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# ON STRUCTURE-RELATED PROPERTIES OF SYNTHETIC ORGANIC CLOT-DISSOLVING (THROMBOLYTIC) COMPOUNDS*

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**Abstract**—Salicylic acid derivatives and various other anionic compounds induce upon dissolution marked fibrinolytic activity in human plasma by activating its fibrinolytic system. Clots formed from such plasma dissolve spontaneously on incubation, even after having been carefully washed. The fibrinolysis-inducing capacity of synthetic organic compounds depends on the type and position of the substitution of the mother compound. The capacity for salicylates and γ-resorcylates increased manyfold with the increase of the bulkiness of the substituent. Above and below an optimal concentration, no fibrinolytic activity is obtained. There is evidence that the synthetic fibrinolytic compounds act basically as inhibitors, possibly by interfering with the activity of an antiactivator, thus freeing the blood activator to activate plasminogen. The synthetic fibrinolytic compounds have a strong affinity for proteins; the major part of them is bound to albumin and lost for the fibrinolysis-inducing process. It is hoped that the synthetic organic fibrinolysis-inducing compounds may be developed into convenient thrombolytic agents.

THROMBOLYTIC therapy with the goal of dissolving intravascular clots, which obstruct vital parts of the circulation, is at present accomplished with the intravenous infusion of enzymes which are activators of the human fibrinolytic system. The use of costly enzymes does not permit the necessary broad application. For inexpensive thrombolytic therapy accessible to every patient who might require it, relatively simple drugs are needed which can be given orally and intravenously and, most importantly, on a preventive basis. Such properties can best be provided by synthetic compounds. Pursuing this line of thought we discovered that certain synthetic organic compounds induce dissolution of human clots in the test tube by activating the fibrinolytic enzyme system. The present paper deals with characteristic properties of such synthetic fibrinolysis-inducing compounds and reports pertinent experimental data.

The clot-dissolving potential of synthetic compounds should be primarily tested on human plasma clots. This is essential because the conventional purified systems have no resemblance to the complex composition of human blood in which the compounds would eventually have to act. Activity against human rather than animal clots was also required, because results obtained with animals cannot necessarily be translated into reliable information concerning the human fibrinolytic enzyme system.

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At the beginning of the studies, it was hoped only to find compounds that enhance the activity of the human plasminogen activator, urokinase. Such compounds were soon discovered with ethylurethan as a characteristic representative.[1] Its enhancing action on urokinase activity was recently confirmed.[2] However, ethylurethan alone, i.e. in absence of urokinase, induces marked fibrinolytic activity in human plasma.[1] The test system used at that time tried to simulate clots occluding narrow vessels: a cylinder-shaped human plasma clot was formed in a graduated glass tube and the solution of substances to be tested was applied on top of the clot in the (slowly rotating) tube, thus permitting the fibrinolytic activity to attack the clot only from one small side.[3] With this particular system it was noticed that the synthetic clot-dissolving agents induce a continuous progressive lysis of the plasma clot which goes on for days, in sharp contrast to the one obtained under the same experimental conditions by addition of fibrinolytic enzymes (in absence of synthetic compounds). Consequently, if the rate of clot dissolution induced by synthetic compounds is plotted against incubation time, a steeply rising straight line is obtained as compared to the rate induced by added fibrinolytic enzymes, which result in a curve that soon completely flattens out.[4]

This original procedure was relatively cumbersome and required skills in techniques with the blood-clotting system. Therefore, a simpler method permitting the easy screening of a larger number of compounds was developed, and the results reported in this paper are essentially based on this particular approach;[5] (see no. 1 under Material and Methods).

There exists a close relationship between the chemical structure of the compounds[6] and their clot-dissolving activity. By subsequent molecular modifications, the activity of the compounds was increased by a factor of approximately 1000 as compared to the compounds at hand at the beginning of the study.

## MATERIAL AND METHODS

*Fibrinolytic assays*

1. "*Hanging clot.*" With this method, a cylinder-shaped clot is formed by recalcification of human plasma at the end of a glass rod. The clot is then suspended on its glass rod in the solution to be tested. Complete dissolution of such a clot on incubation after a 24-hr period indicates a marked fibrinolysis-inducing capacity of the compound tested.[5]

2. "*Dilution clot.*" The compounds are dissolved at various concentrations in human plasma, and the plasma is subsequently clotted with thrombin or by $CaCl_2$ The complete dissolution of the clot on incubation within 24 hr indicates a marked fibrinolysis-inducing capacity.

3. "*Flat clot.*" One ml of human plasma is clotted by recalcification (1 ml plasma + 0·1 ml $CaCl_2$, 0·5 M). The clot is loosened from the test tube walls, 10 ml saline is added, and the test tube with clot and serum is centrifuged for 10 min at 34,000 *g* at 10°. This produces a flat-, disk-like clot which is then twice washed with buffered saline, followed by centrifugation for 10 min at 34,000 *g*.

Method 1 was the standard used throughout the investigation for basic screening and evaluation of each compound. Methods 2 and 3 were used to answer specific questions (see Discussion).

*Plasma.* The plasma was citrated human plasma, either obtained as outdated blood* (1 part ACD-solution to 4 parts blood) or freshly prepared (1 part 3·8% Na citrate USP to 4 parts blood) from healthy donors.

In all experiments with methods 1 and 2, aliquots of the plasma to be tested are run with the standard compound 3-(α, α-dimethylpropyl)salicylic acid. This was done to establish the responsiveness of the particular plasma, which might vary to some extent from plasma to plasma, most likely because of a difference in protein binding.

*Determination of the salicylates.* The binding of 3-(α, α-dimethylpropyl)salicylic acid Na was measured with a modification of the method of Potter and Guy.[7] The maximal fluorescence emission of this compound was at 420 mμ, when excited with a light source emitting a continuum of 300–400 mμ with a peak at 365.

*Compounds.*† All compounds were tested as sodium salts. For the hanging clot, the sodium salts were frequently prepared *in situ*; with the dilution clot method, the sodium salts had first to be prepared in the dry state. For testing, all compounds were dissolved in barbital acetate-buffered saline carefully adjusted to pH 7·4. (One part barbital acetate buffer,[8] pH 7·4, to 4 parts NaCl 0·85%; pH adjusted if necessary.)

## RESULTS

### *Fibrinolytic activity and structure*

Up to the present, over 800 compounds have been tested for their potential fibrinolytic activity. The concentrations required for activity ranged from 1·6 M with the earliest compounds to 0·9 mM with the most recent ones. Various compounds with activity below 50 mM were synthesized for the project with specific modifications of the molecule which, as expected, led to compounds with higher activity or gave additional information on structural requirements. The majority of compounds with high activity were salicylic acid or γ-resorcylic acid derivatives.

Structures and activity of various of these compounds are listed in Table 1, which shows that marked enhancement of fibrinolysis-inducing activity is achieved by appropriate substitution of a poorly active mother compound such as salicylic acid. Salicylic acid is only partially (no complete clot dissolution) active at 150 mM. Introduction of a methyl group in positions 3, 4, or 5 increases the activity at least threefold. The activity increases with the "bulkiness" of the substituent; for instance, it increases by a factor of approximately 150 with the introduction in the 3-position of the bulky 1,1,3,3-tetramethylbutyl group. Furthermore, substitution with halogens, in particular with bromine and iodine, enhances the activity substantially (3–8 times as compared to salicylic acid); indeed, double substitution with halogens in positions 3 and 5 boosts the activity about 100-fold. The increased activity resulting from double substitution in the 3- and 5-positions is also illustrated by the following example (not in the table): 3-isopropyl-6-methylsalicylic acid (*o*-thymotic acid) is active at 10 mM; the double-substituted 3-isopropyl-5-allyl-6-methylsalicylic acid (5-allyl-3-hydroxy-2-*p*-cymene-carboxylic acid) is active at 4 mM.

Substitution in the salicylic acid molecule with ring structures is another good approach to improve the fibrinolysis-inducing capacity, an unsaturated 6-ring in the

* The blood donations by the Belle Bonfils Memorial Blood Bank, Denver, are most gratefully acknowledged.

† The authors will provide the source of any compounds of interest on request.

TABLE 1. FIBRINOLYTIC ACTIVITY AND CHEMICAL STRUCTURES. EFFECT

| Congeners | 200 | 150 | 100 | 90 | 80 | 70 | 60 | 50 |
|---|---|---|---|---|---|---|---|---|
| Salicylic acid | (+) | (+) | − | | | | | |
| γ-resorcylic acid | − | | + | + | + | + | + | + |
| 3-methylsalicylic acid | − | | (+) | (+) | + | + | + | (+) |
| 4-methylsalicylic acid | | | (+) | + | + | + | + | − |
| 4-methyl-γ-resorcylic acid | − | | − | (+) | (+) | + | + | + |
| 5-methylsalicylic acid | − | (+) | + | + | + | + | + | − |
| 5-ethylsalicylic acid | | | (+) | (+) | + | + | + | + |
| 3-isopropylsalicylic acid | | | | | | | | |
| 3-*n*-butylsalicylic acid | | | | | | | | |
| 3-sec-butylsalicylic acid | | | | | | | | |
| 5-sec-butylsalicylic acid | | | | | | | | |
| 3-tert-butylsalicylic acid | | | | | | | | |
| 5-tert-butylsalicylic acid | | | | | | | | |
| 3,5-di-tert-butyl-γ-resorcylic acid | | | | | | | | |
| 3-(α,α-di-methylpropyl)salicylic acid | | | | | | | | |
| 3-(1,1,3,3-tetra-methylbutyl)salicylic acid | | | | | | | | |
| 5-(1,1,3,3-tetra-methylbutyl)salicylic acid | | | | | | | | |
| 5-(2′-cyclopentenyl)salicylic acid | | | | | | | | |
| 3-phenylsalicylic acid | | | | | | | | |
| 3-cyclohexylsalicylic acid | | | | | | | | |
| 4-phenylsalicylic acid | | | | | | | | |
| 5-phenylsalicylic acid | | | | | | | | |
| 5-cyclohexylsalicylic acid | | | | | | | | |
| 3-benzylsalicylic acid | | | | | | | | |
| 3-benzyl-γ-resorcylic acid | | | | | | | | |
| 3-(4-isopropylbenzyl)salicylic acid | | | | | | | | |
| 3-(2′-chlorobenzyl)salicylic acid | | | | | | | | |
| 3-(3′-chlorobenzyl)salicylic acid | | | | | | | | |
| 3-(4′-chlorobenzyl)salicylic acid | | | | | | | | |
| 5-benzylsalicylic acid | | | | | | | | |
| 5-benzyloxysalicylic acid | | | | | | | | |
| 5-(2′-chlorobenzyloxy)salicylic acid | | | | | | | | |
| 5-(3′-chlorobenzyloxy)salicylic acid | | | | | | | | |
| 3-chlorosalicylic acid | | | − | (+) | (+) | + | + | + |
| 4-chlorosalicylic acid | | | − | − | − | − | (+) | (+) |
| 5-chlorosalicylic acid | | | | | | | (+) | (+) |
| 3,5-dichlorosalicylic acid | | | | | | | | |
| 5-bromosalicylic acid | | | | | − | − | (+) | + |
| 3,5-dibromosalicylic acid | | | | | | | | |
| 5-iodosalicylic acid | | | | | | | | |
| 3,5-di-iodosalicylic acid | | | | | | | | |

No lysis, −; partial lysis, (+); complete lysis, +.

TABLE 2. FIBRINOLYTIC ACTIVITY AND CHEMICAL

| Congeners | 400 | 300 | 200 | 100 | 90 | 80 | 70 | 60 |
|---|---|---|---|---|---|---|---|---|
| 1,3-propanediol | − | − | − | − | | | | |
| 2,2-di-ethyl-1,3-propanediol | (+) | + | + | − | | | | |
| aminopyrine | − | − | + | − | | | | |
| 2,4-di-nitrophenol | | | | (+) | (+) | (+) | (+) | + |
| cincophen | | | | − | − | − | − | − |
| 1,2-di-phenyl-3,5-pyrazolidinedione | | | | | | | | |
| oxyphenbutazone | | | | | | | | |
| sulfinpyrazone | | | | | | | | |
| phenylbutazone | | | | | | | | |
| 2-methyl-5-methoxy-3-indolacetic acid Mg | | | | | | | | |
| indomethacin | | | | | | | | |
| di-phenylamine-4-sulfonic acid | | | | | | | | − |
| benzoic acid | − | − | − | − | − | − | − | − |
| 4-methyl benzoic acid | | − | (+) | (+) | | | | |
| 4-(α,α-di-ethyl)ethylbenzoic acid | | | | | | | | |
| 4-iso-propyl benzoic acid | | | | + | + | + | + | + |
| 4-tert-butyl benzoic acid | | | | | | | | |
| 4-iodophenylethoxyethoxyacetic acid | | | | | | | | |
| N-(4-iodophenyl)maleamic acid | | | | | | | | |
| 4,4-methylene-bis(3-hydroxy-2-naphthoic acid) | | | | | | | | |
| 2-naphthoic acid | | | | − | − | (+) | (+) | (+) |
| 3-hydroxy-2-napthoic acid | | | | | | | | |

No lysis, −; partial lysis, (+); complete lysis, +.

OF SUBSTITUTION ON ACTIVITY OF SALICYLIC AND γ-RESORCYLIC ACIDS

| 40 | 30 | 20 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 | 0·9 | 0·8 | 0·7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | − | − | − | | | | | | | | | | | | |
| − | − | − | − | | | | | | | | | | | | |
| − | − | | | | | | | | | | | | | | |
| + | + | (+) | − | | | | | | | | | | | | |
| − | | | | | | | | | | | | | | | |
| + | + | − | − | − | | | | | | | | | | | |
| − | + | + | + | + | + | (+) | − | − | − | | | | | | |
| − | − | − | + | + | + | + | + | (+) | (+) | − | | | | | |
| − | − | − | + | + | + | (+) | (+) | − | | | | | | | |
| − | + | + | + | + | + | + | (+) | − | − | − | | | | | |
| | | − | + | + | + | + | + | + | + | + | − | | | | |
| − | + | + | + | (+) | − | − | − | | | | | | | | |
| | | | | | | | | − | − | − | (+) | (+) | (+) | (+) | — |
| | | | — | (+) | + | + | + | + | + | + | (+) | − | | | |
| | | | | | | | − | − | − | (+) | (+) | (+) | + | − | − |
| | | | | | | | − | − | − | (+) | + | (+) | (+) | − | — |
| − | − | (+) | + | + | + | (+) | (+) | − | − | − | | | | | |
| | − | − | + | + | + | + | − | − | | | | | | | |
| | | | − | − | − | (+) | + | (+) | (+) | − | − | − | | | |
| | | − | + | + | + | + | + | − | | | | | | | |
| | − | − | (+) | (+) | + | + | + | − | | | | | | | |
| | | | | | − | − | + | + | + | + | (+) | − | | | |
| | | − | − | (+) | + | + | + | + | (+) | − | − | − | | | |
| | | | | | − | (+) | (+) | (+) | (+) | + | (+) | − | | | |
| | | | | | | | | − | (+) | + | + | − | | | |
| | | | | | | | | (+) | (+) | + | + | − | | | |
| | | | | | | | | − | (+) | (+) | (+) | − | | | |
| | | | | | | | | − | (+) | + | (+) | − | | | |
| | | − | + | + | + | + | + | + | + | − | − | − | | | |
| − | | (+) | + | + | + | (+) | (+) | − | | | | | | | |
| | | − | − | − | − | (+) | (+) | + | (+) | (+) | − | − | | | |
| | | | − | − | − | − | (+) | + | (+) | (+) | − | − | | | |
| + | + | − | − | | | | | | | | | | | | |
| + | + | + | − | | | | | | | | | | | | |
| + | + | − | − | | | | | | | | | | | | |
| (+) | (+) | − | − | | | | | | | | | | | | |
| | | | − | (+) | (+) | (+) | (+) | + | (+) | (+) | − | − | | | |
| − | (+) | + | − | | | | | | | | | | | | |
| | | | − | − | − | − | − | (+) | + | + | + | − | | | |

"Hanging clot" method; incubation time, 24 hr.

STRUCTURE; ACTIVITY OF VARIOUS COMPOUNDS

| 50 | 40 | 30 | 20 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 | 0·9 | 0·8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | + | − | − | − | | | | | | | | | | | |
| (+) | (+) | + | − | − | | | | | | | | | | | |
| (+) | (+) | (+) | − | − | | | | | | | | | | | |
| − | (+) | (+) | (+) | − | | | | | | | | | | | |
| − | − | − | − | + | (+) | (+) | − | − | | | | | | | |
| | − | − | − | + | + | + | − | − | | | | | | | |
| (+) | (+) | − | − | − | − | − | − | − | − | | | | | | |
| | | | − | (+) | (+) | (+) | (+) | + | + | (+) | − | | | | |
| — | (+) | (+) | (+) | — | | | | | | | | | | | |
| − | − | − | − | − | | | | | | | | | | | |
| | | | − | + | + | + | + | + | (+) | (+) | − | − | | | |
| + | + | (+) | — | | | | | | | | | | | | |
| | − | + | (+) | | | | | | | | | | | | |
| | (+) | + | + | − | | | | | | | | | | | |
| − | (+) | + | (+) | − | | | | | | | | | | | |
| | | | | | | | − | − | − | (+) | + | (+) | − | | |
| − | − | − | − | − | | | | | | | | | | | |
| | | − | (+) | — | | | | | | | | | | | |

"Hanging clot" method; incubation time; 24 hr.

3-position being the most effective substituent of this kind. When, in addition, substitutions are made on the benzene ring itself (3-*o*-chlorobenzylsalicylic acid and 3-*p*-isopropylbenzylsalicylic acid) in order to increase its "bulkiness", a further increase of activity results. One structural example for the increase of the fibrinolysis-inducing capacity of 3-substituted salicylic acid with the increase of the bulkiness of the substituent is shown in Fig. 1.

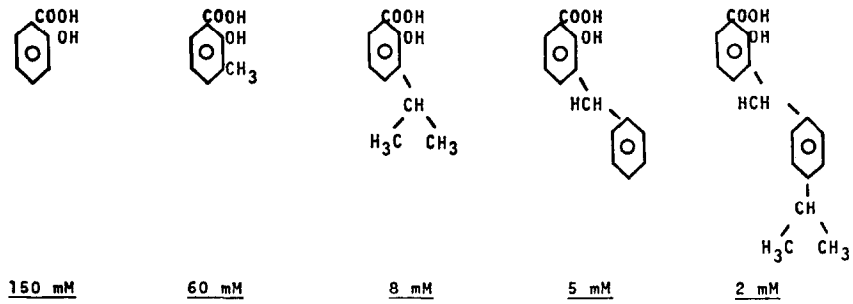


FIG. 1. Reduction of concentration required to induce fibrinolytic activity with increase of bulkiness of substituents. "Hanging clot" method. Incubation time 24 hr. Lowest concentration given at which compound dissolved clot. Salicylic acid dissolved clot only partially.

Introduction of a second hydroxyl group in the 6-position of the salicylic acid molecule leads to threefold increase of activity. The importance of the additional OH-group in the 6-position for enhancement of activity applies also for substituted salicylic acid: 4-methyl-$\gamma$-resorcylic acid is much more active than 4-methylsalicylic acid, and 3-benzyl-$\gamma$-resorcylic acid is more active than 3-benzylsalicylic acid. Of particular interest is the double-substituted 3,5-di-tert-butyl-$\gamma$-resorcylic acid, which is one of the most active compounds (at 0·9 mM) found so far.

The fibrinolysis-inducing capacity of synthetic organic compounds is not restricted to salicylic acid or $\gamma$-resorcylic acid derivatives. In fact, a major part of the earlier research was carried out with urea and urethan derivatives. Based on theoretical considerations (such as assuming hydrotropism as a necessary property of synthetic fibrinolysis-inducing agents[9]) the search was then extended to naphthoates and benzoates.[5, 9] The results with substituted benzoic acid led to investigations of salicylic acid and $\gamma$-resorcylic acid derivatives.

Recently, new groups of compounds have been observed to induce marked fibrinolytic activity. They are structurally not related to salicylates but share with them a number of biological and biophysical properties (see Discussion). Representative results with some of these compounds together with those of some benzoic acid derivatives and other agents are given in Table 2. Again it can be seen that substitution of a poorly active mother compound results in much more active derivatives.

Tables 1 and 2 show quite clearly one prominent feature of the synthetic fibrinolytic agents: no induction of fibrinolytic activity at higher concentration, good activity within a certain lower range, and loss of activity when the concentration is lowered further. The absence of activity at higher concentrations was an important finding which was only gradually appreciated. Indomethacin (Indocin) and phenylbutazone (Butazolidin) (see Table 2), for instance, were tested early in concentrations above 10 mM; no activity was found, and the compounds were thought to be inactive. And

yet, both of these antirheumatics are relatively active synthetic fibrinolytic agents.* This was brought to our attention by Roubal and Nemecek,[10] who found with the hanging clot method that indomethacin and phenylbutazone are active at 6 and 8 mM respectively. We were able, as seen in Table 2, to confirm their observations and to show, moreover, that removal of the butyl substituent in the 4-position of 3,5-dioxodiphenylpyrazolidine (phenylbutazone) reduces the activity to one fifth, and that the introduction of a hydroxy group in the 4-position (Tanduril) of phenylbutazone molecule reduces the activity by about 40 per cent. Likewise, removal of the *p*-chlorobenzo substituent in the 1-position of the indomethacin molecule abolishes its rather high activity altogether. This serves well to illustrate the importance of substitution for the fibrinolytic activity.

*Mechanism of action.* So far it has not been possible to demonstrate that the synthetic fibrinolytic compounds activate purified human plasminogen.[4, 11] However, as shown in Table 3, the antiactivator ε-aminocaproic acid inhibits completely at a

TABLE 3. INHIBITION OF CLOT DISSOLUTION INDUCED BY 3-(α, α-DIMETHYLPROPYL) SALICYLIC ACID BY ε-AMINOCAPROIC ACID

| | mM ε-aminocaproic acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 0·5 | 0·1 | 0·05 | 0·01 | 0·005 | 0·001 | 0 |
| 3-(α,α-dimethylpropyl)-salicylic acid, 6 mM | − | − | − | (+) | (+) | (+) | + | + |

No lysis, −; partial lysis, (+); complete lysis, +. "Hanging clot" method; incubation time; 24 hr.

concentration of 0·1 mM the fibrinolytic activity which is brought about by 3-(α, α-dimethylpropyl)salicylic acid in human plasma in the absence of this synthetic antiactivator.† This observation lends support to the theory that the synthetic fibrinolytic agents act by inducing activator activity in plasma which in turn is followed by plasminogen activation and subsequent proteolytic digestion of the fibrin matrix of the clot (see Discussion).

*Binding of active compounds to protein*

*Clot transfer studies.* When human plasma clots (which contain most of the available serum, in contrast to the "flat clot," see below) are exposed for a given length of incubation time to a buffered solution of synthetic fibrinolytic activity-inducing agents such as 3-(α, α-dimethylpropyl)salicylic acid and they are then transferred into an inert buffered saline not containing the activating compound, the clots dissolve on incubation exactly as if they were being left in the solution of the fibrinolysis-inducing agent. The required preincubation time of the clot in the compound solution decreases as the concentration of the compound increases. A representative experiment is shown in Table 4, from which it can be concluded that the synthetic fibrinolytic compound diffused into the clot and subsequently induced fibrinolytic activity.

* Recently R. J. Gryglewski and T. A. Gryglewska (Biochem. Pharmac. **15**, 1171, 1966) have shown with the hanging clot method[5] that some *N*-aryl-anthranilates which possess antiinflammatory properties also exhibit a marked fibrinolytic activity.

† A reaction of ε-aminocaproic acid Na with 3-(α, α-dimethylpropyl)salicylic acid Na is unlikely. If this had occurred, the fibrinolytic compound, being present in 60-fold excess, would have inactivated all the ε-aminocaproic acid. This was obviously not the case.

*Equilibrium dialysis.* To obtain information on the relation of protein binding to the fibrinolysis-inducing capacity of synthetic compounds, modified equilibrium dialysis experiments were carried out. For this purpose, the active compound was dissolved in human plasma to a concentration known from previous experiments to induce clot

TABLE 4. CONCENTRATION-DEPENDENT INCUBATION TIME REQUIRED FOR CLOT TO ABSORB ENOUGH COMPOUND FOR ITS DISSOLUTION

| Conc. (mM) of compound (3-(α,α-dimethylpropyl)-salicylic acid) | Preincubation (hours) of clot with compound before transfer to compound-free buffered saline | | | | | |
|---|---|---|---|---|---|---|
| | 0 | ½ | 1 | 2 | 3 | 4 |
| 5 | − | − | − | (+) | + | + |
| 6 | − | − | (+) | (+) | + | + |
| 10 | − | − | + | + | + | + |

No lysis, −; partial lysis, (+); complete lysis, +. "Hanging clot" method; incubation time; 24 hr.

dissolution (dilution clot method). Twenty mM of 3-(α, α-dimethylpropyl)salicylic acid was dissolved in human plasma and the plasma dialyzed (Visking tubing, 8 mm diameter) for 5 hr in the cold in a rotation dialyzer against 1000 volumes of citrated buffered saline. After 5 hr an equilibrium was obtained; the compound content of the plasma in the bag remained at about 6 mM and did not change appreciably when the dialysis was continued for 24 hr. The clots of this plasma, from which the unbound compound was removed by dialysis, dissolved on incubation, in spite of the fact that the concentration of 3-(α, α-dimethylpropyl)salicylic acid was only 6 mM, a molarity clearly insufficient to induce clot dissolution in undialyzed plasma.

*"Flat clot" experiment.* To evaluate further the relation between protein binding and induction of fibrinolytic activity by synthetic organic compounds, experiments were carried out with clots which contained a minimum of serum proteins other than fibrin. These clots were obtained, as described, by high-speed centrifugation, thus squeezing out the trapped serum. These flat clots were formed under two contrasting conditions: (a) clots obtained from plasma containing various concentrations of the active fibrinolytic activity-inducing compounds; (b) clots obtained from plasma not containing such compounds. The clots obtained from plasma in which the compounds were previously dissolved were washed and then transferred into buffered saline; the clots obtained from plasma containing no compounds were also washed and then transferred into buffered solutions of the active compound. Both flat-clot varieties dissolved on incubation, but at compound concentrations which differed markedly (Table 5). The concentration of the active compound required in plasma for inducing a dissolution of the isolated flat clot prepared from this compound-plasma is about nine times higher than the concentration required when the clot is first prepared from compound-free plasma and then subsequently exposed to the compound. A typical experiment is shown in Table 5. Both types (a and b) of flat clot had adsorbed the 3-(α, α-dimethylpropyl)salicylic acid, as shown by the fluorescence of the washed clots and by measurements of the compound in the solution after the clot (a) had lysed. Approximately 18·5–23 μg were bound per mg fibrin.

TABLE 5. PLASMA BINDING OF ACTIVE COMPOUNDS INCREASES THE AMOUNT REQUIRED FOR CLOT DISSOLUTION (a) SHARPLY ABOVE THE AMOUNT REQUIRED IN ABSENCE OF PLASMA (b)

| | mM Compound concentration | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 18 | 15 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 |
| (a) Clots made from plasma containing 3-(α,α-dimethylpropyl)salicylic acid transferred into buffered saline | + | + | (+) | − | − | − | − | − | − | − | − | − | − |
| (b) Clots made from compound-free plasma transferred into a solution of 3-(α,α-dimethylpropyl)salicylic acid | − | − | − | − | − | − | − | − | (+) | (+) | (+) | + | − |

No lysis, −; partial lysis, (+); complete lysis, +. "Hanging clot" method; incubation time; 24 hr. "Flat clot" method; 24-hr incubation. There was no lysis of compound-free control clots.

*Anticoagulant action.* All synthetic fibrinolysis-inducing agents checked for this particular feature display anticoagulant activity.[12] This is reflected by a complete inhibition of clotting and, with smaller concentrations, by a concentration-dependent prolongation of the recalcification time of undiluted plasma, or abnormal thrombelastograms. In general, compound concentrations required for anticoagulant activity

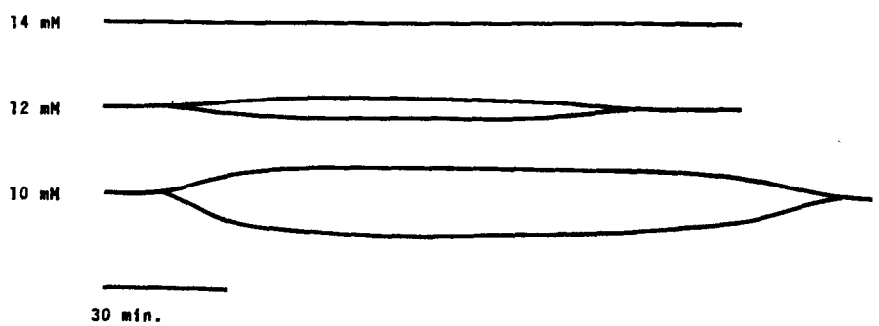


FIG. 2. Serial thrombelastograms of recalcified citrated dog plasma containing 3-(α, α-dimethylpropyl)-salicylic acid at concentrations indicated. Note inhibition of clot formation at 14 mM and clotting followed by clot dissolution at 12 and 10 mM.

are higher than the ones required for fibrinolytic activity. Figure 2 shows thrombelastograms of recalcified dog plasma containing 3-(α, α-dimethylpropyl)salicylic acid-Na which indicate that at 14 mM no clot can be obtained (and consequently no thrombelastogram), but at 12 and 10 mM, clots are formed which then dissolve spontaneously on incubation in a concentration-dependent rate. At the lowest concentration of 8 mM no fibrinolytic activity was evoked.

## DISCUSSION

Certain synthetic organic compounds induce dissolution of clots from human (or dog and guinea pig[13]) plasma by a true activation of the fibrinolytic enzyme system. This is demonstrated (1) by the fact that plasma which has been preincubated with the compounds induces digestion zones on bovine fibrin plates, whereas a solution of the compound alone does not;[4] (2) that such plasma digests casein;[4] and (3) that the anti-activator ϵ-aminocaproic acid abolishes fibrinolytic activity thus induced, probably by blocking a (released) activator.

Since it was not possible to observe a direct activation of human plasminogen by a wide range of compound concentration, the following working theory for a pathway of the activation of the plasma fibrinolysis system by the synthetic organic compounds is offered. The compounds are basically (enzyme) inhibitors. This is shown by inhibition of fibrinolytic activity in higher concentrations, inhibition of coagulation,[12] partial inhibition of antiplasmin,[14] and inhibition of complement C′.[14] The same structural modifications that abolish the fibrinolytic activity (shifting of an effective substituent from para- to ortho-position on the benzene ring, replacement of an effective substituent by an ineffective one, and introduction of a second hydrophilic group[14]), abolish also the effect on antiplasmin and complement C′.

Many of the fibrinolytic synthetic organic compounds inhibit various enzymes or enzymatic processes, among them oxidative phosphorylation,[15] glutamate decarboxylase,[16] and glutamic-pyruvic transaminase.[17] The order of activity in respect to the inhibition of the last two enzymes follows rather closely the order of activity of fibrinolysis induction. This comparison is shown in Table 6. Furthermore, it has been

TABLE 6. COMPARISON OF THE FIBRINOLYSIS-INDUCING CAPACITY OF SALICYLIC ACID DERIVATIVES WITH THEIR INHIBITION OF GLUTAMIN-PYRUVIC TRANSAMINASE AND GLUTAMATE DECARBOXYLASE

| | Fibrinolysis induced at: (mM) | Glutamin-pyruvic transaminase (% inhibition at 5 mM) |
|---|---|---|
| 3-cyclohexylsalicylic acid | 6 | 80·0 |
| 5-phenylsalicylic acid | 6 | 72·0 |
| 3-isopropylsalicylic acid | 8 | 61·5 |
| 6-hydroxysalicylic acid | 30 | 53·6 |
| 5-bromosalicylic acid | 30 | 53·3 |
| 1-hydroxy-2-naphthoic acid | 40 | 49·9 |
| 3-hydroxysalicylic acid | 50 | 42·6 |
| salicylic acid | 150* | 26·0 |
| | | Glutamate decarboxylase (% inhibition at 20 mM) |
| 3-cyclohexylsalicylic acid | 6 | 98 |
| 5-iodosalicylic acid | 20 | 68 |
| 6-hydroxysalicylic acid | 30 | 42 |
| 3-hydroxysalicylic acid | 50 | 11 |
| 3-methylsalicylic acid | 60 | 40 |
| 4-hydroxysalicylic acid | 140 | 16 |
| salicylic acid | 150* | 22 |
| 5-hydroxysalicylic acid | 0 | 7 |

* Incomplete lysis.

Note relationship of inhibitory activity to fibrinolysis-inducing capacity. The inhibition data have been taken from Refs. 16, 17.

pointed out that inhibition of chemical and enzymatic reactions is one of the common denominators of structurally unrelated, nonsteroid antirheumatic drugs.[18] The same compounds, e.g. salicylic acid derivatives, phenylbutazone, and indomethacin, induce fibrinolysis in human plasma.

The fibrinolysis-inducing compounds might inhibit an antiactivator by displacing it from a combination with activators, thus freeing the activator for plasminogen activation. Thirty years ago it was conclusively shown that a fibrin clot absorbs "trypsin"[19] and what was termed at that time a kinase, both of which substances may well be plasminogen activator and plasminogen in modern terms. It appears that adsorbed activator normally cannot activate the plasminogen, because it is inhibited by what is presumably an antiactivator. Since the synthetic fibrinolytic agents also exhibit, at least in the example shown in Table 5, a marked affinity to fibrin, it is likely that the compounds are adsorbed onto the fibrin, displace the antiactivator from adsorbed activator, and the fibrinolytic process begins. Fibrinolytic compounds have been shown, indeed, to displace substances bound to protein, e.g. γ-resorcylic acid displaces thyroxine bound to prealbumin,[20] and *p*-iodobenzoic acid, γ-resorcylic acid, and phenylbutazone all displace dyes bound to albumin.[21]

The binding of the compounds to fibrin appears to be an important feature required for their clot-dissolving activity. However, fibrinolytically active compounds are preferentially bound to albumin, and only when the binding sites of albumin are saturated do the compounds "spill over" to the globulins as shown for fibrinolytically active 3-methylsalicylic acid, 3-isopropylsalicylic acid, and 3-tert-butylsalicylic acid. Only 3-tert-butylsalicylic acid, which is the most active one of these three compounds, was found to bind to fibrinogen.[22] The greater affinity of the compounds for proteins other than fibrin determines how much of the compounds is "lost" for fibrinolysis induction and has a very marked influence on the concentration required for activity.

The reported data show clearly that there are numerous active synthetic fibrinolysis-inducing organic compounds. Further search for more effective fibrinolytic synthetic compounds may center around the following points: (1) synthesis of more potent compounds *per se*, with emphasis on double substitution; (2) discovery of compounds which are specifically bound to fibrin with little or no loss to albumin and other globulins; (3) the characterization of the binding sites of the active compounds both on albumin and on fibrin. This would help in better understanding the mechanism of the fibrinolytic induction by the synthetic organic compounds and thus contribute to the development of therapeutically feasible synthetic thrombolytic agents.

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