mg of platelets from Et_2O–hexane at −5°, mp 125–129°. The melting point, ir spectrum, and pc (system 1) showed this to be identical with dimethyl terephthalate produced synthetically. The distillate (3.7 mg) obtained at a bath temperature of 100–120° yielded approximately 2 mg of crystals from Et_2O–hexane, mp 173–176°. According to the ir spectrum, this substance was identical with the free base of reference substance **6**.

In a second experiment, 1.28 g of the fraction extracted from urine with $CHCl_3$–EtOH (4:1) was separated preparatively in chromatographic system 3. Examination of the distribution of radioactivity in the layers revealed that about 60% was localized in a band between R_f 0.38 and 0.56.[16] This band was divided into zone A with R_f 0.38 to 0.47 and zone B with R_f 0.47 to 0.56; these zones were removed from the plate separately and extracted exhaustively with MeOH. On evaporating to dryness, zone A yielded 66.7 mg (202,000 cpm) and zone B yielded 57.1 mg (167,000 cpm). The residue from zone B was taken up in 1 ml of MeOH, acidified with HCl, then esterified by adding an excess of ethereal CH_2N_2. After concentrating to the consistency of a syrup, it was possible to separate an oil that was sparingly soluble in MeOH. The soluble fraction yielded from MeOH–Et_2O 1.5 mg of yellow crystals and on recrystallization from MeOH–Et_2O colorless platelets, mp 198–208°. According to melting point, mixture melting point, tlc, and ir spectrum, this compound was identical with **6** prepared synthetically. Similarly, 1.0 mg of colorless platelets was obtained from zone A, mp 192–199°, which, according to the same criteria, was identical with reference substance **6**.

Human Experiments. Investigations of the Urine of Humans.—A healthy male trial subject (70 kg) received 60 mg of pyrovalerone·HCl orally. In the subsequent 32 hr the urine was collected in the following fractions which were kept at −5° until used for processing: fraction 1, 0–5 hr; fraction 2, 5–11 hr; fraction 3, 11–25 hr; fraction 4, 25–32 hr.

In each case 500-ml aliquots of these fractions were adjusted with concentrated HCl to pH 2.0–2.5 and concentrated under vacuum at 50° to 40–50 ml. After cooling, a precipitate was filtered off and the filtrate extracted three times with double the volume of $CHCl_3$; the organic phases were combined, dried (Na_2SO_4), and then evaporated to dryness. The residues from each fraction were taken up in a little MeOH and chromatographed in system 4 together with pyrovalerone·HCl as reference substance. In no extract was it possible to detect unchanged pyrovalerone.

The aqueous solutions were then extracted five times with double the volume of $CHCl_3$–EtOH (4:1); the organic phases were combined, dried (Na_2SO_4), and carefully evaporated to dryness. The residues from each fraction were dissolved in a little MeOH and treated with an excess of Et_2O–CH_2N_2. After standing for 24 hr at −5° and for 2 hr at 20–25°, the reaction mixtures were partitioned between Et_2O and 0.1 *N* NaOH. The Et_2O solutions were washed three times with a little H_2O, dried (Na_2SO_4), and evaporated to dryness. In each case, a sample of the residue was chromatographed in system 4. It was evident that the material sought (**6**) was present only in traces in fraction 3 and was absent in fraction 4.

The residues of fractions 1 and 2 were dissolved in a little MeOH, and separated preparatively in chromatographic system 4. Zones around R_f 0.32 were scraped off carefully, placed in small chromatography columns, and eluted with MeOH After evaporating to dryness, the eluates were taken up in Et_2O, washed three times with 1 *N* NH_4OH and once with H_2O, dried (Na_2SO_4), and evaporated to dryness. The residues were dissolved in a little MeOH, converted into the hydrochloride with 15% alcoholic HCl, and crystallized from MeOH–Et_2O. Fraction 1 yielded *ca.* 4 mg of colorless needles, mp 191–193°, and fraction 2 yielded *ca.* 3 mg of colorless needles, mp 193–194°. Ir spectroscopy (KBr) showed both products to be identical with reference substance **6** prepared synthetically.

Acknowledgment.—We wish to thank Mr. E. Schaffer for his careful experimental work.

Structure-Dependent Inhibition by Synthetic Fibrinolytic Anions of Collagen-Induced Aggregation of Human Platelets[1]

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Various organic anions with a large lipophilic moiety induce fibrinolytic activity in human plasma *in vitro*. Some of the fibrinolytic compounds were found to inhibit ADP- and thrombin-induced platelet aggregation. In this study, 38 fibrinolytic congeners were investigated for inhibition of collagen-induced platelet aggregation in human plasma. The parent compounds, anthranilic, salicylic, and thiophene-3-carboxylic acid, had little fibrinolytic and aggregation inhibiting activity; however, unsymmetrical substitution increased both activities. The platelet aggregation inhibition frequently occurred at concentrations at least 10 times lower than those required for fibrinolysis. *N*-(2-Chloro-3-methylphenyl)-4-aminothiophene-3-carboxylic acid was fibrinolytic at 3×10^{-3} *M* and aggregation inhibiting at 5×10^{-5} *M*. The results suggest the theoretical possibility of designing drugs with a dual action: prevention of thrombus formation and induction of thrombolysis.

Many of the compounds which prevent collagen-induced platelet aggregation are nonsteroidal antirheumatic drugs, and it has been suggested that there may be a correlation between antirheumatic activity and aggregation prevention.[3] The antiaggregating antirheumatic drugs are unsymmetrically substituted organic anions. It is the purpose of the present study to determine if related organic anions, which induce marked fibrinolytic activity in human plasma,[4] will also prevent *in vitro* collagen-induced aggregation of human platelets, and if this is the case, to obtain information on the relationship between structure of organic fibrinolytic anions and their ability to prevent collagen-induced platelet aggregation.

Experimental Section

Glassware.—All glassware with the exception of disposable micropipettes was freshly siliconized by vaporized silicone solutions (General Electric Drifilm No. S.C.11994).[5]

Chemicals.—Buffered saline (B.S.): 4 parts of NaCl (0.85%) and 1 part of barbital acetate buffer,[6] pH 7.42. The fibrinolytic

(1) Supported by grants from the American Heart Association and the National Heart Institute (HE 9985).

(2) Student Research Fellow; on leave from the University of Erlangen, Germany.

(3) J. R. O'Brien, *Lancet*, **1**, 894 (1968).

(4) K. N. von Kaulla and G. Ens, *Biochem. Pharmacol.*, **16**, 1023 (1967).

(5) L. B. Jaques, in "The Coagulation of Blood. Methods of Study," L. M. Tocantins, Ed., Grune and Stratton New York, N. Y., 1955, p 3.

(6) L. Michaelis, *Biochem. Z.*, **234**, 139 (1931).

compounds were tested as Na salts. The origin of the compounds indicated in Tables II–V by numbers was as follows: (1) Aldrich Chem. Co., Milwaukee, Wis.; (2) Eastman, Rochester, N. Y.; (3) Dr. C. H. Fuchsman, Ferro Corp., Bedford, Ohio; (4) K & K Laboratories, Plainview, N. Y.; (5) Matheson Scientific, Chicago, Ill.; (6) Dr. J. Meek, Department of Chemistry, University of Colorado, Boulder, Colo.; (7) Dr. J. Sprague, Merck Sharp & Dohme Research Laboratories, Westpoint, Pa.; (8) Miles Laboratories, Elkhart, Ind.; (9) Professor H. Ruschig, Farbwerke Hoechst, Frankfurt-Hoechst, Germany; (10) Dr. C. V. Winder, Parke Davis Research Laboratories, Ann Arbor, Mich.

Collagen was a lyophilized preparation from bovine corium.[7] Approximately 1.5 mg was detached from the bulk of the collagen preparation and placed in a glass homogenizer, covered with 1.5 ml of chilled saline, and homogenized by hand to a fine suspension with a Teflon covered piston, until a light transmission of 40% at 420 mμ was achieved (Coleman cuvette 6-310C, 10 × 7.5 mm.). If, with a very fine suspension the light transmission was less than 40%, the suspension was diluted with chilled saline until the desired transmission was obtained. Distilled water served as blank. The collagen suspension was kept in an ice bath and was not used after 8 hr.

Blood Donors.—Fresh blood was obtained from 7 healthy nonfasting donors who were on no medication for at least 7 days prior to the studies. Lipemic plasma was not used.

Methods. Preparation of Platelet-Rich Plasma.—Human platelets were used, because of their higher tendency to aggregate as compared to animal platelets.[8] Blood was drawn through a 20-gauge hypodermic needle from the cubital vein by the 2-syringe technique with as little stasis as possible. The first syringe was discarded. The second syringe (20–30 ml) which contained 10% of its graduated volume of 3.8% citrate solution, was filled with blood to the mark, the content gently mixed and quickly transferred into centrifuge tubes which were spun for 15 min at 235*g* in the cold room (+4°). The resulting platelet-rich plasma (PRP) was transferred by Pasteur pipettes into one test tube (to assure a homogeneous platelet suspension), then pipetted in aliquots of 0.9 ml into the Coleman cuvettes and stored in the cold until the beginning of the experiment. The longest storage time was 220 min.

Aggregation Determination.—The trend of aggregation of the platelets left in their plasma was recorded according to Born[9] by means of an Evans titrator head which had been modified[10] by mounting a thermostatically controlled (37 ± 0.5°) cell holder in the light path. The output from the photoemissive light cell was fed through a zero suppression circuit to a Heath Servo-Recorder Model EU-20B. An Evans No. 609 dark red glass filter was used. The recorder was adjusted in such a way that the difference of the recordings of the light transmission between nonaggregated PRP and maximally collagen-aggregated PRP was about 80% of the width of the recording paper. The zero position control on the recorder was used to zero in the baseline with individual plain platelet-rich plasma. The paper speed was 2.54 cm 5 min. For aggregation and aggregation inhibition, 0.9 ml of PRP together with 0.08 ml of buffered solution containing the compounds to be tested or no compounds for the controls were preincubated in the cuvette (after addition of the Teflon magnet [7 × 3 × 4 mm.]) for precisely 5 min in a heat block at 37°. The cuvette was then transferred to the titrator head for an equilibration period of exactly 2 min. Immediately thereafter, 0.02 ml of collagen suspension was added with a 20-μl pipet. This brought the volume of the reaction mixture to 1 ml. The plasma specimens were continuously magnetically stirred at 1000 rpm.

All fibrinolytic compounds were dissolved in buffered saline to a 20 m*M* concentration except for concentrations above 1 m*M* where a slightly different procedure had to be used. Of this stock solution, an aliquot was made up to 0.08 ml with buffered saline. The volume of the aliquot was calculated to bring about the desired final compound molarity in the 1 ml reaction mixture.

After 50.5 (S.D. ± 23.1) sec ("lag phase") following addition of collagen the platelets aggregate rapidly and the light transmission increases accordingly. Maximum aggregation is achieved in about 341.5 sec (S.D. ± 105.5). The aggregation span which serves to calculate the aggregation inhibition, is defined as the maximal deflection of the light transmission curve measured as the distance in centimeters from the baseline obtained immediately after collagen addition to the vertex of the curve. In each single experiment, the average of the aggregation spans of the control runs with collagen alone is considered as 100% aggregation. The aggregation span in a run with a compound is expressed as a per cent of the average aggregation of the controls (100%). The difference between the two values represents the aggregation inhibition in per cent. One experiment with platelet-rich plasma of an individual donor consists of a control run with collagen alone followed by several runs with compound-exposed PRP and collagen interspersed with 2–3 additional collagen controls. (Figure 3 illustrates this procedure.) All individual tests (control and compound) are carried out in rapid succession to avoid potential errors introduced by the changing sensitivity of the platelets for collagen and by their increasing tendency to spontaneous agglutination during storage. Platelets counts[11] in PRP and micrographs (Leica Model DBP) were carried out with a Leitz Labolux phase microscope.

pH Measurements. pH was determined with a Corning pH-meter in PRP immediately after runs with collagen alone and runs with compounds plus collagen.

Fibrinolytic Activity.—This has been previously well established for all compounds by the "changing clot method."[12]

Results

Three hundred and thirty runs with compounds and collagen together with 163 collagen controls were carried out in 47 platelet-rich plasma samples from 7 healthy donors.

Influence of Platelet Count on Aggregation Span. The average platelet count of 19 samples of platelet-rich plasma was 547,100 S.D. ± 102,640 (range 395,000–815,000). In these 19 plasmas 69 control runs with collagen were carried out. The average height of the aggregation span was 18.1 cm S.D. ± 3 cm (range 12.4–23.2). There was no correlation between aggregation span and platelet count (correlation coefficient: 0.021). Consequently, standardization of the platelet number was not required in the present study.

pH Measurement. The addition of the compounds did not change the pH of the plasma appreciably: pH of PRP after collagen alone in 24 runs: average pH 7.69 S.D. ± 0.06 (range 7.58–7.84); pH in compound-treated PRP after collagen in 63 runs: average pH 7.73 S.D. ± 0.07 (range 7.65–7.98).

Light Transmission and Platelet Aggregates. The relation of light transmission to size of the platelet aggregates is illustrated by Figure 1. Each of the four panels is composed of a photograph of the same platelet-rich plasma taken under various experimental conditions and a recording of the trend of light transmission. All photographs were taken at 1x magnification at the time of maximum increase of light transmission except for panel 1. Panel 1 shows platelet-rich plasma before addition of collagen. There is no change in light transmission with time. Panel 2 was obtained after addition of 20 μl of collagen suspension (arrow) inducing rapid increase of light transmission and gross aggregates of platelets. The gross aggregates passing through the light path cause the oscillations of the light transmission which are seen near and at maximum aggregation in panel 2. With partial aggregation inhibition, the aggre-

(7) Obtained through the courtesy of Dr. W. R. Thomas, Armour Pharmaceuticals, Kankakee, Ill.

(8) Z. Sinakos and J. Caen, *Nouv. Rev. Fr. Hematol.*, **7**, 120 (1967).

(9) G. V. R. Born, *Nature*, **194**, 927 (1962).

(10) Medical Engineering Department of the University of Colorado Medical Center, Denver, Colo.

(11) G. Brecher and E. P. Cronkite, *J. Appl. Physiol.*, **3**, 365 (1950).

(12) K. N. von Kaudela, *J. Med. Chem.*, **8**, 164 (1965).

gates grow smaller and cannot cause this phenomenon any longer (panels 3 and 4). The photographs in panels 3 and 4 were obtained with 20 μl of collagen (arrow) added to plasma aliquots preincubated with 0.5 and 1 m*M* *N*-(2,4,6-trichlorophenyl)-4-aminothiophene-3-carboxylic acid. There is a clear decrease in the size of the aggregates at the higher concentrations of the compound indicating increased inhibition of collagen-induced platelet aggregation.

Figure 2 shows a micrograph of a PRP in which the platelet aggregation as measured by light transmission was 100% inhibited after preincubation with 0.5 m*M* *N*-(2-chloro-3-methylphenyl)-4-aminothiophene-3-carboxylic acid (right section of Figure 2). The micrograph reveals, however, the presence of small platelet aggregates in this plasma. The light transmission method reflects the formation of larger platelet aggregates and is rather insensitive for aggregates smaller than 20 μ (see scale in Figure 2). In spite of this limitation, the Born-method proves to be an excellent tool for screening compounds for their aggregation inhibition potential.

Reproducibility.—Tables I–V and Figures 1 and 3 show that the aggregation inhibition by the fibrinolytic compounds increases with the compound concentration in plasma. Tables I–V reveal that occasionally considerable variations were observed which did not fit the general pattern. A statistical evaluation of some of the results with lower compound concentrations was difficult due to these variations, whereas with higher concentrations the variations tended to disappear. This fact, along with the larger number of values available for the higher concentrations permitted statistical calculations. There were also some differences in response from plasma to plasma and in the control runs with one individual plasma. Figure 3 depicts one aggregation inhibition experiment with one individual plasma with flufenamic acid as a good example. There are slight differences between the heights of the aggregation span of the four control runs as well as between the height of the aggregation span in the duplicate runs with various compound concentrations. The figure demonstrates also the increasing inhibition of aggregation (shortening of the aggregation span) with increasing molarity. Figure 3 reveals a phenomenon which was reported earlier,[13] the lysis of the platelets with higher compound concentrations. It is reflected by an increase in light transmission clearly evident at the 10 m*M* (progressive lysis) and 15 m*M* (rapid complete lysis) level. Table I gives for flufenamic acid the aggregation inhibition in percent for each of the individual runs with the various concentrations. The variations of the individual values do not affect the general trend of increasing inhibition of collagen-induced platelet aggregation in human plasma with increasing concentrations of flufenamic acid. The aggregation inhibition becomes significant ($P < 0.01$) at the 0.2 m*M* level.

Statistical Evaluation.—For calculations of the statistical significance of some of the inhibition data, the nine lowest aggregation span values among the 163 controls were used. The aggregation inhibition was significant at the 1% level as follows: *N*-(2-chloro-6-methylphenyl)-4-aminothiophene-3-carboxylic acid, 0.1 m*M*; 79.1%; flufenamic acid, 0.2 m*M*: 58.4%; *N*-(2,4,6-trimethylphenyl)-4-aminothiophene-3-carboxylic acid, 0.5 m*M*; 67.6%; *N*-(2-chloro-6-methylphenyl)-4-aminothiophene-3-carboxylic acid, 0.5 m*M*; 83.2%; *N*-(3-trifluoromethylphenyl)-4-aminothiophene-3-carboxylic acid, 0.5 m*M*; 70.5%; 3-(2-chlorobenzyl)salicylic acid, 0.5 m*M*; 59.4%; *N*-(2-dimethylphenyl)-4-aminothiophene-3-carboxylic acid, 1m*M*; 77.9%; *N*-phenylanthranilic acid, 1 m*M*; 78.6%.

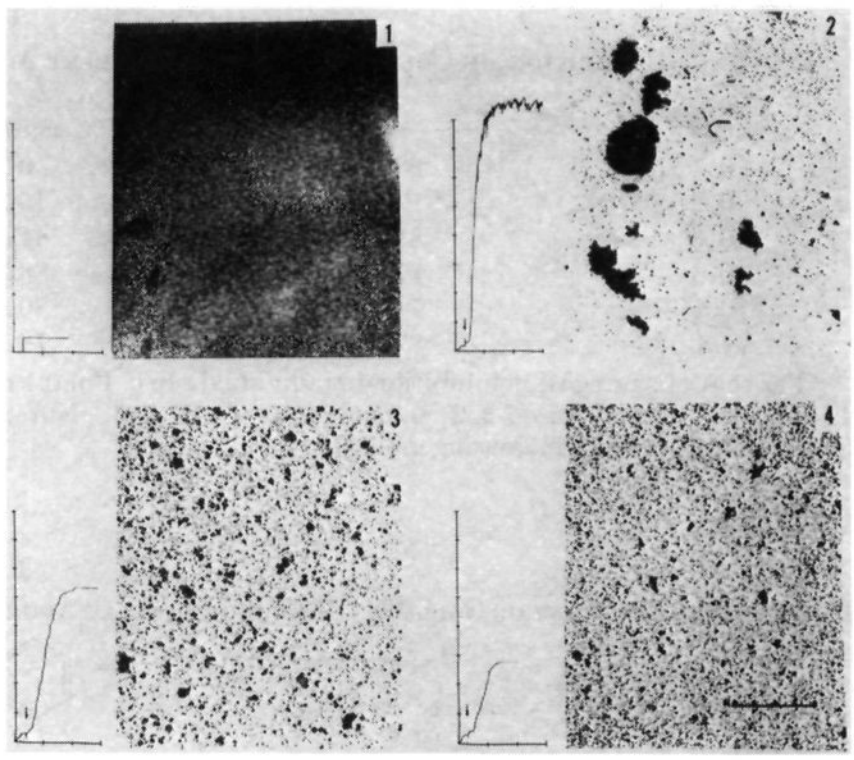


Figure 1.—Effect of *N*-(2,4,6-trichloromethylphenyl)-4-aminothiophene-3-carboxylic acid on collagen (arrow) induced aggregation of human platelets in citrated plasma: left side of each panel, trend of light transmission; right side, photomicrograph of the platelet-rich plasma; Magnification 1×; panel 1, platelet-rich plasma before addition of collagen; panel 2, same after addition of collagen; panels 3 and 4, plasma had been incubated for 5 min with compound before addition of collagen; final concentration of compound in panel 3, 0.5 m*M*; aggregation inhibition 34.7%; in panel 4, 1 m*M*; aggregation inhibition 70.5%. One section of scale in panel 4 is 1 mm. Photographs taken at time of maximum aggregation span.

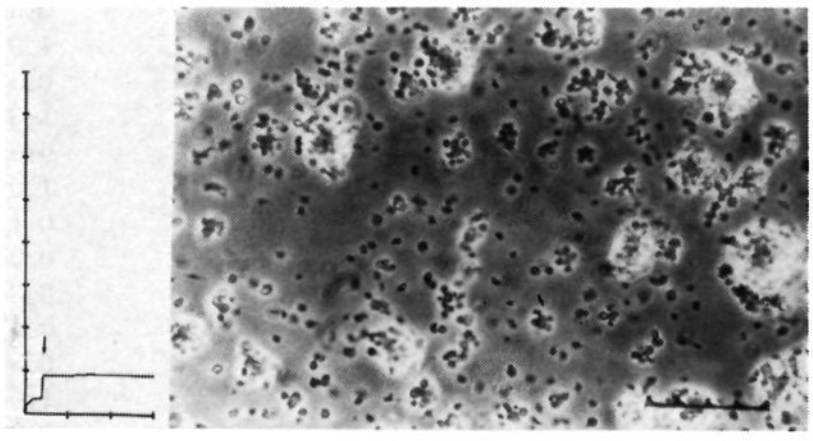


Figure 2.—Micrograph of collagen aggregated platelets in human plasma in which *N*-(2-chloro-3-methylphenyl)-4-aminothiophene-3-carboxylic acid was 0.5 m*M*. The panel of the left side indicates that collagen addition (arrow) does not produce any increase of light transmission. This indicates 100% inhibition of aggregation. Microscopically there are still small aggregates present. One section of scale is 10 μ.

Desaggregation.—*N*-(2-Chloro-3-methylphenyl)-4-aminothiophene-3-carboxylic acid, flufenamic acid, and 3-(2-chlorobenzyl)salicylic acid added to human plasma immediately after maximal aggregation by collagen to make a 1 m*M* final concentration did not induce desaggregation.

(13) F. Molinas and K. N. von Kaulla, *Thrombos. Diath. Haemorrh.*, **21**, 46 (1969).

TABLE I

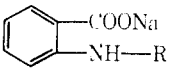
INHIBITION OF COLLAGEN-INDUCED PLATELET AGGREGATION IN HUMAN PLASMA BY FLUFENAMIC ACID

Concn, mM	% aggregation inhibition								Av.
0.1	23.7	19.5	29.9	0					18.3
0.2	12.6	52.5	58.0	45.4	89.4	92.4			58.4
0.3	85.1	88.9	42.8	41.3					64.5
0.4	77.3	38.0	87.8	88.7					72.9
0.5	74.6	69.6	74.7	80.0	78.0	81.5	79.9	96.3	73.3
1.0	87.8	86.8	100.0	73.1					86.9

[a] Per cent of aggregation inhibition in individual runs. For 0.1 mM, 3 plasmas at three occasions from one donor were used; for 0.2 mM, 4/1; 0.3 mM 2/2; 0.4 mM 2/2; 0.5 mM 6/4; 1 mM 3/3. Note: variation of individual values, but clear-cut trends of increasing aggregation inhibition with increasing molarity.

TABLE II

INHIBITION IN PER CENT OF COLLAGEN-INDUCED PLATELET AGGREGATION IN HUMAN PLASMA BY ANTHRANILIC ACID AND DERIVATIVES[a]



R	Fibrinolytic concn, mM	Origin	Concn tested at, mM	n	% aggregation—Range	% aggregation—Average	% aggregation inhibition
H	40	2	1.0	3	98.3–103.4	100.2	0
			2.0	1		85.8	14.2
			4.0	1		70.9	29.1
			6.0	1		50.8	49.2
C_6H_5	20	6	0.1	1		93.8	6.2
			0.2	1		95.8	4.2
			0.5	1		69.8	30.2
			1.0	5	1.1–39.6	21.4	78.6
o-$CH_3C_6H_4$	10	6	0.5	1		79.2	20.8
			1.0	1		9.2	90.8
m-$CH_3C_6H_4$	4	6	0.5	1		43.1	56.9
			1.0	3	6.4–37.1	16.8	83.2
p-$CH_3C_6H_4$	20	6	0.5	1		81.6	18.4
			1.0	1		14.8	85.2
o-$CF_3C_6H_4$	5	6	0.5	1		31.3	68.7
			1.0	1		25.4	74.6
m-$CF_3C_6H_4$	3	10	0.1	4	66.3–100.5	79.4	20.6
			0.2	6	7.6–87.6	41.6	58.4
			0.3	4	11.1–58.7	35.5	64.5
			0.4	4	11.3–62.0	27.1	72.9
			0.5	8	3.7–30.4	20.9	79.1
			1.0	5	0–26.9	10.5	89.5
			2.0	4	17.1–48.2	27.1	72.9
			4.0	1		16.7	83.3
			5.0	1		13.6	86.4
			10.0	1		(L)	
			15.0	1		L	
2,5-$Me_2C_6H_3$	5	6	1.0	2	59.3–67.9	63.6	36.4
2,6-$Me_2C_6H_3$	20	6	0.5	1		103.0	0
			1.0	1		40.1	59.9
3-CF_3-4-ClC_6H_3	2	6	0.5	1		22.4	77.6
			1.0	2	20.9–37.5	29.2	70.8
2-Cl-5-$CF_3C_6H_3$	(2)	6	0.5	2	17.9–33.6	25.8	74.2
			1.0	1		21.6	78.4
2-Br-5-$CF_3C_6H_3$	(2)	6	0.5	2	9.7–17.2	13.5	86.5
			1.0	1		15.6	84.4

[a] Aggregation with collagen alone is 100%. Fibrinolytic activity of compounds with human plasma (hanging clot) is given for comparison; (+): partial fibrinolytic activity. For the origin of compounds, see the Experimental Section. L or (L): total or partial lysis of platelets.

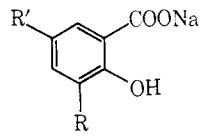
Lag Phase.—There was no correlation between the degree of inhibition of collagen-induced platelet aggregation and the lag phase of compound-plasma and control-plasma. For correlation of aggregation and fibrinolytic activity and for the relationship between structure and aggregation inhibition see Discussion.

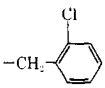
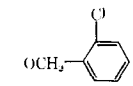
Discussion

Anticipating *in vivo* studies, the effect of the synthetic fibrinolytic compounds on various blood components including platelets was previously investigated, and it was found that some compounds induce in vary-

Table III

Inhibition of Collagen-Induced Platelet Aggregation in Human Plasma by Salicylic Acid and Derivatives[a]



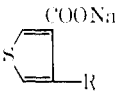
R	R'	Fibrinolytic concn, m*M*	Origin	Concn tested at, m*M*	*n*	% aggregation Range	% aggregation Average	% aggregation inhibition
H	H	(150)	2	0.1	2	92.3–98.3	95.3	4.7
				0.2	2	105.4–107.1	106.3	0
				0.5	2	99.4–101.8	100.6	
				1.0	1		97.0	3.0
				5.0	1		97.0	3.0
				10.0	2	39.9–59.5	49.7	50.3
				15.0	1		17.8	82.2
				20.0	1		19.3	80.7
	H	5	9	0.1	2	106.3–108.0	107.2	0
				0.2	1		101.7	0
				0.5	1		106.3	0
				1.0	1		97.1	2.9
				2.0	1		92.9	8.1
				5.0	4	15.2–49.7	33.8	66.2
				10.0	1		16.6	83.4
				15.0	1		18.4	81.6
H		3	9	0.1	1		105.8	0
				0.2	1		104.9	0
				0.5	1		102.5	0
				1.0	3	98.4–106.4	102.3	0
				2.0	2	41.8–102.5	72.2	27.8
				5.0	1		93.1	6.9
				8.0	2	9.6–13.7	11.7	88.3
				10.0	2	3.9–10.8	7.4	92.6
				15.0	1		2.0	98.0
	H	2	9	0.1	3	84.0–110.1	99.0	0
				0.2	2	84.6–102.4	93.5	6.5
				0.3	3	33.6–62.0	46.0	54.0
				0.4	2	20.6–38.0	29.3	70.7
				0.5	4	17.6–65.7	40.6	59.4
				1.0	1		20.2	79.8
				5.0	2	23.0–34.3	28.7	71.3
				8.0	2	17.4–23.0	20.2	79.8
				10.0	2	0–14.6	7.3	92.7
				15.0	1		18.5	81.5
				20.0	1		(L)	
$CH_2CH{=}CHC_6H_5$	H	3	9	0.3	1		76.8	23.2
				0.5	1		86.2	13.8
				1.0	2	12.3–29.1	20.7	79.3
				2.0	1		4.9	95.1
H		5	7	1.0	2	100.6–101.3	101.0	0
H	I	20	4	0.5	2	63.5–92.6	78.0	22.0
I	H	2	2	0.1	1		100.0	0
				0.5	1		100.0	0
				1.0	2	95.4–97.3	96.4	3.6
				2.0	3	87.0–100.0	96.1	3.4
				4.0	3	0–33.4	14.4	85.6
$-C(CH_3)_3$	$-C(CH_3)_3$	(0.9)	3	2.0	1		63.8	36.2

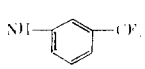
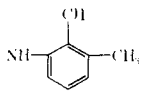
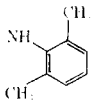
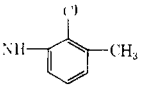
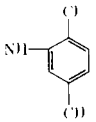
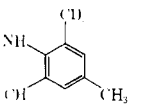
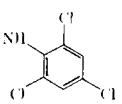
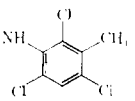
[a] Same arrangement as in Table II.

ing degrees aggregation of platelets. Reduction of the compound concentration by one order of magnitude resulted in a reversal of the effect: the fibrinolytic compounds inhibited ADP- and/or thrombin-induced aggregation of human platelets.[13] This finding indicated an important pharmaceutical and therapeutic potential: development of synthetic organic antithrombotic drugs which act simultaneously by two different pathways: prevention of thrombosis by inhibition of platelet aggregation which is the initial step of formation of the white thrombus and, fibrinolytic dissolution (thrombolysis) of the fibrin part of the thrombus in a manner similar to enzymatic thrombolytic drugs. To explore this double potential further, the present studies were undertaken. Compounds (38) selected for their fibrinolytic features were studied with Born's method for their ability to prevent collagen-induced aggregation of platelets in human plasma. It

TABLE IV

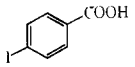
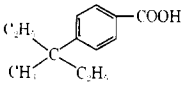
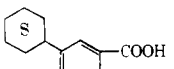
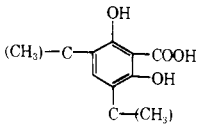
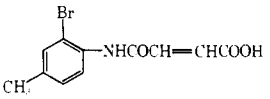
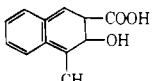
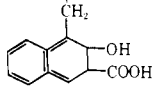
INHIBITION OF COLLAGEN-INDUCED PLATELET AGGREGATION BY THIOPHENE-3-CARBOXYLIC ACID AND ITS 4-AMINOPHENYL DERIVATIVES[a]



R	EDribolytic concn, μM	Origin	Concn tested at, mM	n	% aggregation Range	Average	% aggregation inhibition
	>500	4	0.5	1		102.1	0
			1.0	1		93.1	6.9
			2.0	1		79.0	21.0
			4.0	1		21.1	78.9
			6.0	1		0	100.0
	6	10	0.1	5	12.9–99.0	72.5	27.5
			0.2	2	67.6–91.8	79.7	20.3
			0.3	5	3.4–89.8	52.4	47.6
			0.4	2	34.3–48.8	41.5	58.5
			0.5	7	0–54.6	29.5	70.5
			1.0	3	16.0–17.1	16.6	
	7	9	0.5	2	8.3–18.5	13.4	86.6
			1.0	3	12.5–23.7	17.4	82.6
			2.0	1		20.9	79.1
	5	9	0.1	1		98.1	0
			0.3	1		85.0	15.0
			0.5	6	75.1–96.9	83.9	16.1
			1.0	7	11.6–54.1	22.1	77.9
			2.0	1		14.2	85.8
			4.0	1		13.6	86.4
			6.0	1		11.8	88.2
	3	9	0.005	1		98.6	0
			0.01	2	74.6–87.6	80.1	19.9
			0.02	2	74.6–84.9	79.9	20.1
			0.05	5	21.5–66.7	40.1	59.9
			0.1	5	9.5–35.7	20.9	79.1
			0.2	3	2.9–43.3	25.6	74.4
			0.3	2	2.3–9.4	5.8	94.2
			0.4	2	4.7–5.6	5.2	94.8
			0.5	4	0–13.2	7.5	92.5
			1.0	4	7.7–25.5	15.6	84.4
			2.0	1		23.5	76.5
	3	9	0.5	2	102.6–113.5	108.1	0
			1.0	3	6.9–50.4	23.3	76.6
			2.0	1		15.3	84.7
	4	9	0.1	4	14.9–77.6	54.2	45.8
			0.5	4	7.9–28.5	16.8	83.2
			1.0	3	0–17.5	8.1	91.9
			2.0	1		5.8	94.2
			5.0	2	4.9–6.7	5.8	94.2
			10.0	2	4.9–5.5	5.2	94.8
	3	9	0.1	4	27.5–100.8	80.3	19.7
			0.2	3	77.9–99.2	91.8	8.2
			0.3	2	93.6	93.6	6.2
			0.4	3	9.2–92.5	45.6	54.4
			0.5	6	7.2–98.4	32.4	67.6
			1.0	5	8.8–90.5	35.0	64.0
			2.0	1		27.3	72.7
			5.0	1		69.9	30.1
	(2)	9	10.0	1		8.1	91.9
			0.5	2	22.9–65.3	44.1	55.9
			1.0	2	18.0–29.5	23.7	76.3
	(5)	9	0.5	2	11.1–56.3	33.7	66.3
			1.0	2	11.1–35.8	18.5	81.5

[a] Same arrangement as in Table II.

Table V
Inhibition of Collagen-Induced Platelet Aggregation by Miscellaneous Compounds[a]

	Fibrinolytic concn, mM	Origin	Concn tested at, mM	n	% aggregation Range	% aggregation Average	% aggregation inhibition
	35	2	2.0	1		45.0	55.0
			4.0	1		22.0	78.0
	6	1	2.0	1		64.8	35.2
	3	9	0.1	1		107.7	0
			0.2	1		98.3	0
			0.5	3	96.2–107.8	100.2	0
			1.0	1		101.1	0
			2.0	1		103.8	0
			5.0	1		83.8	16.2
			10.0	1		31.9	68.1
	(0.9)	8	1.0	4	20.2–37.2	26.9	73.1
			2.0	1		18.3	81.7
			4.0	1		22.0	78.0
	30	6	1.0	2		80.0	20.0
	>500	5	0.5	1		110.0	0
			1.0	2	83.0–102.7	93.3	6.7
			2.0	1		100.6	0
			4.0	1		100.6	0
			6.0	1		99.2	0
	2	4	2.0	1		100.6	0
			4.0	2	31.0–42.4	36.7	63.3

[a] Same arrangement as in Table II.

is realized that with complete inhibition of aggregation as indicated by this method, small platelet aggregates may still be detected with the microscope. These may represent, however, loose aggregates.[14] Despite the occasional erratic results with the use of the Born method it is clear, nevertheless, that fibrinolytic organic anions at one concentration or another prevent collagen-induced platelet aggregation.

The pathway of aggregation inhibition by the fibrinolytic compounds is unknown. A common denominator for both activities could be postulated if one assumes that the aggregation inhibition is brought about by inhibition of one or several platelet enzymes, possibly those involved in the release mechanism. Such is the case with the fibrinolytic activity of the compounds where inhibition is the primary mechanism in the induction of this activity. Inhibition of the antiplasmin[15] and antiactivator[16] is a main pathway of fibrinolysis induction by the compounds rather than a compound-induced activation of plasminogen. Synthetic fibrinolytic compounds also inhibit trypsin[17] and other enzymes.[4]

The fibrinolytic activity of the compounds correlated loosely with the aggregation inhibition. The parent compounds which were poor fibrinolytic agents were also poor aggregation inhibitors. When introduction of appropriate substituents increased the fibrinolytic activity, the aggregation inhibitory activity of the compounds rose also. Beyond this, there was no close correlation. Aggregation inhibition was present at approximately $^1/_3$ [3-(cinnamyl)salicylic acid] to $^1/_{60}$ [*N*-(2-chloro-6-methylphenyl)-4-aminothiophene-3-carboxylic acid] of the fibrinolytic concentrations. Three exceptions were noted. 3,5-Diiodosalicylic acid, 5-cyclo-hexylsalicylic acid, and pamoic acid exerted their marked fibrinolytic activity at concentrations lower than those at which they inhibited collagen-induced platelet aggregation.

Tables II–IV list the aggregation inhibition of fibrinolytic derivatives of anthranilic, salicylic, and thiophene-3-carboxylic acids. The parent compound inhibited aggregation more than 50% only above 1 m*M*. Unsymmetrical substitution increases the aggregation inhibition very markedly. As Table II reveals, substitution on N with a nonsubstituted phenyl ring increases the inhibitory action of anthranilic acid at the 1 m*M* level from zero to 78.6%. The phenyl-substituted anthranilic acid has relatively little activity at 0.5 m*M* or lower. Substitution with CF_3 in the 3 position (flufenamic acid) increases the aggregation inhibition by *N*-phenylanthranilic acid (and its fibrinolytic activity). Single or double substitution with Me groups on the N attached phenyl ring appears to result in less active compounds than substitution in 3 with CF_3.

The values for salicylic acid and its fibrinolytic derivatives are listed in Table III. Again, the parent com-

(14) H. Reuter, G. Niemeyer, and R. Gross, in "Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes," E. Deutsch, E. Gerlach, and K. Moser, Ed., G. Thieme, Stuttgart, 1968, p 336.
(15) K. N. von Kaulla, *Thrombos. Diath. Haemorrh.*, **10**, 151 (1963).
(16) N. Aoki and K. N. von Kaulla, *ibid.*, **22**, 251 (1969).
(17) K. N. von Kaulla, in "The Chemical Controls of Fibrinolysis," J. Schor, Ed., Interscience Publishers, New York, N. Y., 1970.

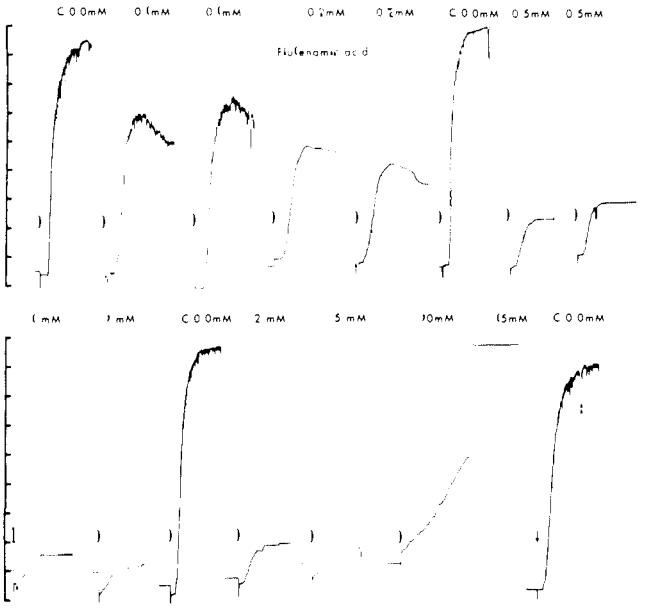


Figure 3.—Inhibition of collagen-induced platelet aggregation in human plasma by flufenamic acid: effect of various compound concentrations; fresh platelet-rich plasma of a male donor. Tests with compounds were run in duplicates. All tests carried out in rapid succession. Individual runs were between 5 and 12 min. Duration of the total experiment was 200 min: *c*, 0.0 m*M*; control. Aggregation inhibition in per cent was: 0.1 m*M*: 23.7; 19.5; 0.2 m*M*: 52.5; 58.0; 0.5 m*M*: 80.0; 78.0; 1 m*M*: 87.6; 86.8. At 2 m*M*, an aggregating effect of the compounds themselves on the platelets appears resulting eventually in partial (10 m*M*) and complete (15 m*M*) thrombocytolysis.

pound is poorly active. Substitution in the 3 or 5 position with cyclohexyl improved fibrinolytic activity but not the aggregation inhibition whereas substitution in 3 with 2-chlorobenzyl increases the fibrinolytic activity and enhances the aggregation inhibition about tenfold.

Table IV lists aggregation inhibition by phenyl-4-amino derivatives of thiophene-3-carboxylic acid. As before, the parent compound shows little activity, but thiophene-2-carboxylic acid (Table V) is even less so. The thiophene analog of flufenamic acid, *N*-(3-trifluoromethylphenyl)-4-aminothiophene-3-carboxylic acid is slightly less active than flufenamic acid. Of the other 4-aminothiophene-3-carboxylic acid derivatives, *N*-(2,5-dimethylphenyl)-4-aminothiophene-3-carboxylic acid appears to be more active than the 2-chloro-5-methyl analogue, which in itself is more active than flufenamic acid. *N*-(2-chloro-3-methylphenyl)-4-aminothiophene-3-carboxylic acid was the most active of all compounds tested with 50% inhibition of collagen-induced platelet aggregation at 5×10^{-5} *M* concentration.

Table V lists the aggregation inhibiting activity of various other compounds. Of the γ-resorcylic acids, the strong fibrinolytic 5-cyclohexyl derivative is non-inhibiting at 1 m*M* similar to 5-cyclohexyl substituted salicylic acid. In contrast, the equally strong fibrinolytic 3,5-di-*t*-butyl-γ-resorcylic acid has good activity at 1 m*M*.

Thus far, all fibrinolytic synthetic compounds tested for this particular feature have exhibited inhibition of collagen-induced platelet aggregation in human plasma. Compounds fibrinolytically active at a few m*M* can be divided in terms of aggregation inhibition into two related categories: (a) aggregation inhibition occurs at concentrations one order of magnitude (or more) lower than fibrinolysis induction; (b) aggregation inhibition occurs at concentrations close to the fibrinolytic one. These observations indicate, at least theoretically, the possibility of developing synthetic drugs which would exert (1) simultaneously thrombolytic effects and aggregation inhibition, and (2) drugs in which these two therapeutic effects could be separated simply by varying the dosage.