β_1C-Globulin and Synthetic Fibrinolytic Agents* (33498)

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Various synthetic organic asymmetric bulky anions will induce fibrinolytic activity when incubated with human or animal plasma (1, 2). The mechanism of this action does not involve activation of plasminogen by the compounds, but rather inactivation of inhibitors of the fibrinolytic enzyme system (1). Early pilot studies indicate that complement C′ activity was lost during this plasma incubation (3). This report concerns the effect of several recently discovered synthetic fibrinolytic compounds on β_1C-globulin (C′3 component of complement).

Material and Methods. Compounds tested. I. Those with no fibrinolytic action: Na salicylate (Merck); 3,5-di-iodo-4-hydroxybenzoic acid Na (Eastman). II. Those with weak fibrinolytic activity: Indomethacin (Merck Sharp & Dohme); phenylbutazone (Geigy). And III. those with strong fibrinolytic activity: (lowest effective fibrinolytic concentration given in m*M*): flufenamic acid Na (10 m*M*, Parke Davis); 3,5-diiodosalicylic acid Na (12 m*M*, Eastman); 3-1,1-dimethylpropyl)salicylic acid Na (14 m*M*, Miles Laboratories); 3-(*o*-chlorobenzyl)salicylic acid Na (14 m*M*, Behring-Werke). Fibrinolytic activity was defined as weak: when active in hanging clot system only (4); strong: when compounds dissolved in human plasma which was then clotted with thrombin, induced subsequent lysis of the clot at 37° in less than 24 hr (1).

Reagents. Epsilon-aminocaproic acid (Pierce Chemicals Co.); Ponceau Red (Buchler Instruments); agarose (Mann Research Laboratories); reagents for disc electrophoresis: Canalco.

Antisera. Antihuman sera from rabbits: Antialbumin, -cerulo-plasmin, -α_1-lipoprotein, -α_1-acid glycoprotein, -α_1-antitrypsin, -α_2-macroglobulin, -α_2-lipoprotein, -β-lipoprotein, -β_2-glycoprotein, -γ-globulin. These antisera were kindly donated by the Behring-Werke. From goats: Antiorosomucoid, Hyland; anti-β_1C/β_1A-globulin, Mann Research Laboratories. Highly specific antihuman β_1C/A serum was also provided by Dr. P. Kohler, Division of Immunology, University of Colorado Medical Center, Denver. Purified β_1C-globulin was donated by Dr. Mueller-Eberhard, Scripps Clinic and Research Foundation, La Jolla, California. Polyacrylamide disc electrophoresis was performed according to Davis (5) using the standard 7% gel and the Canalco-6 apparatus. Immunodiffusion was carried out in 1% agarose prepared in 0.06 *M* phosphate buffer, pH 7.1. Six ml of agarose used for each of the 75 × 50 mm microscopic slides. The agarose plates were kept for 48 hr at room temperature, were washed and then stained with Ponceau Red.

Immuno-electrophoresis was carried out according to Scheidegger (6) with a Buchler Electrophoresis apparatus at 45 V for 80 min in 2% agar Behring-Werke.

Hemolytic complement assay. Serum complement levels were expressed in $C'H_{50}$ hemolytic units according to Kabat and Mayer (7).

Hemolysin and guinea pig complement was kindly supplied by Dr. T. Ishizaka, Children's Asthma Research Institute, Denver, Colorado.

Preparation of serum. Blood was obtained from healthy donors, left standing at room temperature for 90 min and the serum was immediately separated by centrifuging at 1500*g* for 20 min at 4°. Serum was stored at 4° until used. The fibrinolytic compounds were dissolved in this serum at the concentrations indicated, and then incubated at 37° for various lengths of time. They were then immediately dialyzed (Visking cellophane tu-

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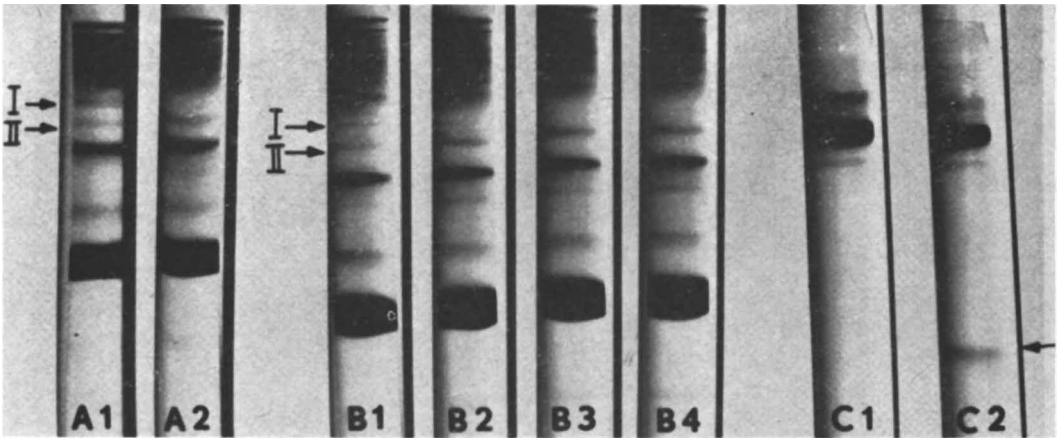


FIG. 1. Polyacrylamide gel disc electrophoresis of human serum and β_1C-globulin preincubated with synthetic fibrinolytic agents: 4 mA per each gel; 45 min run; A1, serum alone; A2, serum plus 3-(1,1-dimyethylpropyl)salicylic acid Na 12 m*M*; B1, serum alone; B2, serum plus flufenamic acid Na 12 m*M*; B3, serum plus flufenamic acid Na together with EACA 40 m*M*. B4, serum plus flufenamic acid Na together with EDTA 25 m*M*; C1, β_1C-globulin alone; C2, β_1C-globulin plus flufenamic acid Na 0.1 m*M*; arrow, the evolving new band. (see text).

bing 8-mm diam) for 16 hr with stirring at 4° against 1000 vol of barbital buffer (1 part)–saline (4 parts), pH 7.4. The control serum lacking fibrinolytic compounds was treated identically. After dialysis all tests were carried out at once.

Heat and ammonia treatment of sera. Serum was either heated for 10 min at 56° and rapidly cooled or treated with ammonia according to Kabat and Mayer (7).

Results. (a) Effect of synthetic agents on disc electrophoresis patterns of human serum. When serum was incubated with the strong fibrinolytic agents for varied time period [not less than approx 7 hr for 3-(1,1-dimethylpropyl)salicylic acid Na 15 m*M* and 3-(*o*-chlorobenzyl)salicylic acid Na 15 m*M* and about 24 hr for 3,5-diiodosalicylicacid Na 15 m*M*] at the concentrations indicated, changes in electrophoretic patterns were consistently the same. These are shown in Fig. 1. A distinct band (I in Fig. 1) located between the haptoglobulin region and the transferrin band faded out and was sometimes barely visible after serum incubation with the fibrinolytic compounds. In addition, another weak band (II in Fig. 1) located under what is left of band I and above the transferrin band became very distinct. These changes were the only distinct ones observed. They did not develop with the weak fibrinolytic agents. Indomethacin (12 m*M*) and phenylbutazone (12 m*M*) or the fibrinolytically ineffective Na salicylate (30 m*M*) and 3, 5-diiodo-4-hydroxybenzoic acid Na (15 m*M*) after 24-hr incubation did not produce any changes of the electrophoretic pattern. In some runs, in addition to the fibrinolytic compounds the serum was also incubated with EDTA 12.5 and 25 m*M* (to prevent inactivation of C'3 by aggregated γ-globulins in the event this should occur) and ϵ-aminocaproic acid 20 and 40 m*M* (to prevent activation of fibrinolysis during incubation). As shown B3 and B4 in Fig. 1, the presence of EDTA and EACA did not interfere with the compound-induced changes of the electrophoretic pattern of human serum.

(b) Identification of altered protein fraction. Pairs of control serum and compound-incubated serum were run as above on disc electrophoresis. One sample of each of them was stained and destained electrophoretically, whereas the twin sample remained unstained in glass sample tubing during the procedure (about 2 hr). The unstained bands do not appreciably diffuse during this time. After destaining, band I and II were located on the unstained samples by comparison with the stained ones, cut out and imbedded in

TABLE I. Effect of Synthetic Fibrinolytic Agents on Serum Complement C'H50 Titers.

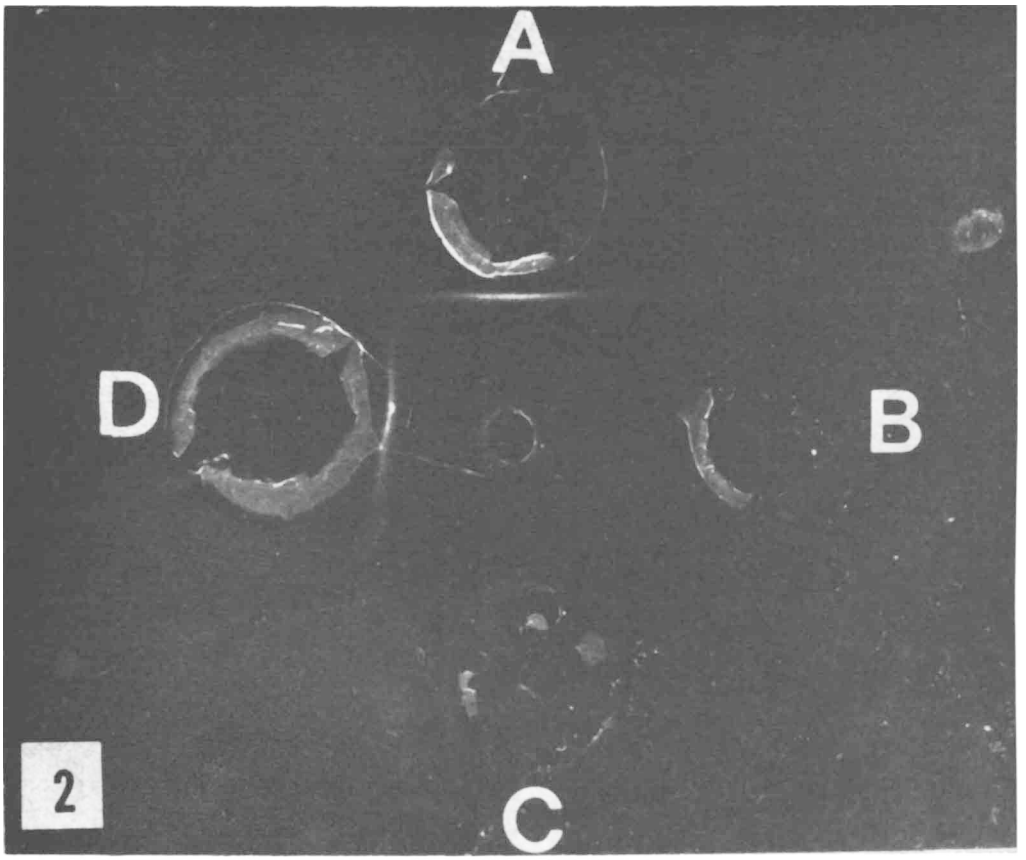
Compound in serum	Conc (m*M*)	Incubation time (hr)		
		0.75	2	7
Flufenamic acid Na	10	19	11	8
Flufenamic acid Na + EACA (20 m*M*)	10	26	23	14
Flufenamic acid Na + EDTA (25 m*M*)	10	26	19	12
3-(*o*-Chlorobenzyl)salicylic acid Na	12	—	—	4
3-(1,1-Dimethylpropyl)salicylic acid Na	12	28	26	20
3,5-Diiodosalicylic acid Na	15	—	30	17
3,5-Diiodo-4-hydroxybenzoic acid Na	15	—	42	38
Salicylic acid Na	30	43	40	39
Indomethacin	12	41	—	35
Phenylbutazone	12	39	—	39
No compound	—	45	40	39

melted agar on slides for immunodiffusion. The remainder of the unstained columns were stained to verify that band I and II were properly obtained and removed for immunodiffusion. All antisera listed under "Material and Methods" were run against band I and II. Only antihuman β_1C/β_1A-globulin reacted and gave precipitation lines with both bands. *Figure 2* gives the representative example for this reaction. Band I of the untreated serum (A in Fig. 2) and band II of the compound-incubated (flufenamic acid Na 10 m*M* 14 hr) serum (D) gave clear precipitation lines with anti-β_1C/β_1A-globulin. Both precipitation lines fused completely with each other. In contrast, band II of the control serum (B) and band I of the compound-incubated serum (C) did not give clear precipitation lines with the anti-β_1C/β_1A-globulin. This strongly suggests that most of the protein fractions located at band I in the control serum have changed their electrophoretic mobility and migrated to the position of band II in the compound-treated serum. Immunoelectrophoresis of the compound (flufenamic acid 10 m*M* 8 hr)-incubated serum and control serum with specific antihuman β_1C/β_1A-globulin confirms the results obtained with polyacrylamide disc electrophoresis. With the compound-incubated serum, there is a diminution of β_1C-precipitation line and the appearance of a new precipitation line, located further towards the anode which fuses with the β_1C-arc (Fig. 3, bottom pattern).

(c) Effect of synthetic fibrinolytic agents on isolated β_1C-globulin: 450 μg/ml of β_1C-globulin were incubated for 1 and 14 hr with 0.1 m*M* flufenamic acid Na and then subjected together with the control to polyacrylamide disc electrophoresis. The low concentration of the compound was adequate because losses from binding to inert proteins was not expected. One-hr incubation was without effect; however, after 14 hr there appeared a new distinct band (arrow, C2 Fig. 1) which migrates much more rapidly than the original major band. Concomittantly, there was a loss of intensity and width of the major band. The control did not exhibit any changes.

(d) Inactivation of serum complement C′ by synthetic fibrinolytic agents. The previous results indicate that the synthetic fibrinolytic agents induce physicochemical changes of β_1C-globulin. They might cause a loss of the complement C′ activity. Indeed, analysis of the compound-incubated sera revealed a marked drop of complement C′ activity with the stronger fibrinolytic compounds. The presence of either EDTA or EACA did not prevent this drop. The results are compiled in Table I.

(e) Partial restoration of complement C′ activity in compound-incubated serum by heat or NH_3-treated serum. Serum incubated for 7 hr with flufenamic acid 10 m*M* was divided after dialysis into three aliquots. To each aliquot either barbital buffered saline, heated fresh serum or ammonia treated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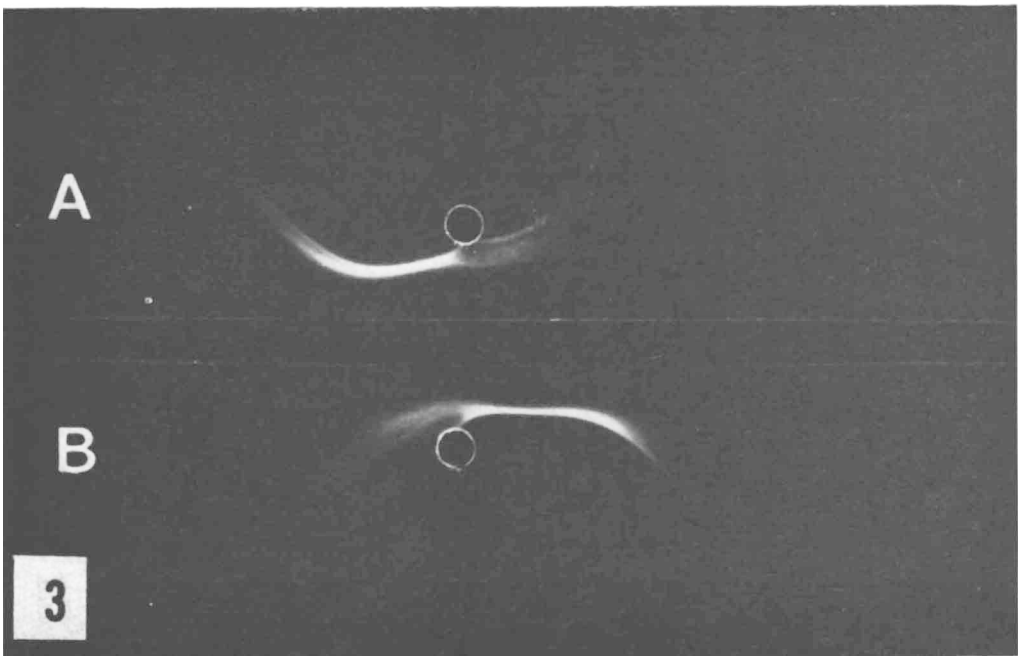
A
B
3

FIG. 2. Immuno-diffusion of protein fraction affected by synthetic fibrinolytic agent: A, Band I of control serum; B, Band II of control serum; C, Band I of serum incubated with 10 m*M* flufenamic acid Na; D, Band II of serum incubated with 10 m*M* flufenamic acid Na; center well, antiserum to human β_1C/β_1A-globulin.

FIG. 3. Immunoelectrophoresis of flufenamic acid incubated serum with specific antihuman B_1C/β_1A-globulin: A, control serum; B, compound serum; central trough, antiserum.

TABLE II. Complement C′ Activity of Compound-Incubated Sera Supplemented by Heat- and NH_3-Treated Sera (compound: flufenamic acid Na 10 m*M*).

Mixtures	C′H50 units/ml of mixtures
Normal serum and buffered saline	16
Compound-incubated serum and buffered saline	4
Compound-incubated serum and heated serum	10
Heated serum and buffered saline	≃0
Compound-incubated serum and NH_3-serum	7
NH_3-serum and buffered saline	1

serum was added in a ratio 1:1. Nontreated, heated, and ammonia-treated serum which served as controls, were mixed with the same proportion of the buffer. In all mixtures complement activity was determined. As shown in Table II, the complement C′ activity which was markedly reduced in the compound-incubated sera, was partially restored by either heat or NH_3-treated serum although these two sera have no complement C′ activity.

Discussion. Except for the induction of fibrinolytic activity and an abolishment of part of the antiplasmin activity, little is known of other effects on serum or plasma of synthetic fibrinolytic agents. The present investigation reveals that the only electrophoretically demonstrable effect of the compounds on serum proteins is an alteration of the β_1C-globulin, either in its native serum or as isolated fraction. β_1C-Globulin, C′3 component of the complement, has been shown by many investigators to be a labile complex protein molecule which is broken up into what appears to be subunits under various conditions (8–10). Therefore, its alteration by fibrinolytic compounds was not surprising. This change is brought about only by potent fibrinolysis-inducing compounds. Weak compounds, or originally potent compounds, transformed into inactive compounds by minor molecular modifications—such as the shifting of an OH group from the 2-position in 3,5-iodo-2-hydroxybenzoic acid (3,5-iodosalicylic acid) to the 4-position in 3, 5-iodo-4-hydroxybenzoic acid—do not cause any alterations. Such results might indicate that the compound-induced fibrinolytic activity in serum is involved in the alteration of the β_1C-globulin. However, the presence of EACA, known to block fibrinolysis induction by synthetic compounds (2) does not prevent this alteration. Furthermore, the compounds might have induced an aggregation of the γ-globulins. Although there is no evidence for this, it cannot be excluded. Such aggregation has been shown to cause C′3 inactivation through a mechanism similar to that of complement inactivation by the antigen–antibody complex (11). This type of inactivation of C′3 is prevented by 10 m*M* EDTA (11). Since the presence of EDTA did not interfere with the effect of the compounds on β_1C-globulin this pathway is unlikely, and a direct action of the compounds on this protein is probable. This conclusion is supported by the fragmentation of the isolated serum-free β_1C-globulin although the new band thus produced moved here faster than the one formed from the serum β_1C-globulin. Differences in the reaction of the isolated and serum β_1C-globulin under various experimental conditions have been described (8–10).

Inactivation of β_1C-globulin in the serum by the compounds without interaction with other components of the complement system was indicated by the partial restoration of total complement C′ activity by heated or ammonia-treated serum. Heated serum has no C′1 or C′2 activity but retains some of the C′3 activity (12). Ammonia-treated

the C'1, C'2 and C'3 activity (13).

Based on the above observations it is tentatively concluded that the synthetic fibrinolytic agents directly affect β_1C-globulin. Whether or not this process has any relation to the induction of the fibrinolytic activity in plasma or serum by these compounds remains an open question. Alteration of β_1C-globulin and induction of fibrinolytic activity require about the same incubation period with the compounds. The same molecular modifications in compounds which abolish their fibrinolytic activity, also abolish the action on complement C' (3) or on β_1C-globulin as shown in this study. Furthermore, it was suggested that some of the components of complement may play a role as activators or kinases in the proteolytic or fibrinolytic system of the blood (14, 15). It was shown that the antiserum against β_1C-globulin can inhibit spontaneous fibrinolytic activity (16). Correlations among these observations will be sought in future investigation. Thus far, there is no indication that modification of β_1C-globulin by these compounds is related in any way to their ability to suppress antiplasmin activity. It is, however, conceivable that the fibrinolytic compounds alter in a similar fashion the inhibitors and reduce their inhibition of fibrinolysis.

Summary. Synthetic organic fibrinolytic agents modify on incubation β_1C-globulin both in its isolated form and in serum. The compounds suppress complement C' activity of serum. Compounds chemically closely related to the fibrinolytic ones but with no fibrinolytic activity themselves do not affect β_1C-globulin or complement C'.

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