MYOCARDIAL NECROSIS PRODUCED IN ANIMALS BY MEANS OF CRYSTALLINE STREPTOCOCCAL PROTEINASE*

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PLATE 15

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Papain and certain other proteolytic enzymes bring about necrosis of cardiac muscle when injected intravenously into animals, as recent observations have shown (1). In the experiments now to be reported, streptococcal proteinase —a crystalline enzyme isolated from filtrates of group A streptococci which resembles papain in a number of respects (2, 3)—was given by vein to rabbits, mice, and guinea pigs. Striking myocardial necrosis followed such injections in each of the animal species used. This finding is of particular interest because of the well known association between rheumatic fever and antecedent streptococcal infections (4), and it suggests the possibility that specific streptococcal products may be directly implicated in the pathogenesis of the anatomical changes present in rheumatic heart disease.

Materials and Methods

The general plan of the experiment was as follows. A single intravenous injection of a solution of crystalline streptococcal proteinase in appropriate dosage was given to groups of rabbits, mice, and guinea pigs. The animals were sacrificed 1 to 3 days later and the heart and other organs examined in the gross and microscopically.

Proteinase.—The enzyme used in these studies was once crystallized streptococcal proteinase generously supplied by Dr. S. D. Elliott of The Rockefeller Institute for Medical Research. For convenience the streptococcal proteinase will hereinafter be referred to as SP. The material, all from a single batch, was a dried, white powder which was prepared for injection by dissolving it in 0.01 M cysteine brought to pH 7.5 with NaOH. Cysteine was used because SP, like papain, requires the presence of sulfhydryl compounds or cyanide for optimal enzymatic activity *in vitro*. In a number of experiments normal rabbit serum or saline was used as the solvent prior to intravenous injection; the effect of the injected SP was found to be independent of the solvent employed, presumably because mammalian blood contains enough sulfhydryl or other reducing substances to activate the enzyme *in vivo*. In the case of each solvent about 10 per cent of the powder failed to go into solution despite prolonged

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stirring, and the insoluble residue was removed by centrifugation. Freshly prepared solutions were employed in each experiment. Enzymatic activity of the proteinase solution was tested by the ability to clot milk (3).

Animals.—The rabbits used were market-bought animals weighing between 2.2 and 3.7 kilos, and were unselected as to breed, color, or sex. The guinea pigs were all young white males weighing from 210 to 330 gm. White male mice were used, ranging in weight from 20 to 30 gm., the majority being about 25 gm.; the mice were from three different strains—A mice raised in the laboratory, and Webster and Rockland mice purchased from a commercial source.

Each animal was given a single intravenous injection of proteinase solution. Rabbits were injected in the marginal ear vein, guinea pigs in the lateral vein of the forepaw, and mice in a tail vein. The injections were made rapidly; they were in almost all instances well tolerated and without obvious reaction by the animals.

Anatomical Studies.—Complete postmortem examination was done on all animals. In the case of the mice and guinea pigs, histological sections were prepared only from the heart; in the case of the rabbits, sections from the abdominal and thoracic viscera and from the diaphragm, the psoas, and masseter muscles were studied as well. Tissues were fixed in Zenker-formol solution, embedded in paraffin, and stained with hematoxylin and eosin. With very few exceptions only a single section was examined from each heart and no section was considered to be positive unless there were at least two characteristic lesions present. The sections were graded on an arbitrary scale from 0 to +++, where 0 represented the absence of lesions or in some instances the presence of only one lesion; +, a few scattered lesions; ++, a moderate number; and +++, numerous lesions.

Tolerance of Rabbits and Mice to Injected SP.—In a preliminary experiment to determine the tolerance of rabbits to streptococcal proteinase, four rabbits weighing between 2.85 and 2.9 kilos were each given a single intravenous injection of a solution containing 20, 20, 10, and 5 mg. of SP, respectively. Of the two animals given 20 mg., one died 95 minutes after injection and the other was found dead the following morning. The animal given 10 mg. died 20 hours after injection, and the remaining animal survived and was sacrificed 48 hours later. In a similar experiment it was found that mice tolerated SP in a dosage range of 0.5 to 1.0 mg. per animal; doses of 1.5 mg. or higher were lethal in most cases.

Clotting Time.—Because of the observation that a blood coagulation defect regularly follows the intravenous injection of papain into rabbits (5), determinations of the whole blood clotting time were made in several rabbits given SP; only very slight and transitory prolongations of the clotting time were noted.

Cardiac Necrosis in Rabbits Given Streptococcal Proteinase Intravenously

In a typical experiment focal necrosis of cardiac muscle—to be described in detail further on—was found in 10 of 13 rabbits given a single intravenous injection of SP in amounts of 1.5 to 2.0 mg. per kilo.

120 mg. of streptococcal proteinase was dissolved in 60 ml. of 0.01 M cysteine. 9 rabbits were given a single intravenous injection of this solution in amounts of 2.0 mg. per kilo and 6 additional rabbits were given 1.5 mg. per kilo. 6 control rabbits were given 2.0 ml. per kilo of this solution previously heated to 90°C. for 30 minutes to inactivate the proteinase, and 6 additional control animals were given 2.0 ml. per kilo of 0.01 M cysteine intravenously. The animals were sacrificed 48 hours after injection and histological sections prepared from the heart, diaphragm, psoas, and masseter muscles in all cases, and also from the liver, adrenal, kidney, and pancreas in the animals receiving proteinase solution. The results of this experiment are given in Table I. Of the 13 surviving animals in groups A and B that were given

TABLE I

*Necrosis of Cardiac and Skeletal Muscle in Rabbits Given Streptococcal Proteinase Intravenously**

Experimental groups	Rabbit No.	Cardiac muscle necrosis	Skeletal muscle necrosis		
			Diaphragm	Masseter	Psoas
A. Experimental rabbits given SP, 2.0 mg./kg., intravenously	1	+++	+	0	0
	2	+++	−	−	−
	3	+	+	0	0
	4	+	+++	0	0
	5	+	+	++	+
	6	0	0	0	0
	7	0	0	0	0
	8	Died	−	−	−
	9	Died	−	−	−
B. Experimental rabbits given SP, 1.5 mg./kg., intravenously	10	+++	+	0	0
	11	++	+	0	0
	12	++	0	0	0
	13	+	+	+	0
	14	+	0	0	0
	15	0	0	0	0
C. Control rabbits given heated SP, 2.0 mg./kg., intravenously	16	0	0	0	0
	17	0	0	0	0
	18	0	0	+	0
	19	0	0	0	0
	20	0	0	0	0
	21	0	0	0	0
D. Control rabbits given 0.01 M cysteine, 2.0 ml./kg., intravenously	22	0	0	0	0
	23	0	0	0	0
	24	0	0	0	0
	25	0	0	0	0
	26	0	0	0	0
	27	0	0	0	0

SP, streptococcal proteinase, 2.0 mg./ml., in 0.01 M cysteine.

−, not examined.

Died, found dead the morning after injection. Time of death not known. Discarded.

In this and in the ensuing tables severity of necrosis is graded according to the following scale: 0, no areas of necrosis; +, few areas of necrosis; ++, moderate number of necrotic lesions; +++, numerous areas of necrosis.

*A single injection was given into the marginal ear vein. Animals sacrificed 48 hours later.

active SP intravenously, 10 had cardiac muscle necrosis, the lesions being extensive in 3; in the 12 control animals, on the other hand, there were no comparable areas of myocardial necrosis. The cardiac lesions even when extensive were not visible in the gross and were detected only after microscopic examination. No changes attributable to the injected SP were observed in the liver, kidney, adrenal, or pancreas in any of the animals in this experiment.

Focal necrosis of skeletal muscle was present in 10 of 36 muscles examined in 12 animals of the experimental groups given SP and in only 1 of 36 comparable muscles examined in 12 animals of the control groups. It is of interest that skeletal muscle lesions were present most often in the diaphragm, though in only one instance were these widespread. Muscle lesions having similar distribution, incidence, and morphological characteristics have been described previously in rabbits given papain solution intravenously (1).

It appeared from this experiment that streptococcal proteinase, like papain and certain other proteolytic enzymes, was capable of causing selective necrosis of the heart and skeletal muscle when given intravenously to rabbits.

Cardiac Necrosis in Guinea Pigs and Mice Given SP Intravenously

A number of experiments were then done to learn whether other animal species were susceptible to the action of SP and whether cardiac changes comparable to those seen in rabbits could also be produced in them, or whether the rabbit was unique in this regard.

16 normal white male guinea pigs were divided into four groups of four animals each. The animals in group A were each given 1.0 mg. of SP intravenously (0.25 ml. of a solution containing 4.0 mg. SP per ml. in 0.01 M cysteine); those of group B were each given 0.375 ml. of the same solution, amounting to 1.5 mg. of SP per guinea pig. Group C served as a control, each animal receiving 0.5 ml. of 0.01 M cysteine intravenously. As an additional control against the possibility that the cardiac lesions might be due to non-specific streptococcal proteins rather than to the action of a specific enzyme, the animals of group D were given an intravenous injection of 2.5 mg. of streptokinase[1] dissolved in 0.5 ml. of saline. Each injection contained about 3,750 units of streptokinase activity, an amount less than that found previously to be necessary for the production of lesions by activation of the plasminogen-plasmin system. The animals were sacrificed 48 hours after injection. At postmortem examination there were no lesions visible in the gross in any of the viscera of the animals in groups A, C, and D. Three of the guinea pigs in group B, however, had striking and easily visible yellow streaks and plaques of necrosis in the myocardium; no other abnormalities were seen in the gross in these animals. Microscopic sections were prepared from a single block cut through the heart in each case. There were focal areas of myocardial necrosis in all four hearts of the guinea pigs in group B, and in two of these the lesions were very extensive. There were no lesions present in the hearts of the animals in group A given the smaller dose of SP nor were any lesions demonstrable in the hearts of the eight controls (see Table II). The distribution and morphological character of the cardiac lesions in the guinea pigs were essentially the same as those seen in rabbits given SP.

36 mice of the A, Rockland, and Webster strains were each given a single intravenous injection into a tail vein of a solution containing 0.5 to 1.0 mg. of SP per animal. The SP was dissolved in 0.01 M cysteine in most cases; in some instances, however, 0.9 per cent saline or normal rabbit serum was used as the solvent. The effect of the injected SP was found to be independent of the solvent employed. 30 mice of the same strains were used as controls, and were given an intravenous injection of cysteine, saline, or normal rabbit serum in amounts comparable to those used in the experimental animals. The animals were sacrificed 24 to 72 hours after injection and blocks for microscopic study were taken from the heart in each case.

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

TABLE II

*Myocardial Necrosis in Guinea Pigs Given Streptococcal Proteinase Intravenously**

Group A SP, 1.0 mg.		Group B SP, 1.5 mg.		Group C 0.01 M cysteine, 0.5 ml.		Group D SK, 2.5 mg.	
Weight	Myocardial necrosis	Weight	Myocardial necrosis	Weight	Myocardial necrosis	Weight	Myocardial necrosis
gm.		*gm.*		*gm.*		*gm.*	
270	0	240	+++	240	0	220	0
330	0	230	+++	285	0	260	0
250	0	230	++	270	0	240	0
245	0	250	+	240	0	210	0

SP, streptococcal proteinase, 4 mg./ml., in 0.01 M cysteine.

SK, streptokinase, 5 mg./ml., in saline.

* A single injection was given into the lateral vein of the forepaw. Animals were sacrificed 48 hours later.

TABLE III

*Myocardial Necrosis in Three Strains of Mice Given Streptococcal Proteinase Intravenously**

A strain		Rockland strain		Webster strain	
SP	Myocardial necrosis	SP	Myocardial necrosis	SP	Myocardial necrosis
mg.		*mg.*		*mg.*	
1.0	+++	1.0	+++	0.75	+
"	++	"	+++	"	+
"	++	"	+++	"	0
0.75	++	"	+++	"	0
0.5	0	"	++	"	0
		"	++	"	0
		0.75	+++	"	0
		"	+++	"	0
		"	++	"	0
		"	++	0.5	+
		0.5	+++	"	0
		"	++	"	0
		"	+	"	0
		"	+	"	0
		"	+	"	0
		"	0		

SP, streptococcal proteinase.

* All injections were made into a tail vein. Animals sacrificed 24 to 72 hours later.

No myocardial lesions were observed in the hearts of any of the control mice. In the animals given SP solutions, on the other hand, 4 out of 5 A mice, 15 out of 16 Rockland mice, and 3 out of 15 Webster mice had gross or microscopic evidence of myocardial necrosis (see Table III). The cardiac lesions in the Webster mice were in all cases small and few in number and were detected only upon microscopic examination; the areas of necrosis in the A and Rock-

land mice were usually quite extensive, in many cases remarkably so, and in more than half the animals they could be seen easily with the naked eye as yellow streaks or plaques on the epicardial surface of the heart. No lesions of any other organs were visible in the gross in these animals.

It is clear from these experiments that guinea pigs and mice, as well as rabbits, are susceptible to the action of intravenously injected SP and develop focal necrosis of the cardiac muscle following such injections. It is of considerable interest, too, that striking differences in the incidence and severity of the lesions were observed in different strains of mice.

Anatomical Characteristics of the Cardiac Lesions

The lesions seen in the hearts of rabbits, guinea pigs, and mice given SP were essentially focal areas of myocardial necrosis (Fig. 1 to 3). These were usually small and fairly well demarcated, though occasionally they coalesced, resulting in quite large areas of necrosis (Fig. 1). In animals sacrificed or dying within 24 hours of the time of injection, the affected muscle fibers had lost their cross-striations and appeared fragmented, swollen, eosinophilic, and granular. In animals killed 2 or 3 days after injection the necrotic muscle fibers had largely disappeared, though bits of necrotic sarcoplasm were still visible in places (Fig. 2). Portions of the endomysium and sarcolemmic sheaths could still be made out after disappearance of the sarcoplasm (Fig. 3). There was little or no inflammatory reaction associated with the early lesions, indicating that necrosis of muscle fibers was the fundamental process involved. Within and about the more advanced lesions, however, there was an extensive infiltration of inflammatory cells consisting of small numbers of polymorphonuclear leukocytes, considerable numbers of mononuclear histiocytes, and scattered multinucleated giant cells (Figs. 2, 3). Proliferating muscle cell nuclei were often present in the necrotic areas, particularly in lesions that were more than 48 hours old. The inflammatory exudate in a number of instances was prominent in the interstitium about the necrotic areas, and occasionally there were collections of inflammatory cells about smaller blood vessels. It should be pointed out, however, that the blood vessels were in all cases patent and in no case was there necrosis or inflammation of the vessel wall itself.

The cardiac lesions appeared in general to have a random distribution. They were present in the muscles of both ventricles, in the papillary muscles, and occasionally also in the auricles, and bore no constant relation to blood vessels. In mice there was some tendency toward subendocardial and subepicardial localization (Fig. 1). Calcification of the areas of cardiac muscle necrosis, as determined by the characteristic appearance and staining reaction in hematoxylin and eosin-stained sections and confirmed by von Kossa stain, was particularly prominent in the mouse hearts (Fig. 1) and perhaps accounts for the frequency with which these lesions could be detected in the gross. No

bacteria, parasites, or viral inclusion bodies were found in any of the involved areas of the myocardium. The cardiac lesions seen in animals given SP were in most details quite similar to those previously described in rabbits given papain solution (1).

With the exception of the cardiac lesions, no visceral lesions were seen in the gross in rabbits, guinea pigs, or mice, nor were such lesions present in sections of the liver, kidney, pancreas, and adrenal of rabbits which were examined microscopically. More detailed studies of the viscera, of smooth and striated muscle, and of serous membranes in these animals are needed, however, to determine whether SP affects only the heart and skeletal muscle or whether other tissues may be affected as well.

It is noteworthy that 2 out of 13 rabbits given SP intravenously and having extensive myocardial necrosis also had verrucous lesions of a heart valve, the tricuspid in each case. (Fig. 4). These lesions were small, measuring about 150 × 100 μ; they were situated near the base of the valve and protruded slightly above the valve surface, and the overlying endocardium was ulcerated. The lesions consisted for the most part of fairly large, closely packed cells having prominent oval or rounded nuclei. In some cases the nuclei were pale and had a central bar of clumped, dark-staining chromatin. There were a few polymorphonuclear leukocytes within the lesions, and the nearby valve tissues were edematous and contained a few inflammatory cells. These lesions, which resembled the verrucous endocarditis seen in acute rheumatic fever, were found by chance in random sections of the heart. Further observations will have to be made before the possible significance of this finding is clear.

DISCUSSION

The experiments herein described make it plain that a single intravenous injection of a solution of crystalline streptococcal proteinase into rabbits, guinea pigs, and mice is followed in a high proportion of cases by the development of focal myocardial necrosis, often remarkably extensive, and an associated inflammatory reaction. Though it has long been known that streptococci and streptococcal filtrates may cause functional and structural changes in the heart (6–8), there has hitherto, to the best of our knowledge, been no report of comparable lesions following the injection into animals of a specific and relatively purified product of group A streptococci, with the possible exception of the cardiac muscle lesions reported recently in rabbits given very large doses of streptokinase (1). It seems likely that the necrosis of cardiac muscle observed following the injections of solutions of streptococcal proteinase was related, not to some impurity which the preparation, though crystalline, may have contained, but rather to the proteolytic activity of the solution. That this is so is suggested by the small amount of proteinase solution required to produce lesions, by failure to find lesions in animals given proteinase solutions the enzymatic activity of which had been destroyed by heat, by the absence of lesions in the guinea pigs given comparable amounts of streptokinase—another enzyme derived from streptococcal filtrates, and particularly, by the striking similarity between the myocardial lesions observed in these studies and those seen in animals given papain solution, an enzyme which is remarkably like SP in many respects (2, 3).

Since proteinase can be produced by many strains of group A streptococci, the question arises whether it plays a role in streptococcal disease of human beings, and particularly, whether it may be involved in the pathogenesis of the cardiac lesions of rheumatic fever. It is probable that conditions favorable for the formation of proteinase exist during streptococcal infection in man, for though proteinase is produced *in vitro* only in media having a slightly acid reaction, such conditions of hydrogen ion concentration have been shown experimentally to be present in areas of inflammation (9). Moreover, it has been shown by Todd (10) that while blood serum of most normal human beings did not contain anti-proteinase, that of many individuals developed measurable quantities of anti-proteinase following infection with group A streptococci; hence, it appears reasonable to assume that this antibody was produced in response to antigenic stimulation by proteinase elaborated during growth of the infecting microorganism *in vivo*. Whether the cardiac tissues of man are susceptible to the action of streptococcal proteinase and whether enough of the enzyme is produced during infection with group A streptococci to induce anatomical changes remains to be determined. The fact that each of three different animal species thus far tested developed myocardial lesions following the injection of SP suggests that man, too, may respond in a similar fashion. It is noteworthy in this connection that rabbits were distinctly more sensitive to the action of injected SP than were mice or guinea pigs and required only one-fourth as much enzyme, on a weight basis, as the latter and only one-tenth to one-twentieth as much as the former for the development of myocardial lesions. It is quite conceivable that human beings may be more sensitive to the action of this enzyme than are rabbits and that even smaller doses may be able to adversely affect cardiac tissues. Such differences in susceptibility to streptococcal enzymes are not without precedent, for the streptokinase produced by group A streptococci is many times more effective as an activator of the plasminogen of man than it is of that of rabbits (11, 12).

The cardiac lesions induced in animals by injected streptococcal proteinase are clearly not identical with those seen in patients with active rheumatic heart disease, nor have Aschoff nodules been observed in these experimental animals. Myofiber necrosis and associated inflammatory changes, however, are frequent findings in acute rheumatic heart disease (13), and further, the experimental and morphological studies of Murphy point to damaged and necrotic muscle fibers as the primary factor in the histogenesis of the Aschoff nodule (14). The experiments thus far performed with streptococcal proteinase have all been short term ones in which relatively large amounts of the enzyme were given at one time. It remains to be seen whether exposure of animals to smaller amounts of the enzyme during prolonged periods of time may result in histological changes closer akin to those seen in naturally occurring rheumatic fever.

SUMMARY

Focal myocardial necrosis that was often extensive was found in a high percentage of rabbits, guinea pigs, and mice given a single intravenous injection of crystalline streptococcal proteinase.

The findings are discussed in relation to their possible implications for the pathogenesis of the cardiac lesions of rheumatic fever.

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EXPLANATION OF PLATE 15

The photographs were made by Mr. Julius Mesiar.

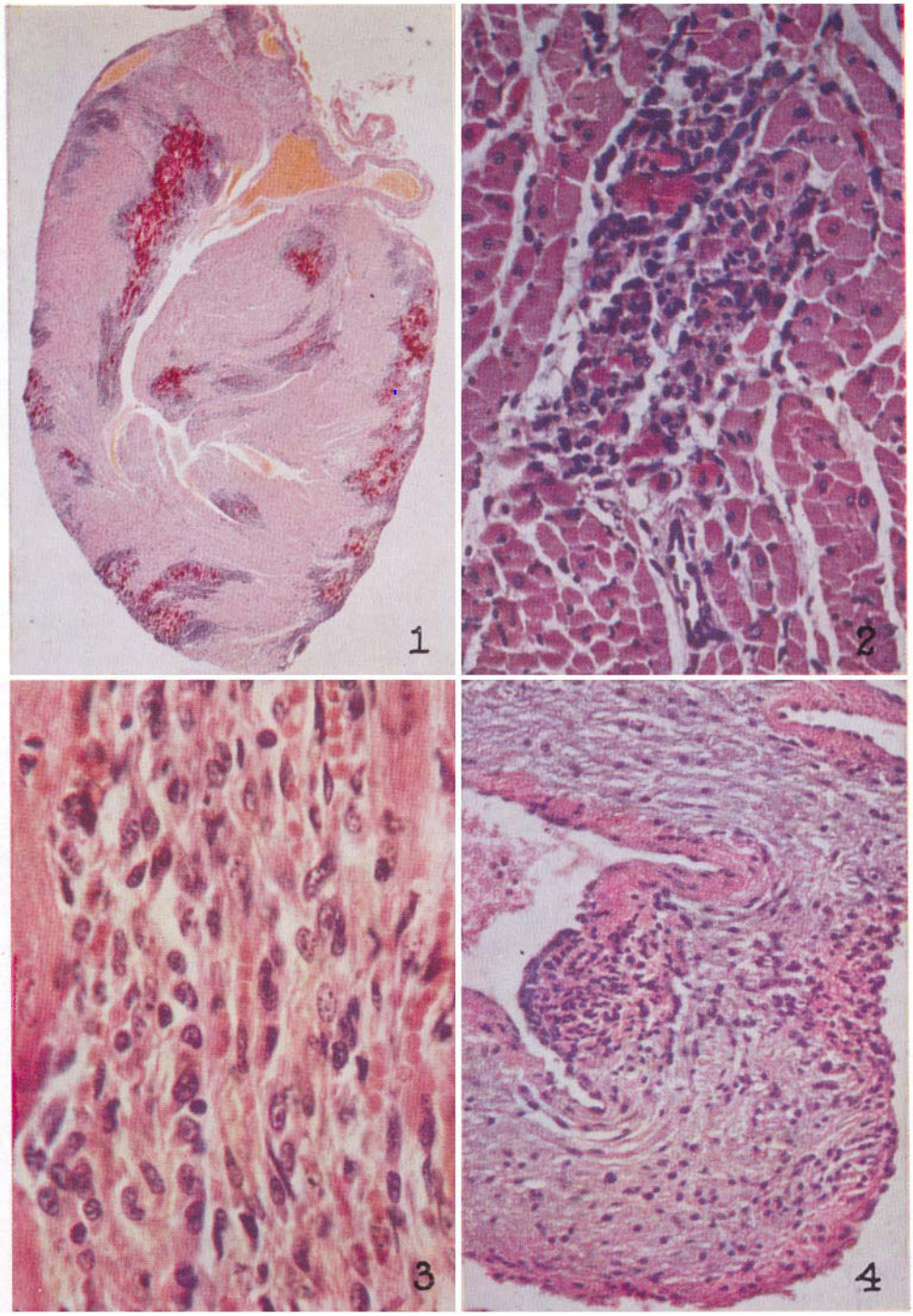
All animals were given a single injection of streptococcal proteinase solution intravenously. Blocks of tissue were fixed in Zenker-formol solution and stained with hematoxylin and eosin.

FIG. 1. Mouse S-1. Heart. 0.5 ml. of a solution containing 2.0 mg. SP per ml. was given intravenously and the animal sacrificed 60 hours later. Numerous coalescent areas of myocardial necrosis with calcification. The necrotic foci tend to localize beneath the endocardium and epicardium. × 25.

FIG. 2. Guinea pig B-1. Heart. 0.375 ml. of a solution containing 4.0 mg. SP per ml. was given intravenously and the animal sacrificed 2 days later. An area of myocardial necrosis in the left ventricle in which bits of necrotic sarcoplasm still remain. There is an inflammatory reaction consisting of a few polymorphonuclear leukocytes and a moderate number of mononuclear histiocytes. × 280.

FIG. 3. Rabbit 15-84. Heart. Given SP, 3.0 mg. per kilo, intravenously and sacrificed 3 days later. Focal area of myocardial necrosis in the left ventricle. The necrotic sarcoplasm has been almost entirely removed, though portions of the endomysium and sarcolemmic sheaths remain and can be seen in places. There are a few mononuclear leukocytes present and proliferating muscle cell nuclei. × 600.

FIG. 4. Rabbit 15-56. Heart. Given SP, 1.5 mg. per kilo, intravenously and sacrificed 2 days later. Small verrucous lesion of the tricuspid valve. This and another similar lesion were found in a random section of the heart in 2 out of 13 rabbits given SP solution. × 240.



(Kellner and Robertson: Streptococcal proteinase)