is secreted into bile, at least in part, as a mercaptide conjugate with glutathione.

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Received December 5, 1960. P.S.E.B.M., 1961, v106.

Urea Derivatives as Fibrinolysis Promoting Agents.* (26392)

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During the search for synthetic compounds to enhance the induction of fibrinolysis in human plasma by urokinase, Cibalgin,® an analgesic drug, was found to be active. Subsequent testing showed that enhancement was derived from 2 components, urethan and ethylurea. A variety of other urea derivatives was then found with similar properties. Controls revealed that some of these compounds slowly dissolved plasma clots in absence of urokinase. We therefore undertook to determine whether small amounts of such non-toxic chemicals could induce activation of the fibrinolytic enzyme system *in vitro*.

Material and methods. The preformed rotating standard clots were prepared from either fibrinogen or human plasma as previously described(1), except that a rotating device was used instead of a rocking device. This arrangement permits study of fibrinolysis promoting agents acting upon one well defined invariable surface of a preformed standardized clot. The disintegration products are continuously removed from the clot surface, and concentration of the agents being tested, in contrast to fibrin plate method, does not vary to a great extent. The progress of the clot lysis is read at intervals in terms of ml of digested clot.

Human plasma clots. Human ACD bank plasma (3 to 5 weeks old) or fresh plasma (1 part Na-citrate USP 0.129 M to 4 parts blood, centrifuged 5 minutes at 1500 g) was clotted by addition of 0.05 ml $CaCl_2$ (0.5 M) per ml for standard clots or by 0.01 ml thrombin for test tube studies. There was no consistent difference in the results of experiments using fresh or stored plasma.

Bovine fibrin standard clots. Armour bovine fibrinogen (fraction I from bovine plasma) was dissolved to 0.4% in borate buffer pH 7.4 and clotted by addition of 0.01 ml thrombin per ml fibrinogen solution.

* Supported by grants-in-aid from Am. Heart Assn., Inc., and Belle Bonfils Memorial Blood Bank, Denver.

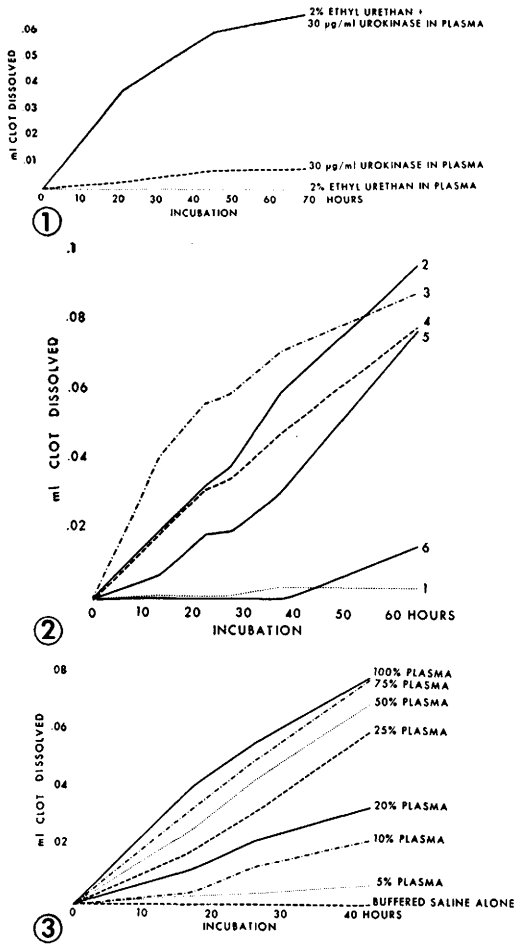

FIG. 1. Enhancement of urokinase-induced lysis of a preformed human plasma standard clot by ethyl urethan.

FIG. 2. Progressive dissolution of preformed human plasma standard clots by urea derivatives: allylurea (1), ethylthiourea (2), ethyl urethan (3), methyl urethan (4), thiourea (5), urethan (6). Note early and marked clot dissolution by ethyl urethan.

FIG. 3. Dissolution of a preformed bovine standard fibrin clot by 4% ethyl urethan in presence of increasing amounts of human defibrinated plasma. Note: Ineffectiveness of ethyl urethan in absence of human plasma and increasing activity with increasing plasma concentrations.

Bovine fibrin plates. Fibrinogen solution was prepared and clotted as above. 8 ml were used in flat bottom Petri dishes 9 cm diameter.

Defibrinated human plasma was prepared by addition of 0.01 ml thrombin per ml plasma. It was used to avoid deposition of fibrin from plasma on the surface of the standard clot, caused by thrombin diffusing out of the clot.

Urokinase was prepared as previously described(1).

Borate buffer. 0.25 M borate-saline buffer, pH 7.4(2).

Buffered saline. One part Na-barbital acetate buffer, pH 7.4, was mixed with 4 parts NaCl 0.85%.

Thrombin. Thrombin Topical Parke Davis, 200 U/ml in 50% glycerol.

Urea derivatives. These compounds, obtained from Eastman Kokak, Rochester, N. Y., or K & K Laboratories, Jamaica, N. Y., were dissolved in defibrinated human plasma or buffered saline and the solutions were adjusted when necessary to pH 7.4.

All studies were done at 37°C.

Results. 1) *Enhancement of action of urokinase on human plasma clots by urea derivatives.* Pilot studies indicated that among the urea derivatives tested, ethyl urethan best enhanced the urokinase-induced lysis of human plasma clots, followed by methyl urethan and ethylthiourea. Enhancement of urokinase-induced fibrinolysis of the preformed plasma clot by 2% ethyl urethan was clearly demonstrable (Fig. 1). The enhancement was also quite evident when plasma clots were formed in presence of 3% ethyl urethan and small amounts of urokinase. Controls, however, revealed that 3% ethyl urethan alone slowly dissolved the clot.

2) *Dissolution of human plasma clots by various urea derivatives.* Since it was observed that human citrated plasma clots formed by thrombin in presence of 3% ethyl urethan dissolved spontaneously after about 18 to 24 hours of incubation, other urea derivatives were similarly tested. Table I represents results of a characteristic experiment with urea derivatives which were effective at a concentration low enough to permit clotting of plasma by thrombin.

At the effective concentrations listed in Table I, only the plasma solution containing 2% ethyl urethan could be clotted with $CaCl_2$. This clot also dissolved as did those clotted with thrombin. Human plasma, freshly drawn with siliconized equipment and al-

TABLE I. Spontaneous Dissolution of Human Plasma Clots Formed in Presence of Various Urea Derivatives.

Compound	Conc., %	Molarity	Dissolution time, hr
Urethan	4	.449	40
Methyl urethan	3	.291	40
Ethylthiourea	3	.288	16
Butylurea	3	.258	16
Allylthiourea	3	.258	21
Ethyl urethan	2	.171	24
None	—	—	>72

lowed to clot spontaneously in glass tubes in presence of 2% ethyl urethan redissolved within 20 to 24 hours.

When preformed standard clot was used, the urea derivatives were dissolved in human defibrinated plasma at a concentration of 5%. The dissolution rates for the most active compounds are shown in Fig. 2. At this concentration, urea, methylurea, ethylurea, 1, 1-diethylurea, 1,3-diethylurea were inactive. In all experiments ethyl urethan exhibited the strongest clot-dissolving activity. It was also observed that ethyl urethan produced a slight turbidity after incubation with plasma, but clearing by centrifugation did not affect the results. The other urea derivatives produced various degrees of turbidity, including those that were inactive. Ethylthiourea, which showed good activity, produced no turbidity.

3) *Dissolution of bovine fibrin clots by ethyl urethan. Requirement of a plasma factor.* The most active compound, ethyl urethan, dissolved to 4% in buffered saline, was completely inactive on bovine fibrin plates or on standard clots formed with bovine fibrinogen. The clot-dissolving property could be restored when ethyl urethan was mixed before application with human plasma instead of buffered saline (other compounds were not tested). Fig. 3 reproduces results of a representative experiment with the bovine fibrin standard clot.

On bovine fibrin plates, the compound also became effective and produced marked lysed zones when mixed with plasma instead of buffered saline. Preincubation of ethyl urethan with plasma for about 4 hours was necessary to produce the maximal effect. Dialysis of the ethyl urethan-plasma mixture against citrated buffered saline removed the ethyl urethan, but only slightly reduced the fibrin dissolving activity of the non-dialysable material.

Discussion. It was first assumed that the plasma clot dissolution by urea derivatives was due to fibrin depolymerization alone. However, the inability of the compounds to dissolve thrombin-clotted fibrin consisting of fibrin s, soluble in 5 M urea(3), in absence of plasma, and dissolution of human plasma clots consisting of fibrin i (urea-insoluble) by some urea derivatives at concentrations much lower than 5 M, suggested that the compounds act by a different mechanism than that of urea. Juehling and Woehlisch (4) demonstrated induction of fibrinolytic activity in equine and porcine plasma incubated with urea and this plasma retained its fibrinolytic activity (demonstrated on clots obtained from pure fibrinogen) after the urea had been removed by dialysis. Identical treatment of a equine or porcine fibrinogen solution with urea did not produce the same phenomenon, so these authors specified the requirement of a plasma factor for this particular urea activity. Their critical urea concentration was 15%, whereas the critical concentrations of the urea derivatives in the experiments reported here were 2 to 4%.

The mechanism and factors involved in this chemically induced fibrinolytic activity of plasma and their relationships to *in vitro* activation of the fibrinolytic system by chloroform(5) or by acetone precipitation at acid pH(6) require further study. Retention of fibrinolytic activity after removal of an active compound by dialysis, and the necessity for presence of human plasma for dissolution of bovine fibrin indicate that a plasma factor is involved which might be related to proactivator.

To our knowledge it has not been previously reported that a non-enzymatic compound added to human plasma before clotting induced spontaneous dissolution of the clot. This experimental observation combined with use of the human standard clot may permit discovery of more active com-

pounds than those described here. Induction of fibrinolytic activity in human plasma by addition of such compounds may lead to new approaches for development of thrombolytic agents.

Summary. A number of urea derivatives added at low concentrations to plasma before or after clotting induce spontaneous dissolution of human plasma clots. They also considerably enhance the activity of urokinase. Ethyl urethan was the most active compound of the group. Bovine fibrin clots are dissolved by ethyl urethan only in presence of human plasma.

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Received December 9, 1960. P.S.E.B.M., 1961. v106.

Metabolism of 5-Hydroxyindole Compounds in Experimentally Produced Phenylketonuric Rats.*† (26393)

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The studies of Jervis(1) have shown that the primary biochemical defect in phenylketonuria consists of an inability to oxidize phenylalanine to tyrosine due to absence of the liver enzyme, phenylalanine hydroxylase. This results in an accumulation of phenylalanine in the blood and excretion of phenylketones in the urine. Armstrong and Robinson (2) and Ferari *et al.*(3) have demonstrated the presence of indolelactic acid in urine of phenylketonuric patients and suggested that there may also be a disorder of tryptophane metabolism in this disease. More recently, 3 different groups(4-6) have observed a significant lowering of 5-hydroxytryptamine in the serum and of 5-hydroxyindoleacetic acid in the urine of phenylketonuric patients. These changes are reversible when the patients are placed on a diet low in phenylalanine content. Pare *et al.*(7) have suggested that this disturbance of 5-hydroxyindole metabolism in phenylketonuria is due to an inhibition of 5-hydroxytryptophan decarboxylase by phenylalanine metabolites, a finding which has been confirmed by both *in vitro*(8) and *in vivo*(9,10) studies.

Auerbach *et al.*(11) showed that phenylketonuria could be produced experimentally in rats by feeding excessive amounts of both phenylalanine and tyrosine. This provided a means for studying the metabolism of the 5-hydroxyindole compounds in an *in vivo* system which is genetically normal.

Materials and methods. Phenylketonuria was produced experimentally according to the procedure described by Auerbach *et al.* (11). Weanling rats of the Sprague-Dawley strain were placed on stock diets and experimental diets as follows: 1) 3.75% L-tyrosine and 3.75% DL-phenylalanine for 3 months, 2) 3.75% L-tyrosine and 3.75% DL-phenylalanine for 2 months, 3) 3.75% L-tyrosine and 2.5% DL-phenylalanine for 2 months, and 4) 2.5% DL-phenylalanine alone for 2 months.

Twenty-four hour collections of urine were obtained weekly and phenylpyruvic acid was determined by the method of Berry and Woolf(12) and 5-hydroxyindoleacetic acid by the method of Udenfriend *et al.*(13). Blood samples were obtained by cardiac puncture at 6½ to 7 weeks and 9 weeks after start of the diet. The plasma was analyzed for 5-hydroxytryptamine using the method

* Aided by grants from Illinois Mental Health Fund and Public Health Service.

† The authors wish to express their appreciation to Dr. Harry Waisman for many helpful suggestions.