raphy of the amine fraction indicated that the same amines were present as in dog urine. Chromatography of the acid fraction indicated that the same acid was present.

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A Simple Test Tube Arrangement for Screening Fibrinolytic Activity of Synthetic Organic Compounds[1]

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It has previously been shown that a variety of synthetic organic compounds dissolve human plasma clots at a rather low concentration. A clear-cut relationship between chemical structure and activity was observed. Testing of activity, however, has required a complicated procedure. To avoid this difficulty, a new, simple, and quantitative screening test has been developed and is described in this paper. It consists essentially of the formation of clots from human plasma obtained from outdated blood bank blood. The clots are suspended in the solution of the compound to be studied and incubated for 24 hr. at 37°. With active compounds, the clots completely dissolved in this time. Based on the new screening test, some examples of the relationship of chemical structure and ability to dissolve clots are given for active benzoic acid derivatives, recently observed to induce fibrinolytic activity.

Development of cheap and reliable thrombolytic (clot dissolving) drugs which are easy to use is one of the great challenges in the field of therapeutics. At present, there are no drugs in wide-spread use which effectively cope with the number-one killer, intravascular clots. One major reason appears to be the lack of participation of the organic chemist in the development of fibrinolytic (thrombolytic) drugs which has been left entirely to the biochemist. The presently available enzymatic thrombolytic agents, though under certain circumstances quite useful, are generally disappointing from the clinical point of view: they are expensive, they require elaborate laboratory procedures for control, and they cannot be used on a preventive basis.

In this laboratory, it was discovered that a variety of organic compounds upon dissolution in human plasma in the test tube induced a marked fibrinolytic activity. This was not true of bovine, canine, and feline plasma. The ability of a compound to exhibit this particular property was structure dependent.[2-6] Minor changes of the chemical structure either enhanced or abolished the ability to produce fibrinolytic activity. The compounds were induced by a yet poorly understood pathway—activation of the fibrinolytic enzyme system in human plasma, demonstrated by fibrinolytic, caseolytic, and esterolytic activity.[2,6] Urethan was the first compound found to induce fibrinolytic activity in human plasma. Variations of the structure showed an increase of activity in this order: urethan < methylurethan < ethylurethan, and (for a related group of compounds) urea (no activity) < thiourea < ethylurea < allyl-2-thiourea.[4] As a working hypothesis it was assumed that hydrotropy could be the common denominator for the activity of these compounds. In order to test the hypothesis, other compounds with known hydrotropic activity but quite different structures were studied. Large asymmetric hydrotropic anions like 2,4-dimethylbenzenesulfonate and 2-naphthalenesulfonate brought about a 5-fold increase in activity as compared to urethan, whereas large asymmetric hydrotropic cations like tetra-*n*-propylammonium bromide were completely ineffective.[5] Compounds with a carboxyl group at the end of the hydrophilic side chain were slightly more active than the ones with sulfonic acid. Therefore, at present, our search for better compounds is concentrated on the former group. It has been possible by varying the structure further to increase the activity by a factor of approximately 40 and to define some additional structural requirements for an active compound (for examples see Discussion).[5,6] It is likely that intensive search for more active compounds will eventually lead to synthetic thrombolytic drugs. For these studies, a quantitative but rather elaborate testing procedure has been used previously.[7]

The investigations were hampered by the lack of quantitative testing methods suitable for screening and, most important, suitable for the researcher unfamiliar with the technique of blood coagulation and fibrinolysis. A new, simpler method, together with some instructive examples of test results, is briefly described in the Experimental section. It is hoped that the procedure will enable the organic chemist to participate in the quest for thrombolytic agents.

Experimental

Principle.—Human plasma clots are suspended at 37° in a solution of a compound to be tested. After 24-hr. incubation,

(1) Supported by a grant-in-aid of the American Heart Association, by Grant HE-05538 of the National Heart Institute, U. S. Public Health Service, and partly by a grant-in-aid of the Wyoming Heart Association.

(2) K. N. von Kaulla, "Chemistry of Thrombolysis: Human Fibrinolytic Enzymes," Charles C Thomas, Publisher, Springfield, Ill., 1963, p. 261.

(3) G. Bugni, "Synthesis of Organic Compounds Which Induce Fibrinolysis," Thesis, Boulder, Colo., 1963.

(4) K. N. von Kaulla and R. L. Smith, *Proc. Soc. Exptl. Biol. Med.*, **106**, 530 (1961).

(5) K. N. von Kaulla, *Arch. Biochem. Biophys.*, **96**, 4 (1962).

(6) K. N. von Kaulla, *Thromb. Diath. Haemorrhag.*, **7**, 404 (1962).

(7) K. N. von Kaulla, *ibid.*, **5**, 489 (1961).

the clots are inspected for the extent of lysis which has occurred.

Material and Methods. Reagents.—(a) Human plasma is obtained by centrifuging outdated (older than 3 weeks) ACD bank blood (from any blood bank), separating the plasma, and freezing it in lots at −15° or lower; the plasma must be frozen before it is ready for use; plasma from animals cannot be used; (b) 0.5 *M* calcium chloride; and (c) buffered saline: 0.85% NaCl and barbital acetate buffer[8] (1:4).

Glassware.—(a) Vials, 1.85 ml., 9 × 30 mm., Kimax Nr. 60930/L; (b) test tubes, 13 × 100 mm. with rim, Kimax Nr. 45042; (c) stirring rods, 3 × 125 mm., Kimax Nr. 40500; (d) pipets, various sizes; and (e) rubber stoppers from Becton and Dickinson vacutainer tubes, series 3204. The stoppers receive a well-centered bore and are slipped over the stirring rods in such a way that the upper end of the rod protrudes 1 in. above the stopper.

Apparatus.—(a) All-glass water bath thermostat set at 37°; (b) aluminum or stainless steel rack to suspend test tubes in water bath thermostat; (c) supporting stand[9] for making cylinder-shaped clots around the stirring rods; and (d) a plastic bar (10.2 × 27.9 × 0.51 cm.). The supporting stand with vials in place (Figure 1) consists of a bottom plate (10.2 × 27.9 × 1.27 cm.),

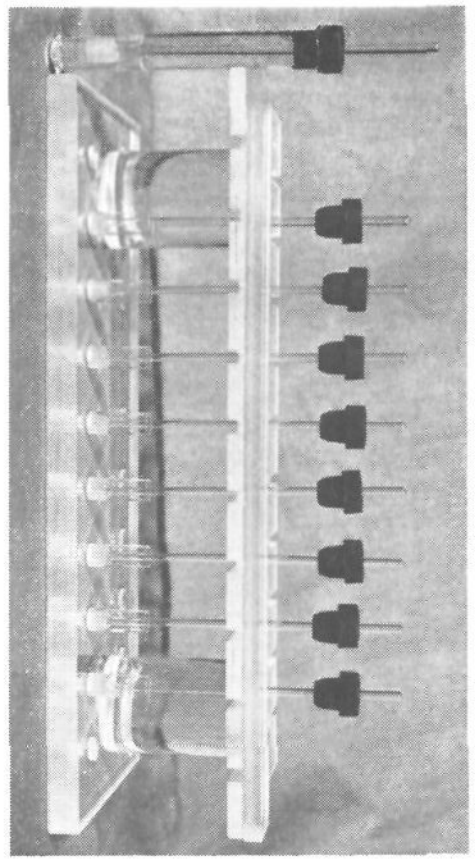

Figure 1.—Stand for production of plasma clots. The clots in the vials and the inserted glass rods around which they have been formed are seen in front. The slots for retaining the rods are to be seen in rear of the top plate. On the left side is a clot inserted into the test solution.

a top plate (10.2 × 27.9 × 0.63 cm.), and two supporting columns (3.7-cm. diameter, 6.99 cm. long, with bosses on each end), all made of acrylic plastic. The bottom plate is drilled with twenty 0.89-cm. holes, 0.8 cm. deep for holding the vials. The top plate has twenty corresponding 0.32-cm. holes to closely approximate the diameter of the rods with 0.5-cm. slots to provide an entry. Both plates have two larger corresponding holes for the bosses of the supporting columns. The stand allows the stirring rods to be placed firmly into the middle of the vials. A plastic bar put on the top plate (see Figure 1) holds the stirring rods in place. This arrangement keeps the rods in a perpendicular position which is an important feature for making suitable clots.

Testing for Fibrinolytic Activity. A. Preparation of Plasma Clots.—The frozen plasma was thawed and brought to 37°. The following steps were carried out at room temperature. To 5 ml. of plasma was added 0.25 ml. of 0.5 *M* $CaCl_2$. The mixture was inverted once for thorough mixing. Next, 0.5 ml. of the recalcified plasma was quickly pipetted into each of the 10 vials which had been previously fitted into the holes of the bottom plate of the stand. Then the stirring rods carrying the stoppers were inserted through the corresponding slots in the upper plate into the center of the vial. Normally a solid clot formed within about 15 min. Thirty to forty-five minutes after addition of $CaCl_2$, vials and stirring rods were both carefully removed from the stand (use both hands). As the next step, the stirring rod was slowly pulled out of the vial together with the plasma clot which stuck firmly on its lower end. Care had to be taken that during this maneuver the clots were not squeezed (by tilting the stirring rod from its vertical axis). The stirring rods with rubber caps and clots were immediately inserted into the solution of compounds to be tested for fibrinolytic activity. The test solu-

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

(9) Manufactured by Simons Engineering Co., Morrison, Colo. 80465.

TABLE I

TEST RESULTS WITH THE SCREENING PROCEDURE FOR FIBRINOLYTIC ACTIVITY OF SYNTHETIC ORGANIC COMPOUNDS. ACTIVITY OF VARIOUS BENZOIC ACID DERIVATIVES[a]

Compd. (Na salts)	Solvent[b]	Molarity 0.4	0.3	0.2	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.009	0.008
Benzoate	BS	−	−	−	−	−	−	−	−	−	−	−	−	−		
p-Isopropylbenzoate[c]	BS						(+)	+	+	(+)	−	−	−	−		
	PL	−	−	+	+	+	+	+	−		−	−	−	−		
p-Butylbenzoate[c]	BS		−	−	−		−		−		−	+	(+)	−		
	PL				−	−	(+)	+	+	+	−	−	−	−		
p-Iodobenzoate	BS				−		(+)		+	+	+	(+)	−			
	PL	−	−	−	+	+	+	+	+	(+)	−	−	−			
p-Iodophenylethoxyethoxyacetate[c,d]	BS										(+)	+	+	−		
	PL							−	(+)	+	+	−	−	−		
Salicylate	BS	−	−	−	−	−	−	−	−	−	−	−	−	−		
2-Hydroxy-4-methylbenzoate[c]	BS	−	−	−	(+)	(+)	(+)	(+)	−	−	−	−	−	−		
	PL	−	−	+	+	−	−	−	−	−	−					
2-Hydroxy-5-methylbenzoate[c]	BS	−	−	−	(+)	(+)	(+)	(+)								
o-Thymotate[c]	BS								−	−	+	+	+	+	+	−
	PL								−	−	(+)	+	−			
p-Thymotate[c,d]	BS				−		−		−	−	−	−	−	−		
2-Hydroxy-*p*-cymene-3-carboxylate[c,d]	BS				−		−		−		−		+	−		

[a] + = complete clot dissolution, (+) = partial clot dissolution, − = no activity. [b] BS = buffered saline, PL = defibrinated human plasma.[6] [c] Compounds not previously studied. [d] These compounds were kindly provided by Dr. J. M. Sprague, Merck Sharp & Dohme Research Laboratories, West Point, Pa.

tion (2.5 ml.) had previously been pipetted into the test tubes which were hanging from the rack into the water bath thermostat. When the stopper was tightly in place closing the tube, the clot was suspended in about the middle of the solution. It is advisable not to recalcify larger batches than 5 ml. of plasma.

B. Preparation of Test Solution.—The compounds to be tested should preferably be Na or K salts if applicable. They were dissolved in buffered saline, pH 7.42. Some compounds not completely soluble in buffered saline were tested as suspensions in the same manner. If necessary, the pH was adjusted after dissolution. Various concentrations were tested such as 0.5, 0.4, 0.3, 0.2, 0.1, 0.075, 0.05, 0.025, and 0 01 *M*. Thus the optimal concentration for activity can be found. The use of various concentrations is essential because a compound might be inhibitory at higher concentration, effective at the middle range, and ineffective at the lower ones. This feature is shown in Figure 2 with *o*-thymotic acid. Inhibition, activity, and ineffectiveness from left to right are clearly seen. Figure 2 shows also how unequivocal the positive reading is.

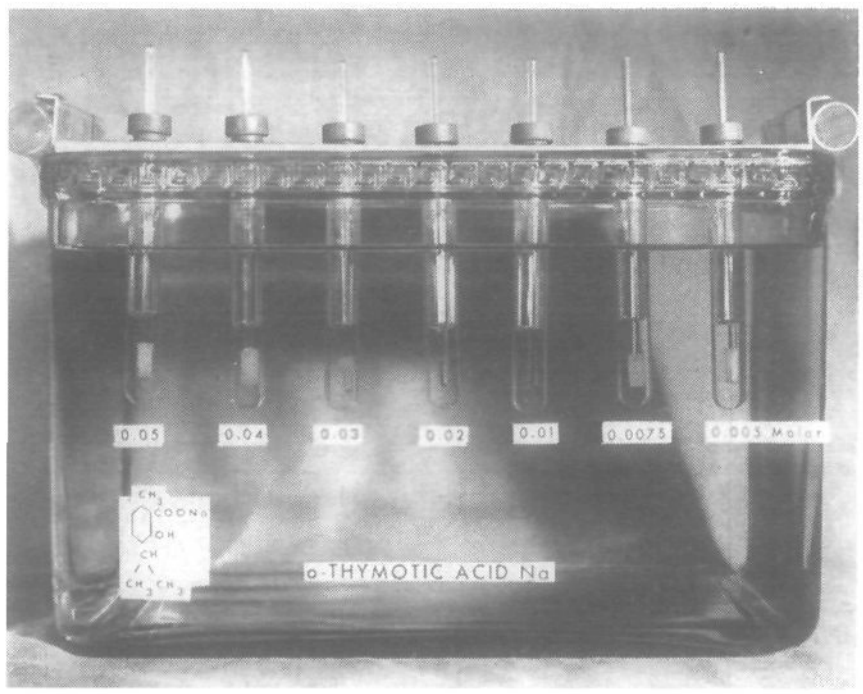

Figure 2.—Water bath thermostat with test tubes containing test solution and clots after 24-hr. incubation. Using *o*-thymotic acid, note partial clot dissolution, (+), at 0.03 *M*; complete dissolution, +, at 0.02 and 0.01 *M*.

Evaluation of Results.—The results are read after 24-hr. incubation. Complete lysis of the clot, 0.02 and 0.01 *M* in Figure 2 is recorded as +; partial, 0.03 *M*, as (+); and no lysis as − (remaining concentrations). Complete dissolution of the clot cannot be mistaken: only a bare glass rod is left; see Figure 2. There is occasionally a slight difference of reactivity from plasma to plasma which, however, does not effect the basic information provided by the test.

Comparing the results obtained with synthetic compounds with those obtained with enzymatic activators, the instability of the latter on incubation, and their susceptibility to plasma inhibitors has to be kept in mind. A complete clot dissolution within 24 hr. was not obtained with as much as 1000 units/ml. of streptokinase in buffered saline, whereas 20–100 units brought about a partial dissolution. However, about 500–1000 units/ml. of streptokinase in plasma were required to induce a partial lysis, smaller amounts being ineffective. Urokinase, 50–100 units (Ploug)/ml., in buffered saline induced a more or less complete lysis.

Discussion

The basic information obtained by the test concerns primarily the detection of the fibrinolysis-inducing capacity of an organic synthetic compound but also shows the striking relationship between the structure of the compound and this capacity. Table I demonstrates such a structural relationship with some benzoic acid derivatives which have been studied with the new test. Benzoic acid is inactive; however, introduction of an isopropyl group into the 4-position produces a rather active compound. Replacement of the isopropyl group by a *t*-butyl group seems to enhance the activity further. Salicylic acid is inactive; introduction of a methyl group into the 4- or 5-position results in a mildly active compound. *o*-Thymotic acid is very active, and switching of its carboxylic and hydroxylic group (2-hydroxycymene-3-carboxylic acid) reduces its activity only moderately. If, however, the hydroxyl group of the *o*-thymotic acid is moved to the *para* position (*p*-thymotic acid), the activity is completely abolished.

Another area of interest is halogenated benzoic acids. It has been shown previously that these are increasingly active in this order: F (no activity), Cl, Br, and I, provided that the halogens are in *para* position. *ortho*-Halogenated compounds are ineffective, and the *meta* ones exhibit an intermediate activity.[6] Table I shows the activity of the *p*-iodobenzoic acid as compared to the *p*-iodoethoxyethoxyacetic acid. Lengthening of the hydrophilic side chain has doubled the activity. Undoubtedly diligent and systematic variation of the molecules will create much more active compounds.

To be suitable for eventual therapeutic use, a compound must be able to produce clot lysis after being dissolved in human plasma (in order to duplicate the condition in the human body). With six compounds (see Table I) a comparison of the activity obtained after their dissolution in buffered saline and in human plasma has been made. By dissolution in plasma the activity of the compounds was slightly to moderately reduced, but not lost.

All compounds checked for this particular feature were inhibitory in higher concentrations. The active range varied from compound to compound. On a tentative basis, it is assumed that the compounds act as inhibitors. They might inhibit an antiactivator at lower concentrations, thus freeing the activators(s) present in human plasma to induce a fibrinolytic reaction. At higher concentrations, the compounds inhibit the fibrinolytic enzyme itself.

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