abundant and readily seen in random sections, the skeletal muscle lesions had to be sought for carefully. It is of some interest that the skeletal muscle lesions in rabbits given papain intravenously were considerably more prominent in an active muscle such as the diaphragm than they were in less active muscles such as those of the abdominal wall or thigh. As in cardiac muscle, the earliest changes, which were noted 6 to 12 hours after injection, consisted of swelling and eosinophilia of a portion of a muscle fiber. In many cases, only a single fiber was involved, the fibers in the surrounding area appearing histologically normal (Fig. 7). The sarcoplasm of the affected fibers became necrotic, and actively proliferating mononuclear cells and a few polymorphonuclear leukocytes were present within and about the sarcolemmic sheath. The necrotic sarcoplasm was removed within 2 to 4 days. Proliferation of muscle cell nuclei and restoration of the muscle fiber then became evident, so that 2 weeks after injection remarkably few altered fibers could be found. Occasionally at this time a few fibers with one or two centrally located nuclei could be seen, and in some animals given larger doses of papain a few small fibrous scars were present.

As a control study histological sections from the heart and skeletal muscles of more than 100 rabbits were examined. These rabbits were for the most part control animals given intravenous injections of saline in the same amounts as that received by the experimental animals given enzyme solutions, and a considerable number were rabbits used in other experiments being done concurrently in the laboratory. Focal myocardial necrosis comparable to that observed following the injection of papain solution was noted in but 4 animals of this series: one was a rabbit that had extensive acute pancreatitis following ligation of the common bile duct; another was an animal that died following a prolonged period of experimental hypotension; the third was a rabbit immunized to bovine gamma globulin and given repeated subcutaneous injections of sodium ribonucleate; and the fourth was an otherwise normal rabbit given saline intravenously. Necrosis of skeletal muscle fibers was seen in 7 animals

of the control series; the lesions observed were generally smaller and less conspicuous than those found in papain-treated rabbits.

Papain Solution Given by Other Routes.—

3 rabbits were each given a single intra-arterial injection of 1.0 ml. per kilo of 5 per cent papain solution, the material being injected into the exposed carotid artery while the animal was under light ether anesthesia. Myocardial lesions were present in each case, though in 2 of the animals these were few in number and quite small. The lesions in skeletal muscles, on the other hand, were more numerous and far larger than those observed in the intravenously injected rabbits, and they were particularly conspicuous in those muscles exposed to direct flooding by the papain solution (Fig. 8). This suggests that the relative paucity of skeletal muscle lesions in the animals given papain by the intravenous route may have been due to dilution of the solution before it reached the peripheral muscles.

3 additional rabbits were given a single dose of 50 to 100 ml. of 5 per cent papain solution orally by means of a stomach tube. The animals tolerated this procedure very well and exhibited no lesions of cardiac or skeletal muscles when examined post mortem.

Injection of Papain Solution into Rats, Mice, Chickens, and Guinea pigs.—A number of experiments were done to determine whether the intravenous injection of papain solutions would produce lesions of cardiac muscle in species of animals other than the rabbit.

Myocardial lesions were observed in each of 7 white rats given 0.6 to 2.0 ml. of 5 per cent papain solution intravenously (Fig. 9). Similarly, 5 white mice were each given a single intravenous injection of 0.15 cc. of a 5 per cent papain solution; these animals were sacrificed 24 hours later and myocardial lesions were found in 3 of the 5 animals examined. On the other hand, no myocardial or skeletal muscle lesions were observed in 4 chickens and 4 guinea pigs each given 1 ml. per kilo of a 5 per cent papain solution intravenously.

Electrocardiographic Studies.—The earliest clearly recognizable morphological changes in the heart and skeletal muscle were observed 6 hours after injection of papain. Since it is well known that a number of hours may elapse following injury to a tissue before anatomical evidence of the injury is apparent by the commonly employed histological techniques, an attempt was made to determine more precisely how soon cardiac muscle fibers were affected by the injected papain solution. To do this electrocardiographic tracings were made from 5 rabbits immediately before and at varying intervals of time following the injection of papain. Changes in the ST segment and abnormal T waves were present 10 minutes after injection; these changes progressed and became quite striking 30 to 60 minutes later. The electrocardiographic findings were interpreted as indicating injury to cardiac muscle fibers.[4] No comparable changes were noted in control rabbits given saline intravenously. It was con-

[4] The authors are indebted for the electrocardiographic tracings to Dr. Jerrold Lieberman and Dr. Alexander Taylor of the Department of Medicine, The New York Hospital—Cornell Medical Center.

cluded from these studies that papain solutions affected the function of myocardial fibers within a few minutes after injection, though anatomical evidence of injury did not become apparent for several hours.

Serum Potassium Levels Following the Intravenous Injection of Papain Solutions.—Since necrosis of cardiac and skeletal muscle has been produced experimentally in rats and dogs by lowering the serum potassium content, usually by means of feeding a potassium deficient diet for several weeks (9, 10), it was considered necessary as a control study to determine whether the intravenous injection of papain solution brought about a hypokalemia of sufficient degree to account for the changes observed.

Accordingly, 6 rabbits were bled to obtain control levels of serum potassium and each was then given a single intravenous injection of 5 per cent papain solution in amounts of 0.7 ml. per kilo. Serum potassium levels were determined again at intervals of 10 minutes to 24 hours following the injection of papain. Only slight and inconstant decreases in serum potassium levels were observed. The control levels had a range of 3.6 to 4.7 m.eq./liter with a mean of 3.9, and the values obtained after injection had a range of 3.4 to 4.7 with a mean of 3.8.

These changes were far less than those reported in animals with muscle lesions due to hypokalemia. All the animals in this experiment had typical focal necrosis of the myocardium upon histological examination, the finding making it seem unlikely that the necrosis of cardiac muscle that followed intravenous injection of crude papain solutions into rabbits could have resulted from a decrease in serum potassium levels.

Attempts to Define More Precisely the Factor in Crude Papain Solutions Responsible for Production of Muscle Lesions.—Since the papain powder used in these studies is prepared commercially by drying crude papaya latex, it almost surely contains substances in addition to the active enzyme papain. A number of experiments were therefore done to see whether the muscle lesions produced in animals by the injection of crude papain solutions were due to the enzyme itself or to some other factor or factors contained in the injected material.

5 per cent solutions of crude papain were heated in a water bath at 55°C., 60°C., 65°C., and 80°C. for 30 minutes and then filtered to remove the insoluble material which precipitated during heating. Total protein determinations by the micro-Kjeldahl technique revealed that the soluble protein content of the solutions had been only very slightly decreased by the heating. A portion of the solution heated at each temperature was tested for enzymatic activity as measured by its ability to clot milk according to the method of Balls and Hoover (5). 2 to 4 rabbits were then given a single intravenous injection of each solution. The animals were sacrificed 2 days later and histological sections made from the heart were examined for the presence of myocardial necrosis. It was found that solutions of crude papain heated to 55°C., 60°C., and 65°C. maintained their ability to clot milk, and also, that animals given these solutions intravenously all developed myocardial lesions. The solution heated at 80°C., on the other hand, lost its ability to clot milk and no myocardial lesions were found in 4 rabbits given this material intravenously.

It is well known that strong oxidizing agents can abolish the milk-clotting activity of the enzyme papain *in vitro* (11).

1 ml. of 20 per cent hydrogen peroxide was added to 50 ml. of 5 per cent crude papain solution and 4 ml. of freshly prepared Lugol's iodine solution was added to 15 ml. of a similar papain solution; both solutions so treated were found inactive when tested for their ability to clot milk. When these solutions were injected into rabbits, however, extensive myocardial lesions were found with both. It thus appeared possible to separate the enzymatic activity of papain from the factor responsible for production of myocardial lesions. Subsequently, however, it was found that the addition of normal rabbit serum *in vitro* to the peroxide-treated or to the iodine-treated papain solutions promptly restored their ability to clot milk, and it appeared probable, therefore, that the solutions inactivated *in vitro* had their enzymatic activity restored *in vivo* upon injection into rabbits.

Since the enzyme papain has been crystallized and shown to be a protein (7), an experiment was done to see whether the active enzyme and the factor in crude papain solutions responsible for muscle necrosis could be separated by dialysis.

Solutions of crude papain were placed in cellophane bags and dialyzed against frequent changes of tap water for 24 to 72 hours. The material remaining in the cellophane bags retained its proteolytic activity and when injected into 4 rabbits resulted in the development of myocardial lesions in each animal.

A number of efforts were made to crystallize the enzyme papain from crude papain powder according to the procedure described by Balls and Lineweaver (12). These were not successful, presumably because liquid papaya latex is required for crystallization of the enzyme and this material was not readily available. It was possible, however, to achieve considerable purification of the enzyme and to concentrate it at least 10- to 15-fold as measured by its milk-clotting ability. 3 rabbits given intravenous injections of this purified material all developed myocardial lesions.

It was evident from these experiments that it was not possible by the methods employed to separate the enzyme papain from the agent in the crude papain solutions responsible for necrosis of cardiac and skeletal muscle. Both were found to be non-dialyzable and to have about the same degree of heat stability. Moreover, purified solutions of the enzyme still were capable of inducing the myocardial lesions. Further studies therefore appeared necessary to determine whether the enzyme papain was in fact the etiological agent responsible for the tissue changes observed.

Cardiac and Skeletal Muscle Lesions in Rabbits Given Solutions of Ficin, Trypsin, and Streptokinase Intravenously

Ficin.—Following the observation that focal necrosis of cardiac muscle could be produced regularly in rabbits by means of a single intravenous injec-

tion of papain solution, it became of interest to see whether another plant proteolytic enzyme, ficin, could induce comparable changes.

10 rabbits were each given a single intravenous injection of 2 per cent ficin solution in amounts ranging from 0.25 to 3.0 ml. per kilo. With doses lower than 0.5 ml. per kilo, no lesions were observed in cardiac muscle, and with doses higher than 2.0 ml. per kilo, the animals died shortly after injection. Within the dosage range 0.5 to 2.0 ml. per kilo, however, 4 out of 5 rabbits injected developed focal lesions of cardiac muscle that were similar to those seen in papain-injected rabbits. (Fig. 10).

As a control against the possibility that non-specific plant proteins present in the papain and ficin solutions were responsible for the lesions observed, a solution of a plant protein free of proteolytic activity was prepared.

5.0 gm. of commercial white flour was added to 100 ml. of 0.9 per cent saline, shaken for several minutes, and then filtered through a Seitz pad. This solution, which did not clot milk nor digest hemoglobin, contained considerable soluble protein as evidenced by a heavy white precipitate upon the addition of trichloroacetic acid. Injection of the flour solution intravenously in large dosage into 2 rabbits—10 ml. and 20 ml. per kilo, respectively—did not result in lesions of the heart or skeletal muscles in either animal.

Trypsin.—The finding that selective necrosis of cardiac muscle could be produced in rabbits by the injection of solutions of the plant proteolytic enzymes papain and ficin raised the question whether similar changes could also be induced by proteolytic enzymes of animal origin. To gain more information on this point a number of experiments were done in which solutions of trypsin were given intravenously to rabbits.

In preliminary experiments it was found that rapid injection of trypsin solutions even in amounts as low as 5 mg. resulted in the death of almost all the injected animals within a very few minutes. Postmortem examination of these animals revealed the presence of large blood clots in the right side of the heart in most cases. This was a curious finding in view of the fact that the blood in other parts of the body did not clot spontaneously and remained fluid even after the addition of thrombin, and in several instances there were extensive hemorrhages beneath the endocardium and epicardium and into the myocardium in these animals. This suggested that death following rapid administration of trypsin solutions was caused by intravascular thrombosis, the thrombi formed in the venous circulation being swept into the right side of the heart, and the remaining blood becoming, in effect, defibrinated. Because of this experience trypsin solutions were diluted to 150 ml. in saline and infused at a very slow rate, 10 to 15 drops per minute. Even with such slow rates of administration, however, there was still a mortality of about 50 per cent in these experiments. Animals died at varying times during the infusion, some after only 5 to 10 ml. of the solution had been given, and others shortly after the entire infusion had run in. In these animals, too, death appeared to be associated with intravascular thrombosis and hemorrhage.

22 rabbits were each given a single infusion of trypsin solution (enzar) in amounts ranging from 25,000 to 200,000 units by slow intravenous drip. 9 animals died during or immediately following the procedure; the remaining 13 were sacrificed 24 to 72 hours after the injection. On postmortem examination, there was myocardial necrosis in 10 of these 13 animals, and in 2 there were also small areas of skeletal muscle necrosis (see Table I). The histological characteristics of these lesions were identical in most respects to those seen in papain-injected rabbits, the only notable difference being that the areas of necrosis tended to be somewhat more diffuse in the animals given trypsin, due perhaps to the fact that the trypsin solutions were administered slowly over a period of several hours. No lesions attributable to the trypsin were found in any of the other viscera. It is of interest, too, that there was no evidence of intravascular thrombosis or hemorrhage into the heart muscle in the animals that survived the injection of trypsin.

5 additional rabbits were each given an intravenous injection of 200,000 units of enzar to which had been added 100 mg. of crystalline soy bean trypsin inhibitor (Worthington Biochemical Co., Freehold, New Jersey). There were no deaths during these injections, even when given rapidly, and none of these animals developed myocardial necrosis. It is of considerable interest, too, that all 5 of these rabbits exhibited focal necrosis of the skeletal musculature, suggesting that enzar, admittedly an impure enzyme preparation, contains in addition to trypsin some other agent that is not affected by trypsin inhibitor and that is capable of causing lesions in skeletal muscle.

Similarly, 17 rabbits were each given a single slow intravenous injection of a solution containing 15 to 50 mg. of crystalline trypsin. 8 of these animals died during the course of the injection. The 9 survivors were sacrificed 48 hours later (see Table I). No areas of necrosis of the heart or skeletal muscle were observed in 5 rabbits given 15 to 35 mg. of crystalline trypsin. 3 out of 4 rabbits that received 50 mg. of crystalline trypsin, however, showed extensive necrosis of cardiac muscle (Figs. 11 and 12), and one of these also had necrosis of skeletal muscle. 4 additional rabbits were each given a single rapid intravenous injection of a solution containing 50 mg. crystalline trypsin plus 100 mg. crystalline soy bean trypsin inhibitor. There were no reactions to these injections, and none of the animals developed lesions of the heart or skeletal muscles.

To rule out further the possibility that the cardiac lesions might be due to foreign protein rather than to the proteolytic enzyme, 6 rabbits were each given a single intravenous injection of sterile normal horse serum in amounts of 10 ml. per kilo and 10 rats were each given 2.0 ml. of the same material intravenously. No myocardial necrosis was observed in any of these animals when examined post mortem 1 to 6 days after injection.

Because of the observation by Tagnon that injections of trypsin were better tolerated by heparinized animals (13), a few rabbits were given 100 mg. of heparin intravenously just prior to the administration of trypsin. The series was too small to determine with certainty whether the mortality associated with trypsin injections was appreciably reduced; focal necrosis of the myocardium, however, was present in heparinized rabbits that survived the injection of trypsin (Fig. 13).

It is clear from these experiments that the intravenous injection of solutions of trypsin into rabbits resulted in the development of necrosis of the myocardium in a high proportion of cases, and also, but to a much lesser extent, in focal necrosis of skeletal muscle. Moreover, specific inactivation of the

tryptic activity of the solution by means of crystalline soy bean trypsin inhibitor was effective in each of nine instances in preventing the development of myocardial lesions.

Streptokinase.—The blood serum of mammals contains a proteolytic enzyme precursor, plasminogen, which can be converted to the active enzyme, plasmin, by a number of agents, the most effective of these being streptokinase, an enzyme found in filtrates of cultures of hemolytic streptococci. Since streptokinase is capable of activating plasminogen *in vivo* as well as *in vitro*, an experiment was done in which streptokinase was injected intravenously into rabbits in order to learn whether the *in vivo* liberation of a proteolytic enzyme would result in lesions of the heart and skeletal muscles comparable to those observed following the injection of papain, ficin, and trypsin.

22 rabbits were each given a single intravenous injection of 250,000 to 2.0 million units of streptokinase dissolved in 15 ml. of saline. Doses of 1.0 million units or higher were not well tolerated; 3 rabbits given 1.5 to 2.0 million units and 3 out of 12 rabbits given 1.0 million units died immediately after the injection. There were no fatalities with doses of less than 1.0 million units. Surviving rabbits were sacrificed 48 hours after injection (see Table I). 5 out of 9 rabbits given 1.0 million units of streptokinase had focal necrotic lesions of the myocardium (Fig. 14) and 2 also had small areas of necrosis of skeletal muscles. 4 rabbits received 500,000 units of streptokinase, and 2 of these had lesions of cardiac muscle. The myocardial lesions in these rabbits were very similar to those seen in papain-injected rabbits, though they were fewer and somewhat smaller. No cardiac muscle lesions were observed in 3 rabbits given 250,000 units of streptokinase.

To demonstrate that the blood of rabbits given streptokinase solution intravenously did in fact have increased proteolytic activity, the following experiment was performed.

2 rabbits were each given a single injection of 1.0 million units of streptokinase solution intravenously. 0.5 ml. samples of blood were withdrawn from an ear vein into a small test tube 15, 30, and 60 minutes and also 3 hours after injection. The blood was permitted to clot at room temperature and the clot lysis time observed. The whole blood-clotting time was normal in each case. The clots from the samples drawn at 15, 30, and 60 minutes, however, were completely lysed within 5 to 20 minutes. Following dissolution of the clots, the fluid blood could not be clotted again upon the addition of thrombin. The clots from the two specimens drawn at 3 hours, on the other hand, were still intact 24 hours later and normal clot retraction had taken place. It is evident from this experiment that the injection of a large amount of streptokinase into rabbits resulted in a striking increase in the proteolytic activity of the blood for a period of at least 1 hour.

Since the streptokinase preparation employed in these studies also contained considerable quantities of desoxyribonuclease, it was felt necessary to determine whether the changes observed in cardiac and skeletal muscle were due to streptokinase or whether they were in part or in whole due to the desoxyribonuclease content of the injected material.

Accordingly, 3 rabbits were each given a single intravenous injection of 15 mg. of crystalline desoxyribonuclease (Worthington) dissolved in 10 ml. of saline, an amount of desoxyribonuclease comparable to that contained in the streptokinase preparation used, and the animals were sacrificed 48 hours later. Examination of histological sections prepared from the heart revealed no areas of necrosis.

It was concluded from these experiments, therefore, that the intravenous injection of large amounts of a streptokinase preparation into rabbits resulted in focal necrosis of the myocardium and in a few animals also of skeletal muscle, and that the development of these lesions appeared to be due to the streptokinase rather than to the desoxyribonuclease content of the injected material.

DISCUSSION

The studies herein described demonstrate that solutions of the enzymes papain, ficin, trypsin, and streptokinase contain an agent that when given intravenously to rabbits and other animals is capable of selectively causing injury or death to discrete portions of the cardiac and skeletal musculature. Similar lesions of cardiac and skeletal muscles have been described by Hicks in rats given repeated sublethal injections of the enzyme inhibitors plasmocid and fluoroacetate (14, 15). These agents, however, did not exhibit the remarkable specificity of the enzyme solutions, for Hicks reported lesions in the liver, kidney, testes, and central nervous system of the injected rats as well as in the heart and skeletal muscle. Necrosis of specific tissues, including the myocardium and coronary arteries, has also been reported by Stetson (16) and by Thomas and Good (17), during the generalized Shwartzman reaction; these lesions were found to be accompanied by a profound thrombocytopenia and granulopenia and to be associated with leukocyte-platelet thrombi in the capillaries of the affected tissues. No comparable reductions in circulating platelet and leukocyte levels have been observed in papain-injected rabbits (18), and no thrombi have been found within the blood vessels in the areas of cardiac and skeletal muscle necrosis. Moreover, Good and Thomas have reported recently that heparin prevents the anatomical lesions associated with the Shwartzman phenomenon (19). The administration of heparin just prior to the injection of trypsin, however, failed to interfere with the development of myocardial or skeletal muscle lesions.

Particular attention was paid to control studies in these experiments because of the reports by Miller (20) and Loewe and Lenke (21) that spontaneous myocarditis may be a common occurrence in rabbits. Miller found interstitial myocarditis, as evidenced by small focal collections of leukocytes, in the hearts of 60 per cent of normal rabbits, and Loewe and Lenke reported an epizootic pancarditis of rabbits that was often fatal and that affected about one-third of their animals within a short period of time. Changes such as those described

by Miller were seen in a number of hearts both from experimental and control animals; these interstitial collections of leukocytes could be readily distinguished from the obvious myofiber necrosis seen in the enzyme-injected rabbits. The cardiac lesions reported by Loewe and Lenke—verrucous endocarditis, mononuclear inflammation of the pericardium, and interstitial myocarditis—are also quite different morphologically from those herein described. There was no serious epizootic disease among the many rabbits housed in the animal quarters during the 2 year period that these experiments were in progress nor was there any unusual mortality among control rabbits used in these and other studies. The finding of cardiac and skeletal muscle necrosis in a small percentage of the control animals is not surprising in view of the well known fact that such lesions may follow certain dietary deficiencies (22) and virus infections (23) of rabbits. There was no evidence, however, that dietary deficiency or virus infection played a role in the lesions seen in the enzyme-treated rabbits.

It is of interest that the muscles of rabbits given enzyme solutions intravenously were in general affected in the order of their contractile activity. Thus the constantly active myocardium was most often involved, and a very active skeletal muscle such as the diaphragm was affected more frequently than were less active muscles such as the femoral or psoas. The areas of necrosis in any one muscle had a random distribution and bore no obvious relation to blood vessels or to other structures; the lesions were focal in nature involving a portion of a muscle fiber or a small group of fibers while adjacent areas appeared to be morphologically intact. Electrocardiographic changes interpreted as being due to myocardial damage were obtained within a few minutes after injection of papain solutions, indicating that changes in the electrical activity of cardiac muscle fibers occurred almost immediately after injection though morphological evidence of damage was not apparent for 6 hours or more. In addition, the muscle lesions in any one animal all appeared to be of the same age suggesting that the noxious agent affected the susceptible muscle fibers at the same time and then was removed or rendered inactive. Taken together, these findings suggest that the active principle in the enzyme solutions exerts its influence by interfering with a specific and vital metabolic process within the muscle fiber itself. The precise point of chemical injury is not evident from these studies, but since smooth muscles and other tissues were not involved, it would appear to be a metabolic process unique to striated muscle or one for which striated muscle has no alternate metabolic pathway.

Despite the fact that all but one of the enzyme solutions used in these studies were relatively crude preparations, it appears likely that it was the proteolytic enzyme in the solutions (or in the case of streptokinase, the proteolytic activity induced in the blood) that was responsible for the specific

effect on cardiac and skeletal muscle. Firstly, it would be unusual indeed if materials prepared from such widely different sources as the papaya (papain), the fig tree (ficin), hog pancreas (trypsin), and streptococcal filtrates (streptokinase) were to have, in addition to their enzyme content, another agent capable of causing a specific lesion of heart and skeletal muscle. Secondly, a purified and twice recrystallized preparation of trypsin produced the same lesions as did the more crude enzyme solutions. Thirdly, in the case of papain, the fact that only solutions having proteolytic activity were capable of inducing the muscle changes, plus the further fact that it was not possible by a number of different procedures to separate the enzyme papain from the factor affecting cardiac and skeletal muscle points to an intimate association between the two, if they are not actually identical. Fourthly, specific inactivation of the tryptic content of enzar and crystalline trypsin solutions by means of crystalline soy bean trypsin inhibitor effectively prevented the development of myocardial necrosis. And finally, the injection of non-enzymatic solutions containing proteins of plant and animal origin failed in every instance to induce cardiac muscle necrosis. Taken together, these findings indicate that the lesions observed were the result of the action of the proteolytic enzymes.

That selective necrosis of muscle can be produced by proteolytic enzymes is also suggested by the studies of Robb-Smith who showed by means of *in vitro* experiments that trypsin can dissolve myoplasm while leaving reticulum and collagen more or less intact (24). The fact that necrosis of cardiac and skeletal muscle was produced more regularly by papain solutions than by solutions of trypsin or streptokinase is not inconsistent with the idea that the lesions were enzyme-induced, for it is well known that mammalian serum and tissues may contain potent inhibitors of trypsin, streptokinase, and plasmin (25, 26), whereas papain is not so inhibited; indeed, it is activated by rabbit serum. The presence of such inhibitors of trypsin and streptokinase-activated plasmin would explain why very much larger doses of these enzymes were required for the production of cardiac muscle lesions than was the case with papain.

The finding that proteolytic enzymes injected intravenously are capable of causing selective necrosis of cardiac and skeletal muscle has interesting implications for the pathogenesis of the morphological changes in rheumatic fever and certain hypersensitivity states. There is now considerable clinical and experimental evidence to indicate that rheumatic fever is causally related to antecedent streptococcal infections (27), and it is well known that streptococci are capable of producing a remarkable number of different enzymes. Thus, it is perhaps noteworthy that a papain-like proteolytic enzyme and its precursor have been crystallized from streptococcal filtrates by Elliott (28). Also, streptokinase, another enzyme elaborated by streptococci, is a powerful

activator of the protease precursor plasminogen normally present in plasma, and there is evidence at hand to show that streptokinase is a more effective activator of the plasminogen-plasmin system of human beings than that of rabbits (29). The unopposed action on a susceptible tissue of such proteolytic enzymes or of other enzyme systems liberated by the microorganisms locally or into the blood stream may help explain the selective morphological changes in the heart valves, myocardium, and other tissues seen in this disease. It may be pertinent, too, that Murphy and Swift have produced by means of repeated streptococcal infections in rabbits a lesion resembling the Aschoff nodule (30, 31), and recent studies by Murphy have pointed to altered cardiac muscle fibers as being implicated in the histogenesis of these nodules (32). Furthermore, the anatomical changes of glomerulonephritis, which usually develop 1 to 2 weeks after infection with certain types of streptococci (33) and which have been produced experimentally in animals by the injection of crystalline foreign proteins (34, 35), have been etiologically associated with the development of hypersensitivity to streptococcal products or to the injected protein (36); and similarly, the selective necrosis of blood vessels that is characteristic of periarteritis nodosa has been linked causally with sensitivity to sulfonamides and to horse serum and has been produced experimentally by the injection into rabbits of large doses of horse serum or other proteins (35, 37, 38). The mechanism, however, whereby hypersensitivity results in the development of such specific morphological changes remains obscure. In this connection, the observations herein recorded have added interest in light of the findings of Ungar that antigen-antibody reactions result in the liberation of measurable proteolytic activity (39–41).

SUMMARY

Focal necrosis of cardiac and skeletal muscle was produced regularly in rabbits by means of a single intravenous or intra-arterial injection of a solution of crude papain. Similar lesions were produced in rats and mice injected with this material. The intravenous injection of solutions of ficin, trypsin, and streptokinase also resulted in comparable lesions of cardiac and skeletal muscle in rabbits.

The lesions in the myocardium became apparent within 6 hours after injection of the enzyme; they consisted essentially of focal degeneration and necrosis of the sarcoplasm and myofibrils within a segment of muscle fiber. An inflammatory reaction consisting of a small number of polymorphonuclear leukocytes and considerable numbers of mononuclear cells, and often multinucleated giant cells, was present within the lesions. In some instances severely damaged fibers were replaced by fibrous tissue and in others proliferation of

muscle cell nuclei and restitution of the fiber appeared to take place. Similar changes of a lesser degree were also observed in skeletal muscle.

The findings are discussed in connection with the pathogenesis of the anatomical lesions of rheumatic fever, periarteritis nodosa, and other hypersensitivity states.

BIBLIOGRAPHY

1. Mirsky, I. A., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60,** 143.
2. Schlamowitz, S. T., and De Graff, A. C., *Circulation*, 1950, **1,** 816.
3. McCarty, M., *Bull. New York Acad. Med.*, 1952, **28,** 307.
4. Geiger, W. B., *J. Immunol.*, 1952, **68,** 11.
5. Balls, A. K., and Hoover, S. R., *J. Biol. Chem.*, 1937, **121,** 737.
6. Anson, M. L., *J. Gen. Physiol.*, 1939, **22,** 79.
7. Balls, A. K., and Lineweaver, H., *Science*, 1937, **86,** 379.
8. Christensen, L. R., *J. Clin. Inv.*, 1949, **28,** 163.
9. French, J. E., *Arch. Path.*, 1952, **53,** 485.
10. Smith, S. G., Black-Schaffer, B., and Lasater, T. E., *Arch Path.*, 1950, **49** 185.
11. Bergmann, M., and Leonidas, Z., *J. Biol. Chem.*, 1936, **114,** 711.
12. Balls, A. K., and Lineweaver, H., *J. Biol. Chem.*, 1939, **130,** 669.
13. Tagnon, H. J., *J. Clin. Inv.*, 1945, **24,** 1.
14. Hicks, S. P., *Arch. Path.*, 1950, **49,** 111.
15. Hicks, S. P., *Arch. Path.*, 1950, **50,** 545.
16. Stetson, C. A., Jr., *J. Exp. Med.*, 1951, **93,** 489.
17. Thomas, L., and Good, R. A., *J. Exp. Med.*, 1952, **96,** 605.
18. Kellner, A., and Robertson, T., unpublished observations.
19. Good, R. A., and Thomas, L., *J. Exp. Med.*, 1953, **97,** 871.
20. Miller, C. P., Jr., *J. Exp. Med.*, 1924, **40,** 543.
21. Loewe, L., and Lenke, S. E., *J. Exp. Med.*, 1940, **71,** 89.
22. Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.*, 1931, **54,** 145.
23. Pearce, J. M., *Arch. Path.*, 1942, **34,** 319.
24. Robb-Smith, A. H. T., *Lancet*, 1945, **2,** 362.
25. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28,** 559.
26. Kaplan, M. H., *J. Clin. Inv.*, 1946, **25,** 347.
27. Swift, H. F., *Ann. Int. Med.*, 1949, **31,** 715.
28. Elliott, S. D., *J. Exp. Med.*, 1950, **92,** 201.
29. Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H., *J. Exp. Med.*, 1953, **97,** 573.
30. Murphy, G. E., and Swift, H. F., *J. Exp. Med.*, 1949, **89,** 687.
31. Murphy, G. E., and Swift, H. F., *J. Exp. Med.*, 1950, **91,** 485.
32. Murphy, G. E., *J. Exp. Med.*, 1952, **95,** 319.
33. Rammelkamp, C. H., Jr., and Weaver, R. S., *J. Clin. Inv.*, 1953, **32,** 345.
34. More, R. H., and Waugh, D., *J. Exp. Med.*, 1949, **89,** 541.
35. Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, **85,** 571.
36. Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1950, **91,** 505.

37. Rich, A. R., *Bull. Johns Hopkins Hosp.*, 1942, **71,** 123.
38. Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1944, **75,** 115.
39. Ungar, G., *Lancet*, 1947, **1,** 708.
40. Ungar, G., and Mist, S. H., *J. Exp. Med.*, 1949, **90,** 39.
41. Ungar, G., Damgaard, E., and Hummel, F. P., *J. Exp. Med.*, 1953, **98,** 291.

EXPLANATION OF PLATES

The photographs were made by Mr. Julius Mesiar.

All animals were given a single intravenous or intra-arterial injection of an enzyme solution. The time of sacrifice in each instance is recorded in the figure legend. Blocks of tissue were fixed in Zenker-formol solution and stained with hematoxylin and eosin.

PLATE 11

Lesions in heart and skeletal muscle in rabbits given 5 per cent papain solution intravenously.

FIG. 1. Rabbit 14-03. Heart. Sacrificed 48 hours after injection. Numerous focal areas of myocardial necrosis, with calcification, in wall of left ventricle, interventricular septum, and papillary muscle. × 4.

FIG. 2. Rabbit 6-84. Heart. Sacrificed 12 hours after injection. Swelling, fragmentation, and eosinophilia of myofibers with beginning infiltration of polymorphonuclear leukocytes. × 360.

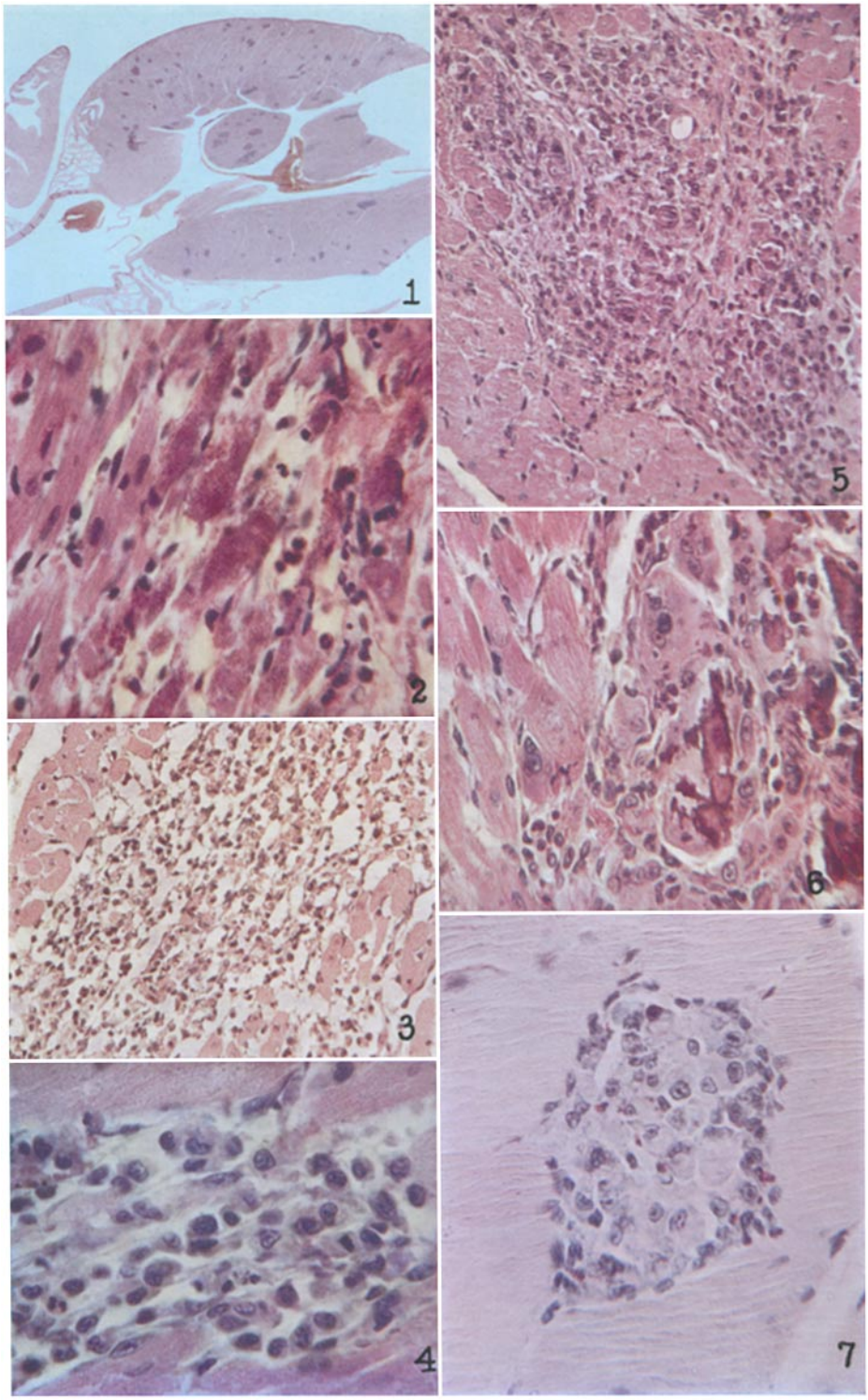
FIG. 3. Rabbit 6-87. Heart. Sacrificed 2 days after injection. Large area of necrosis of cardiac muscle fibers with inflammatory reaction consisting predominantly of edema and mononuclear cells. × 190.

FIG. 4. Rabbit 7-60. Heart. Sacrificed 3 days after injection. An area of myocardial necrosis with the necrotic sarcoplasm largely removed and an infiltration of mononuclear cells and a few polymorphonuclear leukocytes. × 500.

FIG. 5. Rabbit 6-24. Heart. Sacrificed 4 days after injection. An area of myocardial necrosis with beginning proliferation of fibroblasts and muscle cell nuclei. × 225.

FIG. 6. Rabbit 6-55. Heart. Sacrificed 7 days after injection. An area of myocardial necrosis with multinucleated giant cells and calcification. × 360.

FIG. 7. Rabbit X 5. Diaphragm. Sacrificed 3 days after injection. Necrosis of a single skeletal muscle fiber with phagocytosis of sarcoplasm and proliferation of muscle cell nuclei. × 360.



(Kellner and Robertson: Proteolytic enzymes)

PLATE 12

FIG. 8. Rabbit 8-57. Left sternocleidomastoid muscle. Sacrificed 48 hours after injection of 5 per cent papain solution into left common carotid artery. Large area of necrosis of skeletal muscle fibers and interstitial inflammatory reaction. × 95.

FIG. 9. Rat 1-06. Sacrificed 24 hours after injection of papain solution. A subendocardial area of acute myocardial necrosis with edema and inflammatory reaction. × 190.

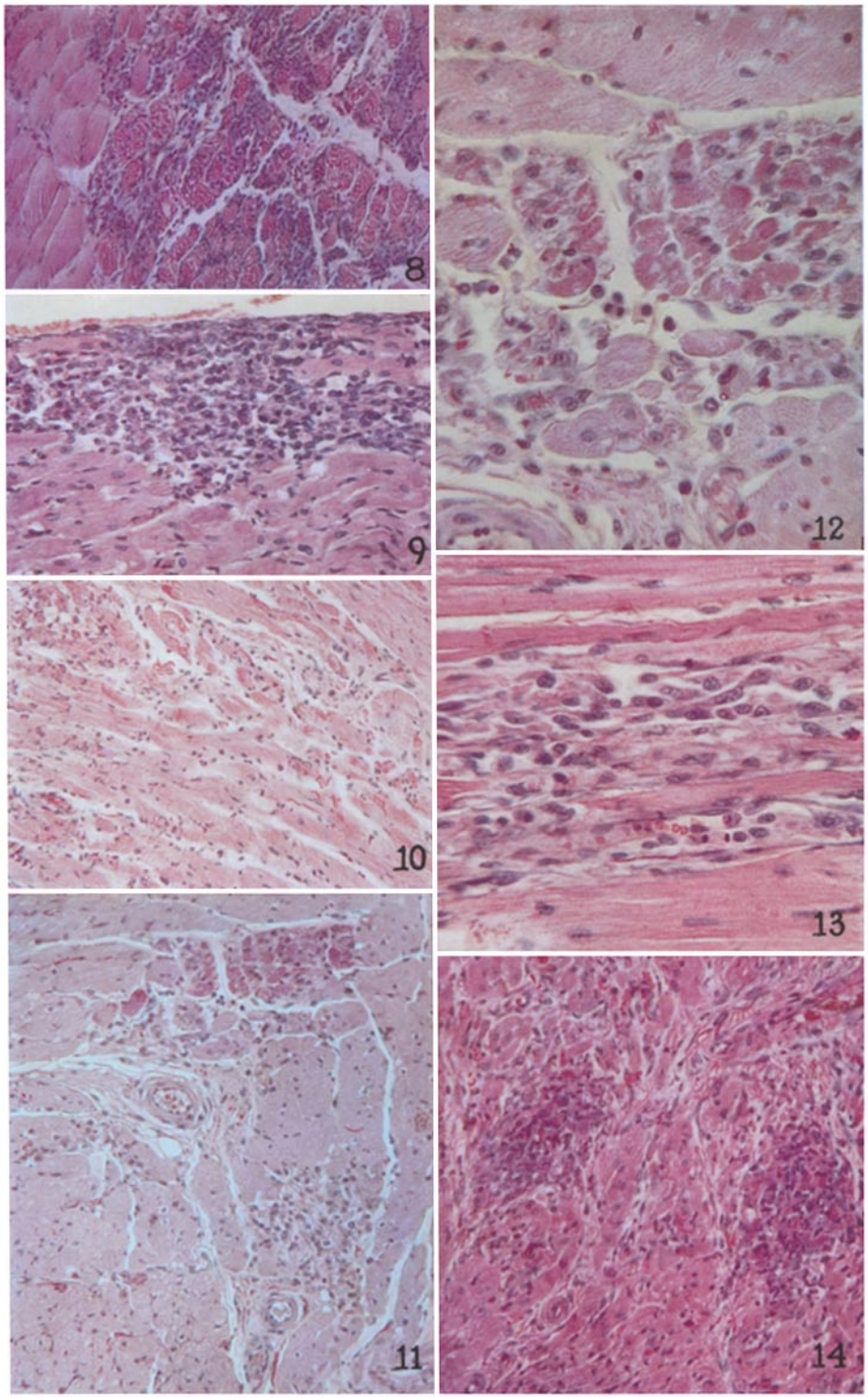
FIG. 10. Rabbit 11-70. Heart. Sacrificed 24 hours after an intravenous injection of 2 per cent ficin solution. Swelling, fragmentation, and eosinophilia of myocardial fibers with infiltration of moderate numbers of polymorphonuclear leukocytes. × 145.

FIG. 11. Rabbit 14-11. Heart. Sacrificed 48 hours after an intravenous injection of 50 mg. of crystalline trypsin. Two focal areas of myocardial necrosis near small coronary arteries. The arterial walls are not involved in the necrotic process and there are no thrombi present. × 145.

FIG. 12. Rabbit 14-11. Heart. Higher magnification of focal area of necrosis from Fig. 11. × 360.

FIG. 13. Rabbit 12-43. Heart. Sacrificed 48 hours after an intravenous injection of 100 mg. of heparin and 120,000 units of trypsin (enzar). Area of myocardial necrosis showing focal dissolution of sarcoplasm with persistence of muscle cell nuclei. × 360.

FIG. 14. Rabbit 14-19. Heart. Sacrificed 3 days after intravenous injection of 1,000,000 units of streptokinase solution (varidase). Two focal areas of myocardial necrosis. × 190.



(Kellner and Robertson: Proteolytic enzymes)