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Animal Plasma as Substrate for Synthetic Fibrinolysis Inducers.* (30693)

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Fibrinolytic, caseolytic and esterolytic activity is generated in human plasma in which certain synthetic organic compounds are dissolved and incubated. There is a close relationship between the chemical structure of a compound and its fibrinolysis-inducing capacity(1,2,3). All studies were carried out in human plasma. Attempts to evoke fibrinolytic activity in animal plasma with synthetic organic compounds gave either equivocal results or failed completely with the methods used(3,4). Animal plasma with a fibrinolytic system sensitive to synthetic organic compounds similar to that of human plasma would be of great assistance in the development of synthetic thrombolytic agents. This need, together with the availability of compounds more potent than the previously reported structures, prompted us to reinvestigate the use of animal plasma.

Material and methods. Plasma: Citrated plasma obtained from citrated blood (one part sodium citrate U.S.P. 3.8%, four parts blood) by spinning for 5 minutes at 1480 *g* was frozen in lots at —25°C until use within 3 weeks.

Buffered saline: One part barbital acetate buffer(5) pH 7.4 to 4 parts NaCl 0.85%.

Thrombin solution: 1000 units thrombin (Parke Davis) dissolved in 5 ml 50% glycerol.

Test of animal plasma for fibrinolytic activation by synthetic organic compounds: 3-isopropyl-6-methylsalicylic acid Na (o-thymotic acid Na) is dissolved to make a 20 mM concentration in a 0.4% solution of bovine fibrinogen Armour in buffered saline pH

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TABLE I. Effectiveness of Various Animal Plasmas in Inducing Lysis Zones After 24 hr Incubation on Unheated Bovine Fibrin Plates Containing 20 mM 3-isopropyl-6-methylsalicylic Acid Na (o-thymotic Acid Na). Average diameter of lysis zone in mm.

Animal	n	Run	Lysis zones			a.v. diameter
			Complete	Partial	None	
Dog	6	14	14	–	–	12.3
Guinea pig	4	13	13	–	–	12.8
Rat	4	11	7	1	3	6.2
Mouse	3*	5	2	1	2	9
Cat	3	10	3	5	2	11
Pigeon	3	9	1	7	1	14
Rabbit	3	8	–	–	8	–
Golden hamster	3	5	–	1	4	–
Goat	3	3	–	–	3	–
Ox	2	2	–	–	2	–
Horse	2	2	–	–	2	–
Monkey	2	6	–	–	6	–

* Pooled

7.4. The pH is adjusted to 6.9. The mixture is then clotted by a thrombin solution in a 9 cm-diameter flat-bottom Petri dish to give unheated fibrin plates. The plates are incubated for 24 hours at 37°C, after 0.03 ml of plasma to be tested have been applied. Positive results are indicated by lysis zones on the plates.

Tests for fibrinolysis-inducing capacity of synthetic organic compounds: a) Preformed standardized plasma clots obtained from recalcified citrated plasma(3) are introduced into a buffered saline solution of the compounds to be investigated and incubated for 24 hours at 37°C. Clot lysis is recorded as +: complete lysis; (+): partial lysis; —: no lysis. b) Clots formed in presence of the compounds: The compounds are dissolved in plasma to the maximum concentration shown for each compound (Table II). This plasma is then serially diluted with compound-free plasma to the desired range of concentration. 0.3 ml of each concentration is clotted with 0.01 ml thrombin solution and incubated for 24 hours at 37°C. Grading of lysis as for a.

Synthetic organic compounds: 3-isopropyl-6-methylsalicylic acid Na (o-thymotic acid Na) donated by Dr. James Sprague, Merck Sharp & Dohme Research Laboratories, West Point, Pa.

3-benzylsalicylic acid; donated by Dr. F. G. Henderson, Eli Lilly Co., Indianapolis.

3-tert-butylsalicylic acid and 3(α,α'dimethylpropyl)-salicylic acid; donated by Dr. O. E. Fancher, Miles Laboratories, Elkhart, Ind.

Sodium salts of the compounds were prepared.

Results. a) o-thymotic acid fibrin plates: Dog and guinea pig plasma invariably produced digestion zones when placed on fibrin plates containing 20 mM o-thymotic acid. Rat, mouse, cat and pigeon plasmas gave equivocal results, the main reason being that the digestion zones if they occurred at all were often incomplete with a non-lysed center and a lysed ring around it. The plasma of rabbit, golden hamster, goat, ox, horse and monkey (one Java monkey, one baboon) never produced any complete or incomplete lysis zones. The fibrin plate results are compiled in Table I. For each experiment controls with all plasmas were run on fibrin plates without o-thymotic acid, and they gave consistently negative results. Based on these observations, dog and guinea pig plasmas were chosen to be further evaluated for use as animal plasma in investigation of synthetic fibrinolysis-inducing agents.

b) Preformed standardized plasma clots: In our laboratories dissolution of preformed human plasma clots is a standard screening procedure for testing synthetic organic fibrinolysis-inducing compounds. This technique permits studies of any desired concentrations of the compound even when clotting would be affected by high concentrations of the compound. The effects of fibrinolysis-inducing salicylic acid derivatives on preformed standardized plasma clots made from dog plasma are shown in Table II. The re-

TABLE II. Fibrinolysis of Preformed Standard Clots of Recalcified Citrated Dog and Human Plasma Induced by 3-Substituted Salicylic Acid Derivatives. Incubation time 24 hours. Complete lysis: +; partial lysis: (+); no lysis: —.

Compound	Concentration in millimols of compound in buffered saline										Plasma clot source
	40	30	20	10	8	7	6	5	4	3	
3-isopropyl-6-methyl-salicylic acid Na (o-thymotic acid Na)	—	+	+	—							Dog 517
	+	+	+	—							" 563
	—	+	+	—							" 1616
	—	+	+	—							" 723
	—	+	+	(+)	—						Man
3-tert-butylsalicylic acid Na			—	+	+	+	(+)	(+)	—		Dog 517
			(+)	+	+	+	(+)	—	—		" 563
			(+)	+	+	(+)	—	—	—		" 1616
			(+)	+	+	+	+	+	—		" 723
			—	+	+	+	(+)	(+)	—		Man
3-benzylsalicylic acid Na						—	+	(+)	—	—	Dog 517
						—	+	(+)	—	—	" 563
						—	+	(+)	—	—	" 1616
						—	+	+	—	—	" 723
				+	+	+	+	+	+	(+)	Man
3-(α,α'dimethylpropyl)-salicylic acid Na			—	(+)	(+)	+	+	+	+		Dog 517
			—	(+)	(+)	+	+	+	(+)		" 563
			—	(+)	(+)	+	+	(+)	(+)		" 1616
			—	—	(+)	+	+	+	+		" 723
				(+)	+	+	+	+	(+)	—	Man

sults with human plasma using the same compounds are given for comparison. All four 3-substituted salicylic acids at concentrations of a few millimols induced complete dissolution of dog (and human) preformed plasma clots. There is also some relation between the structure of the substituent and its activity, indicating that the activity increases with the "bulkiness" (so far tested) of the substituent.

c) Clots formed in presence of the compounds: Guinea pig clots could not be tested with the preformed clot technique because it was not possible to remove them from the stand used to produce preformed clots of uniform size(3). Consequently, another technique had to be applied. As described in *Material and methods,* various concentrations of the compounds in dog and guinea pig plasmas were prepared and the plasma then clotted and incubated. The results were clear-cut. Dog or guinea pig plasma which clotted in the presence of 3-substituted salicylic acid derivatives dissolved on incubation. As with the preformed clot, 3-(α,α'dimethylpropyl)-salicylic acid Na was at least twice as active as 3-isopropyl-6-methylsalicylic acid Na. With the higher concentrations clot dissolution with dog plasma occurred earlier (not shown in Table III) than with guinea pig plasma. The concentrations required to induce dissolution of plasma clots formed in presence of the compounds were greater than the ones required for dissolution of preformed clots in which the compounds to be tested were dissolved in buffered saline rather than in plasma.

Discussion. The dissolution of human plasma clots by various synthetic organic compounds was previously studied primarily on preformed clots (clots obtained from plasma in absence of the compound). Clotting of plasma in presence of the compounds (*i.e.,* compounds dissolved in plasma prior to clotting) was in many instances not feasible because the required concentrations interfered with the clotting. With the growing insight into the relationship between chemical structure of an organic compound and its fibrinolysis-inducing capacity, compounds with increasing activity were found; 3-substituted salicylic acids proved to be one particularly promising group. The low, yet fibrinolytically active concentrations of these derivatives did not interfere with the clotting of plasma either by recalcification or by thrombin. This

TABLE III: Fibrinolysis of Dog and Guinea Pig Plasma Clots Formed in Presence of 3-Substituted Salicylic Acid Derivatives. Incubation time 24 hr. Complete lysis: +; partial lysis: (+); no lysis: —.

Compound	Concentration in millimols of compound in plasma													Plasma source	
	40	36	33	30	24	21	20	18	15	12	9	8	7		
3-isopropyl-6-methylsalicylic acid Na				+	+	—		—	—	—	—			Dog	000
				+	+	+		+	+	+	—			"	723
				+	+	+		—	—	—	—			"	517
	(+)	+	+		+		+	+		—		—		Guinea pig	204
(o-thymotic acid Na)	(+)	+	+		+		+	—		—				" "	512
				+	+	(+)	—							Man	
3-(α,α'dimethyl-propyl)-salicylic acid Na						(+)		(+)	+	+	(+)			Dog	736
								(+)	+	+	+			"	1564
								(+)	+	+	+	—	—	"	1443
						(+)		(+)	+	+	+	—	—	"	868
							+	+	+	+	(+)	—	—	Guinea pig	204
							(+)	(+)	+	+	+	+	—	" "	512
					(+)	+		+	+	+	—			Man	

feature had two advantages in testing animal plasma as a substrate for synthetic fibrinolysis inducers: the compounds could be incorporated into bovine fibrin plates, and the animal plasma in which they were dissolved was clottable. Unheated bovine fibrin plates containing a fibrinolytically active compound were instrumental in the screening of plasma of various species. The digestion zones obtained with dog and guinea pig plasma indicated that these species have a fibrinolytic system which is activated by the synthetic compounds. It is assumed that the compounds diffuse from the plate into the plasma, activate its fibrinolytic system which then, in turn, produces a digestion zone. Once suitable plasmas were found, they were investigated with the two methods used for evaluating prospective synthetic organic fibrinolysis inducers. Of these, the screening method based on the preformed plasma clot is more sensitive: complete clot dissolutions are obtained at lower concentrations of the compounds than with the plasma method. This is most likely due to the binding of the compounds to plasma proteins.

The results obtained with the dog and the guinea pig indicate that the plasmas of these two species are suitable as substrates for the fibrinolysis-inducing activity of synthetic compounds. These findings will be of importance when, with the development of more active compounds, the time will be ripe for *in vivo* studies. Dog and guinea pig plasmas react to the fibrinolysis-inducing synthetic compounds in a manner similar to human plasma. Both compounds also exhibit a particular phenomenon previously observed with human plasma: the absence or inhibition of fibrinolysis with higher compound concentrations. As with human plasma, fibrinolysis is induced in dog and guinea pig plasma within a certain range of concentration of the compounds; above and below this range no activity can be observed.

As a working hypothesis, it is thought that the synthetic fibrinolysis inducers are generally inhibitors. In higher concentrations, they may inhibit the fibrinolytic system in its entirety including the natural inhibitors. By lowering the concentration, plasminogen and plasmin activity are restored, but the inhibitors still are ineffective (inhibition of anti-activator?) and a fibrinolytic reaction results.

Summary. Dog and guinea pig plasmas are suitable substrates for the test tube investigation of the fibrinolysis-inducing activities of synthetic organic compounds. Four 3-substituted salicylic acids were used as synthetic fibrinolysis inducers. They produce a marked fibrinolytic activity in plasmas of both species at a concentration of a few millimols.

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