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Chymotrypsin Inhibition of Muscular Contraction

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Abstract □ The effects of chymotrypsin and its various pharmaceutical preparations on skeletal, smooth, and cardiac muscles were tested. Chymotrypsin inhibited the development of adenosine 5′-triphosphate-induced tension by glycerol-extracted fibers and caused the relaxation of contracted fibers. When adenosine 5′-triphosphate-contracted muscle fibers were exposed to chymotrypsin and then washed, the muscle fibers failed to respond to the addition of the nucleotide triphosphate. If added to Tyrode's solution containing strips of tensed guinea pig gut, crystalline chymotrypsin or its various pharmaceutical preparations caused loss of tone, tension, and peristaltic movements. The enzyme preparations also inhibited the response of the gut strip to histamine or serotonin. When a perfusing rat heart was subjected to chymotrypsin, the enzyme caused an immediate short-term increase in cardiac tone and a series of abnormal contractions. These changes continued until the heart failed. Ultrasonication of chymotrypsin increased its hydrolytic activity on low molecular weight substrates and abolished its effects on muscle contraction.

Keyphrases □ Chymotrypsin—inhibition of adenosine 5′-triphosphate-induced muscular contraction, effect of ultrasonification on physicochemical and pharmacological properties □ Muscular contraction, adenosine 5′-triphosphate induced—inhibition by chymotrypsin □ Ultrasonication—effect on physicochemical and pharmacological properties of chymotrypsin

Chymotrypsin, a proteolytic enzyme, is of therapeutic interest as an anti-inflammatory agent. It is extensively employed to reverse inflammation, resorb edema, and liquify or localize suppurative exudation (1–6). For intramuscular administration, it is used as a suspension in sesame oil or as an aqueous solution. For oral administration, a purified enzyme concentrate is incorporated into tablets. The action of chymotrypsin has been attributed to the catalytic conversion of profibrinolysin to fibrinolysin (4, 5), a characteristic reaction of both chymotrypsin and trypsin (6).

Studies from these laboratories led to the isolation of a toxic glycoprotein from *in vitro* scalded human skin, which was shown to inhibit specifically the formation of adenosine 5′-triphosphate-induced contraction by glycerol-extracted rabbit muscle fibers (7, 8). This inhibitory activity of the glycoprotein was destroyed by incubating the glycoprotein with collagenase. The enzyme itself did not influence the development of adenosine 5′-triphosphate-induced tension. To extend these studies, the effects of chymotrypsin and trypsin were investigated. In these experiments, crystalline chymotrypsin and its various pharmaceutical forms were tested *in vitro* on the physiological properties of glycerol-extracted rabbit psoas muscle fibers, guinea pig gut, and isolated rat heart. When added to the bathing medium, chymotrypsin or its various pharmaceutical forms inhibited the formation of adenosine 5′-triphosphate-induced tension by muscle fibers. If added to the medium with adenosine 5′-triphosphate-contracted fibers, chymotrypsin or its various pharmaceutical forms caused immediate loss of tension. They also inhibited the response by guinea pig gut strips to histamine or serotonin and resulted in loss of tone, tension, and peristaltic movements. If perfused into the rat heart, they caused an immediate short-term increase in cardiac tone, a series of abnormal contractions, and an enhanced heart failure.

Sonication of the enzyme increased its hydrolytic activity on low molecular weight, synthetic substrates and abolished its effect on muscular contraction.

EXPERIMENTAL

Materials—α-Chymotrypsin[1] was crystallized (9) three times to obtain a preparation with an activity of 1200 units/mg. on *N*-acetyl-l-tyrosine ethyl ester and of 25 units/mg. on *N*-benzoyl-l-arginine ethyl ester. A sterile solution[2] was obtained containing 5000 units of purified chymotrypsin/ml. of 0.9% sodium chloride. A purified enzyme concentrate[3] containing a chymotrypsin mixture with a potency on hemoglobin of 3065, on *N*-acetyl-l-tyrosine ethyl ester of 495, and on *N*-benzoyl-l-arginine ethyl ester of 2625 units/mg. was employed as supplied. Trypsin[4], with a potency of 4067 units/mg. on *N*-benzoyl-l-arginine ethyl ester, was used.

The glycerol-extracted rabbit psoas muscle fibers were prepared according to the method of Szent-Gyorgyi (10). The guinea pig gut strips were obtained from freshly sacrificed animals, cleaned, and washed thoroughly with Tyrode's solution. The toxic glycoprotein was prepared as reported earlier (7, 8).

Methods[5]—An assay system was developed to measure the action of drugs on muscular activities. For the skeletal muscle, the system employed glycerol-extracted rabbit psoas muscle fibers. The fibers were suspended from a 0.3-force transducer in 62 m*M* tromethamine–phosphate buffer of pH 7.5 containing 2.5 m*M* $MgCl_2$. In this chamber the fibers were allowed to incubate at 26° for 60 sec.; then adenosine 5′-triphosphate was added in 0.10-ml. aliquots of 3×10^{-2} *M* solution to induce tension. The tension was maintained for 2 min.; then chymotrypsin was added in the amounts stated for each experiment.

[1] Armour lot K450038.
[2] Chymar injectable, Armour Pharmaceutical Co.
[3] Armour lots 582 and 583.
[4] Armour lot K152045.
[5] In all these experiments, the tension developed was monitored with a model Grass polygraph with 7 P 1 preamplifier and recorded.

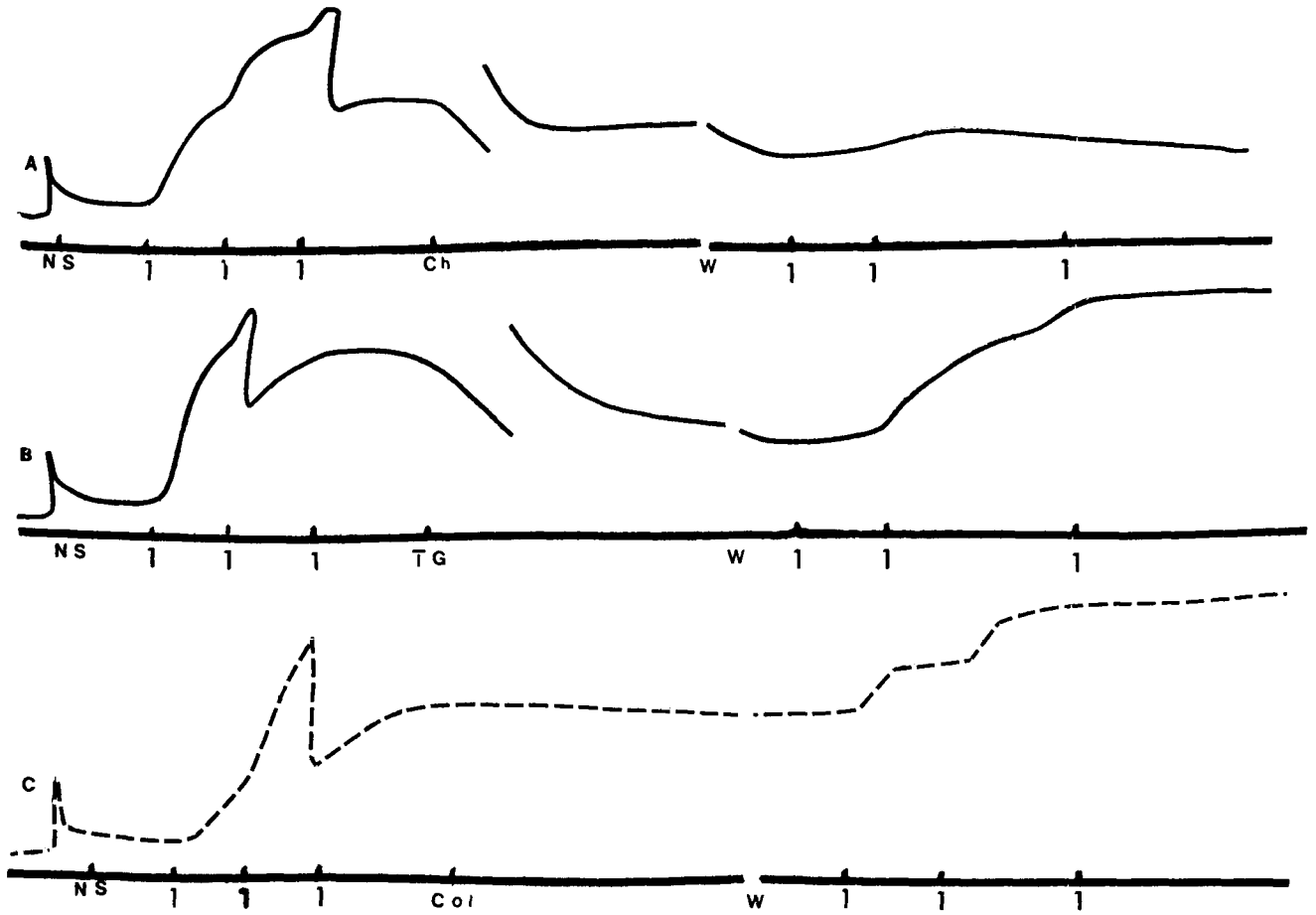

Figure 1—*Tracings of the action of chymotrypsin, toxic glycoprotein, and collagenase on adenosine 5′-triphosphate-induced tension by glycerol-extracted muscle fibers. A stretch of 40-mg. tension was set for on the fiber, and 0.1 ml. of 0.9% sodium chloride (NS) was added; 2 min. later, three aliquots of adenosine 5′-triphosphate (1) were used to develop tension. Then 5 units/ml. of chymotrypsin (Ch) (A), 0.5 mg. of the toxic glycoprotein (TG) (B), or 0.5 of* Clostridium *collagenase (Col) (C) was added, and incubation was continued for 3 min. The bathing medium was drained out, the fibers were washed (w), and fresh buffer and adenosine 5′-triphosphate (1) were added to develop tension. The first break in the tracings indicates the change in sensitivity of the polygraph from 0.02 to 0.05 v./cm. The second break in the tracings during relaxation of the fiber is the change in sensitivity back to 0.02 v./cm.*

For the smooth muscle, freshly washed and prepared strips of guinea pig gut were employed. The 2.5-cm. long strips were suspended from the 0.3-force transducer in Tyrode's solution of pH 8.2. Tension was induced by adding 0.05 mcg. of either histamine or serotonin to the incubating medium containing the strip of gut.

For the cardiac muscle, isolated rat hearts were employed. Basically a Langendorf procedure (11) was used for the isolation and perfusion of the rat heart. The perfusion solution was standard Locke's solution buffered at 37° with bicarbonate to pH 7.4. A reservoir of perfusion solution was under constant gassing with 100% oxygen at approximately 1 l./min. Perfusion pressure was maintained at 55–65 mm. Hg. Changes in the force of contraction of isolated hearts were monitored by a 0.03-force transducer[6] attached to a balanced aluminum lever connected to the apex of the heart with a braided silk thread.

Determination of Hydrolytic Activities—Measurements of chymotrypsin and trypsin hydrolytic activities were carried out using the synthetic substrates *N*-acetyl-l-tyrosine ethyl ester (12) and *N*-benzoyl-l-arginine ethyl ester (13, 14), respectively.

Analytical Procedures—Physicochemical characteristics of chymotrypsin and its sonicated products were determined by polyacrylamide gel electrophoresis, analytical ultracentrifugation, and chromatography on a diethylaminoethane column under the experimental conditions reported earlier (15).

RESULTS

Effect of Chymotrypsin on Glycerol-Extracted Skeletal Muscle—Chymotrypsin inhibited the development of adenosine 5′-triphosphate-induced tension when fibers were treated with the enzyme prior to adenosine 5′-triphosphate. When the fibers were contracted by adenosine 5′-triphosphate under standard conditions and chymotrypsin was added after tension development, an immediate loss of tension occurred (Fig. 1). The chymotrypsin-treated fibers lost their ability to contract again, even after removal of the enzyme. The activity of crystalline chymotrypsin was compared with that of a pharmaceutically used enzyme concentrate and product[2]. When applied at equal activities (hydrolytic on synthetic substrate), the crystalline preparation had the least activity.

To test the specificity of the reaction, trypsin, collagenase, and the toxic glycoprotein were tried. Trypsin caused similar effects as chymotrypsin. The toxic glycoprotein caused relaxation or prevented contraction; however, this was reversible. Collagenase had no effect.

The rate of relaxation by chymotrypsin on adenosine 5′-triphosphate-contracted fibers increased linearly with the applied enzyme concentration (Fig. 2). This rate was also dependent on the degree of tension of the fiber (Fig. 3). The crystalline enzyme had less effect than the various pharmaceutical forms when compared at equal activities.

Effect of Chymotrypsin on Smooth Muscle—Crystalline chymotrypsin and its pharmaceutical forms caused loss of tone, tension, and peristaltic movements of the tensed strip of gut. Chymotrypsin also prevented the development of tension caused by microgram quantities of histamine or serotonin (Table I). This inhibition increased at higher enzyme levels.

Ultrasonic treatment was shown to cause altered physiological properties for some proteins (12). To see whether the effects of chymotrypsin could be altered, the crystalline enzyme and its phar-

[6] Grass FT.

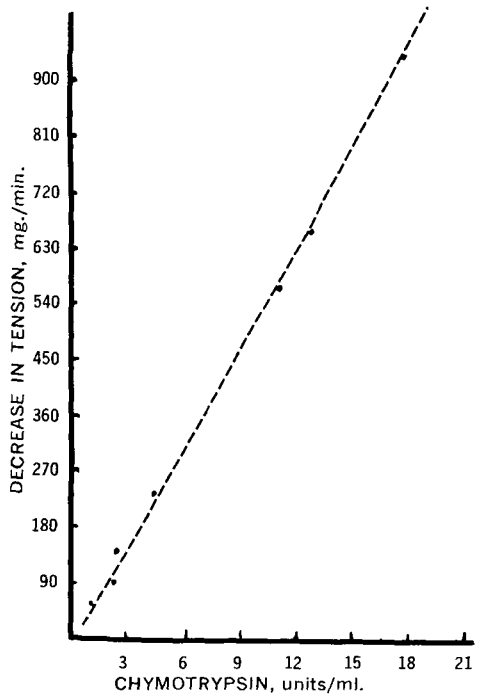

Figure 2—*Relaxation of adenosine 5-triphosphate-contracted muscle fiber with chymotrypsin. The abscissa indicates the final concentration in units of chymotrypsin per milliliter. The ordinate indicates the rate of relaxation, in milligrams per minute decrease in tension, caused by the enzyme.*

maceutical forms were subjected to ultrasonic vibration. Chymotrypsin lost its inhibitory activity in the guinea pig gut system (Table I). Surprisingly, the enzyme activity on *N*-acetyl-l-tyrosine ethyl ester increased rather than decreased (Table II).

Effect of Ultrasonic Frequencies on Chymotrypsin—The effect of ultrasonication on some physicochemical characteristics of the

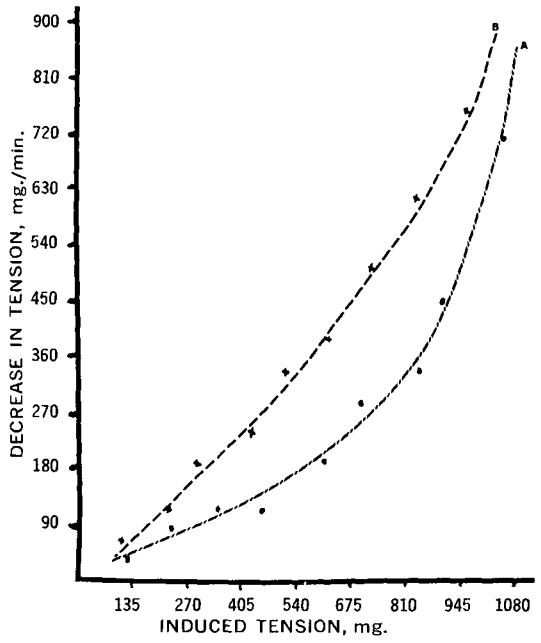

Figure 3—*Relaxation of adenosine 5'-triphosphate-contracted fiber with 3 units of chymotrypsin. The degree of tension shown on the abscissa was developed with adenosine 5'-triphosphate. The ordinate shows the rate of relaxation of the fiber, in milligrams per minute decrease in tension, caused by 3 units of chymotrypsin. Curve A shows the effect of 3 units of 3x crystallized enzyme. Curve B shows the effect of 3 units of chymotryptic activity in the purified enzyme concentrate.*

Table I—Effect of Chymotrypsin on Guinea Pig Gut Response to Histamine and Serotonin[a]

Ultrasonication Period, min.	Preparation	Concentration, mcg.	—Response to— Histamine, %	Serotonin, %
—	0.9% NaCl	—	100	100
—	Chymotrypsin	0.1	51	62
—	Chymotrypsin	0.3	29	44
—	Chymotrypsin	0.5	1	6
5	Chymotrypsin	0.5	88	91
5	Chymotrypsin	10.0	84	93
5	Chymotrypsin	100.0	79	90
15	Chymotrypsin	0.5	99	99
15	Chymotrypsin	10.0	95	95
15	Chymotrypsin	100.0	86	89
—	P.E.C.[b]	0.1	68	67
—	P.E.C.	0.3	50	54
—	P.E.C.	0.5	33	42
—	P.E.C.	1.0	17	22
—	P.E.C.	10.0	4	6
5	P.E.C.	0.5	82	88
5	P.E.C.	10.0	73	82
5	P.E.C.	100.0	72	76
15	P.E.C.	0.5	93	97
15	P.E.C.	10.0	87	91
15	P.E.C.	100.0	84	90

[a] Chymotrypsin (5 mcg./ml.) was added to 10 ml. incubation medium containing strips of gut and allowed to incubate at 26° for 2 min. Then 0.10 ml. of 0.05 mcg./ml. of histamine dihydrochloride or serotonin (adjusted to pH 8.2) was added. Tension was monitored with a model Grass polygraph with 7 P 1 preamplifier and recorded. The experiments were run in triplicate. The 0.1 ml. of histamine solution added to the 10 ml. incubating medium produced 415, 478, and 505 mg. tension in three separate experiments. The 0.10 ml. of serotonin solution added to the incubating medium resulted in 225, 247, and 238 mg. tension in three separate experiments. [b] P.E.C. is purified enzyme concentrate.

enzyme was also tested. The ultrasonicated enzyme gave one single sharp protein band; it gave one sharp homogeneous peak when resolved by polyacrylamide gel electrophoresis and when passed through a diethylaminoethane chromatography column, and it sedimented on the analytical ultracentrifuge as a single homogeneous peak with a velocity of $S_{20,w}$ of 2.5 *S*.

Brief periods of ultrasonication (1, 2, 5, or 15 min.) of the purified enzyme concentrate caused an approximate 80% increase in its specific enzyme activities if assayed against *N*-acetyl-l-tyrosine ethyl ester or *N*-benzoyl-l-arginine ethyl ester. When resolved by polyacrylamide gel electrophoresis, the ultrasonicated purified enzyme concentrate gave two protein bands, and it produced two peaks

Table II—Effect of Ultrasonic Frequencies on Chymotrypsin Hydrolytic Activities

Ultrasonication Period, min.	Preparation	—Hydrolytic Activities on— BAEE[a], units/mg.	ATEE[b], units/mg.
0	Chymotrypsin	45	1072
1	Chymotrypsin	60	1638
2	Chymotrypsin	76	2077
3	Chymotrypsin	82	2278
5	Chymotrypsin	85	2345
10	Chymotrypsin	58	1675
15	Chymotrypsin	51	1340
20	Chymotrypsin	48	1265
30	Chymotrypsin	43	1065
0	P.E.C.[c]	2650	495
1	P.E.C.	3135	615
2	P.E.C.	3265	725
3	P.E.C.	3455	795
5	P.E.C.	3935	965
10	P.E.C.	3115	610
15	P.E.C.	2950	565
20	P.E.C.	2715	505
30	P.E.C.	2545	475

[a] BAEE is *N*-benzoyl-l-argenine ethyl ester. [b] ATEE is *N*-acetyl-l-tyrosine ethyl ester. [c] P.E.C. is purified enzyme concentrate.

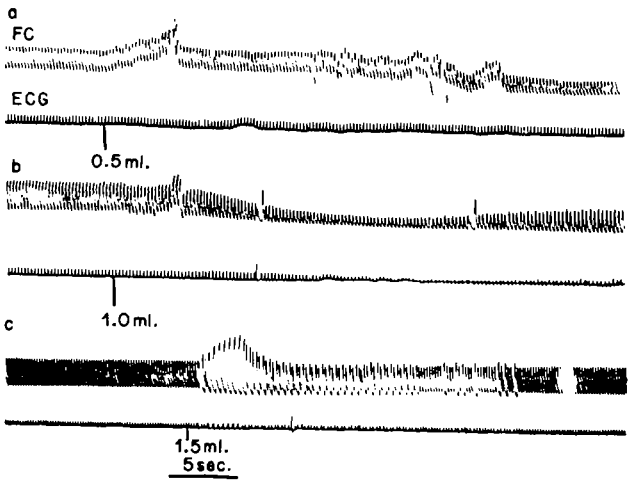

Figure 4—*Action of chymotrypsin on cardiac muscle—tracings of the response to* in vitro *perfusion of rat heart with a sterile chymotrypsin solution with 5000 units/ml. Key: a, tracing of the response to 0.5 ml. of the sterile chymotrypsin solution diluted 1:100 with 0.9% sodium chloride; b and c, the responses to 1.0 and 1.5 ml. of the diluted sterile chymotrypsin solution, respectively. FC is the tracing of force of contraction. ECG is the tracing of the cardiac electrical signals. The chart speed was 10 mm./sec.*

when passed through a diethylaminoethane chromatography column. These results were similar to the properties of the enzyme preparation prior to exposure to ultrasonic frequencies.

Effect of Chymotrypsin on Cardiac Muscle—Chymotrypsin in a pharmaceutical product[2] caused a series of abnormalities in cardiac activities. The effects caused by the product increased with the amount of the perfused enzyme. At 0.5 ml., the product caused an immediate short-term increase in the force of contraction, followed by a decrease in cardiac tone and rate (Fig. 4). At levels of 1.5 ml. and above, the pharmaceutical product caused a noticeable slowing in the cardiac rate accompanied by abnormal contractions leading to an enhanced heart failure.

DISCUSSION

Glycerol-extracted rabbit psoas muscle fibers, prepared by the method of Szent-Gyorgyi (10), contain all the contractile elements and exhibit most of the key functional characteristics of intact muscle. Addition of adenosine 5′-triphosphate to the fiber bathing medium causes development of tension for such muscle fibers (16).

Chymotrypsin similar to trypsin inhibited the formation of adenosine 5′-triphosphate-induced tension when fibers were treated with the enzyme prior to adenosine 5′-triphosphate. Morita (17, 18) found that addition of adenosine 5′-triphosphate causes configurational changes associated with the active sites of the myosin molecule which temporarily buries some tyrosyl and tryptophyl chromophores. Further studies are needed to show that the enzymes, chymotrypsin or trypsin, did hydrolyze these bonds and thus altered the configurational changes necessary for development of tension. In cases of adenosine 5′-triphosphate-contracted fibers, chymotrypsin caused an irreversible relaxation of the muscle fibers.

When actomyosin was interacted with 2% of trypsin at 25° for 20 min., heavy actomeromyosin was produced (19–21). Under these conditions, trypsin caused the splitting of myosin A into light meromyosin and heavy meromyosin without splitting the complex with actine (18). The enzyme-specific hydrolytic activity of chymotrypsin differs from trypsin (it hydrolyzes different peptide bonds), but both enzymes are proteolytic-esterolytic in action. Collagenase, a peptidase, did not cause the relaxing effect. These observations suggested that the action of chymotrypsin on the adenosine 5′-triphosphate-contracted fiber might be related to the catalytic specificity of the enzyme.

Earlier studies (16) showed that ultrasonic treatment of purified 95% clottable protein, human fibrinogen, altered its physiological properties without appreciable changes in the sedimentation rate, electromobility, or chromatographic behavior of the native clottable protein. Ultrasonic frequencies were shown also to alter the physicochemical characteristics of human serum (22–24). The data presented in this article indicate that the physiological action of chymotrypsin or the purified enzyme concentrate on smooth muscle was caused either by specific enzyme activities or by a contaminating factor present in the chymotrypsin preparation employed. Since exposure to ultrasonic frequencies reduced the physiological action of chymotrypsin on muscular contraction while enzyme activity increased, the possible role of a contaminating factor in the enzyme preparation is favored. Further studies are in progress to determine the nature of the factor.

Chymotrypsin in a pharmaceutical product[2] caused a series of abnormalities in cardiac activities. The effects caused by the product with a single passage through the heart were unpredicted because of the low enzyme concentration perfused and the short period that the enzyme solution remained in the cardiac muscle. The effects of recirculation of the enzyme solution through the heart could yield more permanent effects on the cardiac activities. Further studies along these lines are in progress.

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