teins, although the possibility exists that the hydrocarbons interacted with the -SH or S-S groups of the proteins in a manner which prevented the detection of these groups by the analytical procedures which we employed. The binding of the hydrocarbons to the proteins, particularly in liver, could conceivably be reflected in the alteration of their metabolic availability to other organs and in the protein content of these organs.

Summary. Phenacyl bromide, p-chlorobromobenzene, or naphthalene tetrachloride inhibit growth of rats which ingested an 8% casein diet. The inhibition was alleviated by supplementary cystine, or by a 27% casein diet. Nitrogen balance remained positive in rats which ingested the 27% casein diet or the 8% casein diet which was supplemented with cystine. Intraperitoneal injection of naphthalene tetrachloride into adult rats on the high or low protein diet induced a negative nitrogen balance, accompanied by a decrease in the -SH and S-S content of the whole blood and in the protein content of practically all tissues. The implication of these observations is discussed in terms of possible interference of the hydrocarbons with the protein metabolism of the rat via direct binding of the hydrocarbons to the proteins through the -SH or S-S groups.

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Coagulation-Promoting and Inhibitory Properties of Modified Thrombin Preparations.‡ (22780)

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The fibrinogen-clotting activity of thrombin is known to be destroyed by heating above 60°C(1). As will be shown here, such heated preparations have profound effects on various phases of the coagulation mechanism.

Materials and methods. Tropical thrombin;* bovine fibrinogen, Fraction I;† human plasma; bovine plasma; human platelet material; degradation products of thrombin. Human plasma was prepared from normal blood which had been treated with a solution of sodium oxalate (1 part of 0.1 M oxalate solution in water to 9 parts of blood) and was centrifuged at 32,000 g for 30 minutes at room temperature. Bovine plasma was prepared in a similar manner. Human platelet material was prepared by the method described previously(2). Inhibitors of thrombin were prepared by heating solutions of thrombin in isotonic saline in the waterbath for varying periods at temperatures ranging from 40°C to 100°C. In most of the work reported here, the material studied was ob-

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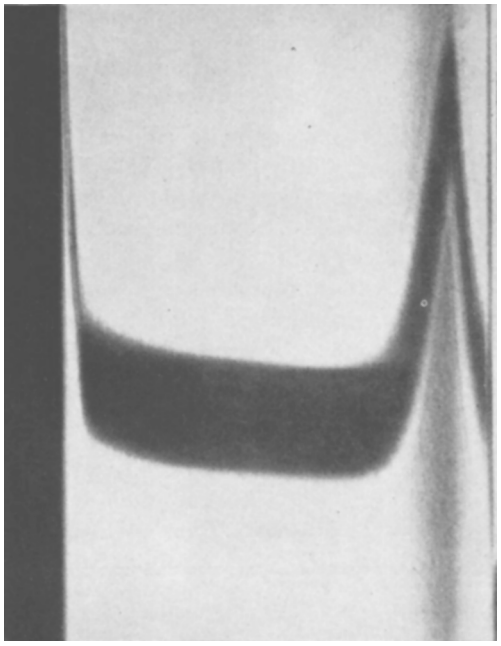

FIG. 1. Ultracentrifugal pattern of supernatant obtained from boiled thrombin preparation.

tained by heating thrombin at 100°C for 30 minutes. The thick coagulum which formed was removed by centrifugation. The supernatant (B.T.) was precipitated with 9 volumes of 95% ethyl alcohol. The white precipitate (A.T.) was collected by centrifugation, dried *in vacuo* and redissolved in water, physiological saline or imidazole buffer at a pH of 7.4. The activity obtained by treating one unit of thrombin as described above will be referred to as one unit of B.T. or A.T., respectively.

The following determinations were carried out on plasma to which B.T., A.T. or a blank (saline or buffer) had been added: recalcification time according to a modification(3) of the method of Quick(4); thromboplastin generation test(5) modified as described previously(6). The thrombin time was determined by forcefully ejecting 0.1 cc of thrombin solution into test tube (7 mm) containing 0.1 cc of buffer or solution of heated thrombin preparation and 0.2 cc of a solution of 400 mg % fibrinogen in imadazole buffer. The first appearance of a fibrin thread was taken as the end-point. Thrombin generation, estimated by the method previously described (7), was modified in the following manner. 4 cc of a 0.25 M solution of calcium chloride in water were added to 2 cc of oxalated plasma at 37°C. Aliquots of 0.1 cc of this mixture were aspirated into tuberculin syringes containing 0.1 cc of B.T., A.T. or a

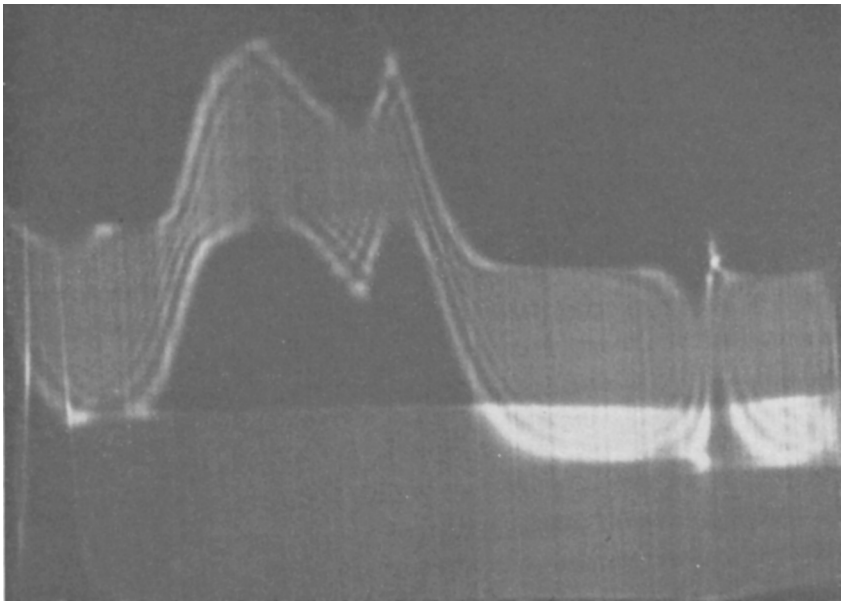

FIG. 2. Free boundary electrophoretic pattern of supernatant obtained from boiled thrombin preparation.

TABLE I. Effect of Varying Concentration of A.T. on Recalcification Time of Bovine Plasma.

A.T. units*	Clotting time, sec.
0	157
1	165
10	172
100	176
500	192
1000	265
5000	600
10,000	600

* A.T. = Alcohol precipitate. One unit of A.T. is the amount obtained from one unit of thrombin.

blank at minute intervals. The contents of the syringe were ejected forcefully into test tube (7 mm) containing 0.2 ml of a solution of 400 mg% fibrinogen in buffer. The tube was agitated by manual rotation at 37°C. The first appearance of fibrin strands was recorded as the end-point.

Results. Chemical characteristics: Activities in supernatant (B.T.) obtained after centrifugation of heated bovine thrombin solution, and the precipitate (A.T.) obtained by adding ethyl alcohol to B.T. were non-dialyzable, contained peptide bonds (Biuret test), carbohydrate (Orcinol test) and amino sugars (Morgan Elson reaction). Free reducing groups were not detected. In the analytical ultracentrifuge, a uniform peak is observed (Fig. 1) which differs markedly from that given by the starting material. On free boundary electrophoresis a pattern is obtained which resembles the starting material in terms of the general relation of the major peaks to each other, but the rate of migration is considerably reduced in the modified preparation (Fig. 2).

The yield of A.T. amounts to approximately 5% of the weight of the starting material.

Coagulation studies: High concentrations of heated thrombin or the alcohol precipitate inhibited the clotting of recalcified bovine plasma. Acceleration was not observed at any concentration of these materials (Table I).

Coagulation of recalcified human plasma, however, was accelerated by B.T.‡ or A.T.§ except at high concentrations, which may cause inhibition. Low concentrations were ineffective. (Table II). It appears that these effects are more marked with stored plasma.

TABLE II. Effect of Varying Concentrations of A.T. on Recalcification Time of Human Plasma.

Duration of storage of plasma	Control (Imidazole buffer)	500 units* of A.T.†	250 units of A.T.	125 units of A.T.	62.5 units of A.T.	31 units of A.T.
	(sec.)					
Fresh plasma	220	485	310	175	210	240
Stored plasma (24 hr)	185	215	100	97	125	155
" " (1 wk)	240	305	270	186	197	236
" " (2 ")	190	115	115	90	130	150
" " (3 ")	160	118	115	110	128	—

* 1 unit of A.T. is the amount obtained from 1 unit of thrombin.
† Alcohol precipitate.

It can be seen from Table III that the accelerating activity of B.T.‡ decreases as the lengths of time for which it is kept at 100°C increases. Addition of calcium ion leads to partial reactivation.

TABLE III. Effect of Varying the Period for Which Thrombin Is Heated at 100°C on Activity of A.T. in the Clotting of Human Plasma. (Control without addition of thrombin derivative—347 seconds.)

Time of heating thrombin at 100°C, min.	Clotting times (sec.) Without added calcium	With added calcium
0	1	1
5	85	30
10	116	57
20	600	105
30	600	132
60	600	210

‡ Supernatant obtained from heated thrombin preparation.

§ Alcohol precipitate from supernatant of heated thrombin preparation.

TABLE IV. Effects of Pre-incubating Human Plasma with 300 Units of A.T. for Varying Periods Prior to Addition of Ca^{++}.

Time of pre-incubation, min.	Clotting times, sec.
0	190
2	18
4	9
6	5
10	2

Rate of thromboplastin generation was increased by B.T.‡ or A.T.§ except at high concentration of these materials, which caused inhibition. (Table V).

TABLE V. Effect of Varying Concentration of A.T.* on Generation of Thromboplastin in Human Plasma.

Time of incubation of mixture, min.	Control	A.T.* 300 units†	A.T. 60 units	A.T. 10 units
	(sec.)			
1	88	58	42	50
3	43	30	12	12
4	—	—	11	11
5	13	18	11	11
6	10	17	11	11
7	10	16	11	12
8	11	15	12	—
9	—	16	—	—

* Alcohol precipitate obtained from supernatant of heated thrombin preparation.
† One unit of A.T. is the amount obtained from one unit of thrombin.

Inhibitory effects of heated thrombin preparations on coagulating activity of native thrombin. When bovine thrombin was used in "thrombin time" determinations on human and bovine plasma, its coagulating activity was inhibited by B.T.‡ and A.T.§

TABLE VI. Effect of Varying Concentration of A.T.* on Activity of Bovine Thrombin Added to Human and Bovine Plasma.

A.T. units*	Human plasma, sec.	Bovine plasma, sec.
10,000	—	255
5000	—	160
1000	47	28
500	38	25
100	29	19
10	25	16
1	25	16
0	25	16

* Alcohol precipitate obtained from supernatant of heated thrombin preparation. One unit of A.T. is the amount obtained from one unit of thrombin.

In the thrombin generation test, B.T.‡ and A.T.§ produced inhibition in the bovine test system, but not in the human system. Representative findings are tabulated in Table VII.

TABLE VII. Effect of A.T. on Activity of Thrombin Generated in Bovine and Human Plasma.

Time of incubation, min.	Bovine plasma		Human plasma	
	Control	500 units* A.T.† added	Control	500 units* A.T.† added
	(sec.)			
2	205	—	212	240
3	63	—	135	173
4	30	313	43	38
5	30	240	32	30
6	24	176	30	26
7	24	99	30	25
8	24	72	25	25
9	25	65	25	22
10	29	69	30	25
12	—	75	30	27

* One unit of A.T. is the amount obtained from one unit of thrombin.
† Alcohol precipitate obtained from supernatant of heated thrombin preparation.

Discussion. The exposure of thrombin to temperatures above 60°C has been found to lead to loss of its ability to clot fibrinogen. The data presented here refer to the alcohol precipitate obtained from the supernatant of heated bovine thrombin preparations, but essentially the same results were obtained when the supernatant prior to the addition of alcohol was used. The data indicate that the redissolved alcohol precipitate (AT) obtained from heated thrombin solution has a number of effects on the various stages of the coagulation mechanism. None of these effects is due to a true thrombin activity, as evidenced by failure to clot fibrinogen. 1. The effects of the alcohol precipitate obtained from heated thrombin preparations on the recalcification time may be related to the increased rate of the generation of thromboplastin. This aspect of thrombin activity appears to differ qualitatively from its coagulant effects on fibrinogen and is more stable than the latter. 2. The effects of incubation of heated thrombin preparations with plasma prior to the addition of calcium are not understood. 3. The alcohol precipitate obtained from solutions of heated thrombin preparations inhibited bo-

vine thrombin. This inhibitor appears to be different from the antithrombic activities described so far(8). It should be noted that the considerably lower concentrations of thrombin derivatives prepared from bovine thrombin were sufficient to inhibit the latter, than were required to inhibit thrombin. This species-specific inhibitory effect may be related to differences between bovine and human thrombin and to the ease with which the respective enzymes can be inhibited by the same inhibitor. On the other hand, the possibility may also be considered that the modification of an enzyme may result in a derivative which will inhibit preferentially the specific enzyme from which it has been prepared.

The heated thrombin preparations and the redissolved alcohol precipitates have been administered intravenously to rabbits and guinea pigs without toxic or thromboembolic sequelae.

Summary. An alcohol precipitate obtained from heated bovine thrombin preparations promoted the early phases of the coagulation mechanism, but did not clot fibrinogen in the manner of the unheated preparation. These materials inhibited bovine thrombin at lower concentrations that human thrombin in the clotting of fibrinogen.

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Antifibrinolytic Activity of Derivatives of Fibrinolysin.‡ (22781)

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Fibrinolysin and its inhibition and activation have been intensively investigated in recent years in relation to coagulation and hemostasis. The clinical investigation of bleeding patients(1) as well as the fractionation of lyophilized human platelet material suggested the presence of an inhibitor of fibrinolysin in these materials. Antifibrinolysin, moreover, has been demonstrated previously in bovine platelets(2).

In the search for specific inhibitors of fibrinolysin, an attempt was made to produce an inactive derivative which would specifically inhibit the enzyme.

Materials and methods. Bovine fibrinolysin* was suspended in water, saline or buffer (imidazole pH 7.2), and heated at 90°C for periods varying from 5 to 30 min. Coagula formed during the heating were removed by centrifugation at 3,000 g for 10 min. The supernatant (M.F.) was precipitated with 3 volumes ethyl-alcohol to yield a white, water-soluble material (MFP). The amount of MFP obtained was approximately 4.5% of the weight of the starting material. The fibrin-plate method previously described (3) was used for the determination of fibrinolytic activity. The method was modified, however, in regard to times and temperatures of incubation. Human fibrinolysin activity was obtained by activating normal human serum with Streptokinase (Varidase).*

Results. The soluble material remaining after heating bovine fibrinolysin at 90°C was

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