yield decisive information in this regard.

Summary and conclusions. 1. Studies have been made of the urinary and fecal excretion of coproporphyrins I and III administered intravenously to rats. In the normal animal, no increase in urinary excretion occurred; approximately one-half of the injected material was excreted in the feces. 2. Coproporphyrin administered intravenously to rats was rapidly excreted in the bile via an external biliary fistula, or in the urine when complete biliary obstruction existed. Orally administered coproporphyrin did not appear to be absorbed, and approximately half was recovered from the feces. Regardless of the route of administration, only half of the coproporphyrin could be recovered. 3. In acute liver injury due to carbon tetrachloride, intravenous administration of coproporphyrin resulted in an increased urinary porphyrin excretion, with a relative reduction in the amount excreted in the feces. 4. In rats with chronic liver injury due to a choline-deficient diet virtually none of the injected porphyrin could be recovered in either urine or feces. The reason for this is unknown. 5. Rats made anemic by acute bleeding exhibited normal (*i.e.*, increased) fecal excretion of coproporphyrin III, following its intravenous administration.

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Proteolytic, Hypotensive and Thromboplastic Properties of Hypochlorite Treated Trypsin.* (20319)

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It is well known that the parenteral administration of trypsin induces a reaction similar to that of an antigen-antibody response with a sharp fall in blood pressure resulting from intravenous administration(1). That the inhibition of the proteolytic activity of the enzyme does not necessarily diminish its hypotensive effect was pointed out earlier(2). It was also reported that iodination(3,4) and treatment with formaldehyde(2) serve to decrease the hypotensive activity of trypsin more than its proteolytic or thromboplastic effects. Since the proteolytic and hypotensive properties are separable more advantageous means of accomplishing this separation were

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sought. This was done, in part, with the view of attempting to improve the enzyme as a possible agent for the elimination of protein debris and the digestion of fibrin *in vivo*.

The present report deals with proteolytic, hypotensive and thromboplastic properties of trypsin treated with sodium hypochlorite(5). In a subsequent report its use in combating fibrosis and the promotion of healing will be described.

Methods. Sodium hypochlorite was added to a 0.4% solution of commercial trypsin. After standing 18 hours at 5°C the solution was dialysed in distilled water at 5°C for 24 hours and either lyophilized or precipitated with 80% ethyl alcohol, washed in absolute alcohol and dried *in vacuo*. In order to minimize denaturation of crystalline enzymes 90 ml of a cold solution of NaOCl was added to 0.25 g of the enzyme in 10 ml of cold water. After 5 hours at 5°C the preparation was dialysed at the same temperature in a mechanical dialyser for 4 hours and lyophilized. After dialysis tests for available chlorine were negative. Proteolytic activity was usually estimated by the procedure described by Kunitz(6) based on the increase in optical density of a filtrate of a casein digestion mixture. Changes in proteolytic activity were estimated by determining the amount of the treated enzyme required to cause the same change in optical density as a given amount of untreated trypsin with the same solution of casein. The relative proteolytic activity of acetone treated plasma due to added protease was estimated by an adaptation of a method of Schmitz(7). One ml of plasma was precipitated in 95% acetone. The precipitate was washed with acetone, centrifuged and dried in vacuum. The precipitate was thoroughly ground with the aid of a glass rod in the centrifuge tube and one ml of 0.08 M phosphate buffer, pH 7.4, containing 10 mg of merthiolate per 100 ml was added. After standing at 5°C for one hour with the rod remaining in the tube the contents were thoroughly mixed and 9 ml of buffer added. The tubes were stoppered and incubated at 38°C for 48 hours. After precipitating with 10% trichloroacetic acid the non-protein nitrogen of the filtrate was estimated by the micro-Kjeldahl procedure. Values given were corrected for the original non-protein nitrogen and that due to the activity of the partially activated natural protease of the plasma. All glassware coming into contact with the blood or plasma was siliconed. Hypotensive activity was estimated employing dogs and the usual methods of carotid cannulation and recording. After the repeated initial injection of a standard fixed dose of 0.1 to 0.5 mg of untreated trypsin per kg a reasonably reproducible response was obtained in a given dog with a return to the control blood pressure level following each injection. Changes in hypotensive effect were determined by observing the amount of the treated enzyme required to produce approximately the same degree and duration of decreased blood pressure as a standard dose of untreated trypsin injected before and after the treated enzyme. In observing coagulation time plasma containing 1.34 mg of sodium oxalate per ml was diluted with 2 ml of saline. To 0.5 ml of diluted plasma at 38°C was added 0.5 ml of a solution prepared by adding 2 ml of saline or enzyme solution to 5 ml of 0.222% $CaCl_2$. The tubes were gently tilted 40 times per minute at 38°C until gel formation was observed and verified by turning the tube horizontally. The plasma was obtained from blood drawn from a cannulated carotid artery discarding the first blood obtained. After centrifuging at 15,000 rpm for 30 minutes at 18°C the plasma was drawn off to within one cm of the cells. All glassware was siliconed. The term trypsin refers to commercial trypsin unless otherwise indicated. Rabbits were used in most of this work because of the marked susceptibility of this species to the thromboplastic effect of trypsin and trypsin embolism.

Results. Suitable amounts of hypochlorite markedly reduce the hypotensive activity of commercial trypsin while ability to digest casein is actually increased (Table I). With larger amounts of hypochlorite proteolytic activity is also diminished. In a large series of preparations such changes have been relatively reproducible. In spite of efforts to minimize denaturation of crystalline chymotrypsin and crystalline trypsin marked loss o

TABLE I. Influence of Sodium Hypochlorite on Hypotensive and Proteolytic* Activities of Trypsin and Chymotrypsin.

g NaOCl/ g enzyme	% of control activity: Commercial trypsin Hypotensive	Commercial trypsin Proteolytic	Crystalline trypsin Hypotensive	Crystalline trypsin Proteolytic	Crystalline chymotrypsin Hypotensive	Crystalline chymotrypsin Proteolytic
.007			50	49	50	60
.014			33	39	22	45
.028	5	143	16	19	12	36
.055	5	150	†	11	11	17
.11	4	145	†		†	
.22	3	108	†	Inactive	†	Inactive
.44	.75	25				

* Casein digestion.
† Low solubility prevented preparation of solutions practical for inj.

solubility with probable serious degradation resulted from hypochlorite treatment. However, to some extent crystalline chymotrypsin undergoes relative changes similar to those of commercial trypsin. Approximately equal losses in hypotensive and proteolytic activities occurred in crystalline trypsin. Effort is being made to further minimize the possible obscuring effect of denaturation.

When added to plasma *in vitro* hypochlorite treated trypsin imparts greater proteolytic activity to the acetone precipitate of the plasma than does untreated trypsin (Table III). The activity of such an acetone precipitate has been attributed to free protease of the plasma by some investigators; however, proteolytic activity of the precipitate is routinely observed with plasma having a net inhibitor effect prior to precipitation. We have used acetone precipitation as a reasonably reproducible means of revealing content of readily activated protease. Subsequent further treatment with 10% trichloroacetic acid, chloroform and streptokinase in this order results in progressively greater activity. That the mechanism of the action of hypochlorite may be to a large extent oxidative in nature is indicated by the differential effect of hydrogen peroxide on the proteolytic and hypotensive activities of commercial trypsin (Table II). Treatment with peroxide was similar to that with hypochlorite.

Spontaneous changes in trypsin which is allowed to stand in aqueous solution for 5 to 25 hours at 5 to 37°C also involve greater loss of hypotensive activity. In some cases the latter may fall to 20% of the original level

TABLE II. Influence of Hydrogen Peroxide on Hypotensive and Proteolytic Activities of Commercial Trypsin.

g H_2O_2/g trypsin	% of control activity: Hypotensive	Proteolytic
.075	33	162
.15	29	144
.225	20	137
.33	15	130

TABLE III. Increase in Proteolytic Activity of Acetone Treated Rabbit Plasma Following Addition of Trypsin and Hypochlorite Treated* Trypsin *in Vitro*.

Material added	Amt added to plasma, mg/ml	Increase in N.P.N. in digestion mixture due to activity of added enzyme, mg/100 ml
Trypsin, untreated	.10	7.4
	.20	15.9
	.08	10.3
Trypsin, hypochlorite treated	.10	20.1
	.20	27.3

* .11 g NaOCl/g trypsin.

TABLE IV. Thromboplastic Activity of Hypochlorite Treated Commercial Trypsin *In Vitro*.

mg enzyme/ ml plasma	% of control coagulation time
.00025	85
.001	68
.002	50
.012	22
.020	15
.072	7.4
.010	6.7
.020	6.5
.024	5
.048	4
.072	6
.096	5
1.3	Incoagulable

TABLE V. Influence of Heparin on Trypsin Embolism in Rabbits.

No. of animals	Substance inj.*	No.	No. with gross emboli	Amt of trypsin inj., mg/kg		
				Avg total	Max	Min
		Fatal cases				
44	Trypsin	40	38	8	19	2
20	Cl-trypsin	14	14	18	31.5	3
20	Cl-trypsin and heparin	0				
		Sacrificed cases				
44	Trypsin	4	0	6	6	6
20	Cl-trypsin	6	3	23.3	24	20
20	Cl-trypsin and heparin	20	0	25	25	25

* Each substance inj. at rate of 1 mg/kg/15 min.

while the proteolytic activity is altered very little; however, such changes are unpredictable and difficult to reproduce.

Trypsin treated with 0.11 g NaOCl per g of enzyme has marked thromboplastic activity when added to plasma *in vitro* in suitable amounts (Table IV). An excess interferes with blood coagulation through digestion of fibrinogen as indicated by the subsequent addition of thrombin. Except in concentrations causing maximal clot acceleration the coagulation time is approximately equivalent to that obtained with 50% more untreated trypsin.

The coagulation of plasma obtained from rabbits after injecting hypochlorite treated trypsin is also accelerated. In a typical case 5, 60, and 180 minutes after injecting 2.5 mg/kg of plasma coagulation times were respectively 22, 33, and 49% of the control time. However, as with untreated trypsin intravenous administration involves considerable risk of fatal embolism in rabbits. Such embolism is prevented by the simultaneous injection of an equal amount of heparin (Table V).

As estimated by the non-protein nitrogen procedure the injection of 25 mg/kg of treated trypsin at the rate of 1 mg/kg every 15 minutes with an equal amount of heparin resulted in an increase in proteolytic activity of acetone precipitated plasma which was slightly greater than that observed upon adding 0.1 mg of the treated enzyme alone to 1 ml of plasma *in vitro*.

Heparin does not alter the hypotensive effect of hypochlorite treated trypsin and in a 1:1 ratio, at pH 7.4, does not diminish the digestion of casein. However, with the same proportions and pH, heparin decreased the proteolytic activity of acetone precipitated plasma containing 0.1 mg of treated trypsin per ml to about half of the non-protein nitrogen index obtained without heparin.

Even with the injection of 25 mg of hypochlorite treated trypsin in rabbits at the rate of 1 mg/kg every 15 minutes the plasma trypsin inhibitor level remains essentially unchanged.

Summary. Treatment of commercial trypsin with suitable amounts of sodium hypochlorite greatly diminishes the hypotensive effect of the enzyme while its proteolytic activity is increased by approximately 50%. Similar changes result from treatment with hydrogen peroxide. Hypochlorite treated trypsin possesses a marked thromboplastic effect. Heparin prevents fatal embolism which otherwise results in a high percentage of cases following the intravenous injection of treated trypsin in rabbits. An increase in the readily activated protease fraction of plasma is observed after adding the treated enzyme to plasma *in vitro* and after injecting it intravenously.

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