–SH occurring in severely injured rats (and mice) are not brought about by the transfer to, and storage of, such compounds in unaltered chemical form in tissues other than the liver.

Leaf and Neuberger(10) and Edwards and Westerfeld(11) have reported that by 24 hours after rats were placed on a low protein diet, a considerable decrease had occurred in the concentration of glutathione in the liver. Since animals subjected by us to scalding, trauma, or hemorrhage did not eat for several hours thereafter, it might be postulated that the low liver non-protein –SH values observed by us were due to protein deprivation. However, the changes observed by us have been most pronounced at three and six hours after infliction of injury or hemorrhage, whereas in the experiments of Leaf and Neuberger rats deliberately starved did not exhibit any appreciable decrease in liver glutathione concentration even at 24 hours after the beginning of the starvation.

It is hoped that future experimentation will elucidate some of the metabolic and/or hormonal mechanisms responsible for the decreases in concentration of non-protein –SH compounds which occur in certain tissues, especially the liver, following severe injury.

Summary. 1. Significant decreases in concentration of non-protein –SH were noted in rat liver following tumbling trauma, and in rat liver and kidney following severe scalding. 2. In analyses confined to the liver, a significant decrease in liver non-protein –SH was noted following severe hemorrhage. No appreciable change was noted in either liver protein –SH or extractable liver protein. 3. Blood non-protein –SH concentrations were practically identical for control and tumbled rats. Scalded rats exhibited a moderate increase in concentration of blood non-protein –SH, paralleling the hemoconcentration.

1. Beck, L. V., and Linkenheimer, W., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 291.

2. Grunert, R. R., and Phillips, P. H., *Arch. Biochem.*, 1951, v30, 217.

3. Weissman, N., Schoenbach, E. B., and Armistead, E. B., *J. Biol. Chem.*, 1950, v187, 153.

4. Peterson, R. D., Beatty, C. H., and West, E. S., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 747.

5. Robinson, H. W., and Hogden, C. C., *J. Biol. Chem.*, 1940, v135, 707.

6. Noble, R. L., and Collip, J. B., *Quart. J. Exp. Physiol.*, 1942, v31, 187.

7. Rosenthal, S. M., *Pub. Health Rep.*, 1942, v57, 1923.

8. Tabor, H., Kabat, H., and Rosenthal, S. M., *ibid.*, 1944, v59, 637.

9. Wiggers, C. J., *Physiology of Shock*, 1950, Harvard Univ. Press, Cambridge, Mass.

10. Leaf, G., and Neuberger, A., *Biochem. J.*, 1947, v41, 280.

11. Edwards, S., and Westerfeld, W. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 57.

Received May 21, 1954. P.S.E.B.M., 1954, v86.

Activation of Bovine Plasminogen by Trypsin.*† (21244)

H. D. JACKSON AND E. T. MERTZ. (Introduced by W. A. Hiestand.)

From the Department of Biochemistry, Purdue University, Lafayette, Ind.

Following the early observation that chloroform treatment increased the proteolytic activity of serum(1), chloroform was found to function by reacting with a protease inhibitor, thus allowing an autocatalytic type activation of the protease(2). The inactive zymogen of this protease system has been named plasminogen (profibrinolysin) and the active enzyme, plasmin (fibrinolysin). Plasmin functions in the blood coagulation scheme by splitting fibrinogen and fibrin to smaller fragments. Since chloroform, first used to activate plasminogen, is foreign to a biological system, attempts have been made to find biological

* Journal Paper No. 792, Purdue Univ. Agri. Exp. Station.

† This work was supported by contract between Purdue University and the Department of the Army.

activators of plasminogen. Activators of human plasminogen have been found in streptokinase(3-6), staphylokinase(7,8), various mammalian tissues(3,5,9), human urine(10), human blood(6,11), and trypsin(8,12,19). Activators of dog plasminogen include staphylokinase(5,7,13), various mammalian tissues (5), dog serum(14), but not dog plasmin (5,8,13,14), and trypsin(8). Bovine plasminogen is not activated by bacterial kinases (5,7), but is activated by kinases found in human milk(15), human urine(10), human blood(6,11), lung tissue of several species (5,16), pig heart(3,17,18), and trypsin (12,16).

Trypsin can activate bovine prothrombin (20) as well as plasminogen and thus appears to function as a kinase for 2 important zymogens of the blood. In the present report trypsin is compared with other proteases and with chloroform as an activator of bovine plasminogen. Several of the bovine plasmins were partially purified and obtained in lyophilized form.

Methods and materials. Crude plasminogen. Beef plasma was obtained frozen from a commercial source or prepared from fresh blood. In either case the blood was collected into oxalate solution(21) and centrifuged. The oxalated plasma was converted to serum by stirring 30 ml of 5.3% calcium chloride solution (added dropwise) into a liter of oxalated plasma. Fibrin precipitated in strands and stirring was continued for 30 minutes(22). The fibrin was removed on cheesecloth and the resulting serum was frozen in plastic containers at −20°C. Crude plasminogen was obtained from thawed serum by fractionation with ammonium sulfate at 0°C. Two hundred ml of ammonium sulfate solution (saturated at 25°C) was added dropwise to a chilled mixture containing 500 ml serum and 300 ml distilled water giving a solution of 0.82 M ammonium sulfate. If a precipitate formed, it was removed by centrifugation and discarded. Eighty grams of solid ammonium sulfate were then dialyzed into the chilled serum solution. After equilibrium, the contents of the dialysis tubing were added with stirring to the serum solution giving a final concentration of 1.38 M ammonium sulfate. The solution was stored overnight at 5°C. Following centrifugation the supernatant was discarded and the precipitate was taken up in distilled water, yielding approximately one-fifth of the original serum volume. This ammonium sulfate fraction was frozen until further use. Upon thawing, 100 ml of this fraction was mixed with 20 ml of pH 7.2 imidazole buffer(23). The buffered product was called crude plasminogen solution. These procedures were based in part on those of Loomis *et al.*(22). In our work, freezing the serum and the ammonium sulfate fraction was convenient, and in addition, yielded a more active product than could be prepared without these freezing steps. The frozen products were stable for as long as 4 months. *Activation to plasmin.* To cause an autocatalytic type of activation, or to increase the efficiency of a protease activator, chloroform was used as follows: 100 ml of crude plasminogen solution (pH 7.2) was mixed with 20 ml reagent grade chloroform in a separatory funnel and the mixture shaken intermittently for an hour at room temperature. The solvent layer was then drawn off, and excess chloroform and denatured protein removed in an angle centrifuge. In the chloroform treatment, the critical factor appears to be adequate mixing of chloroform and crude plasminogen during the one-hour period. After such treatment, the chloroform may remain in contact with the supernatant liquid, be drawn off, or removed with denatured protein by centrifugation, with no effect on the activation. Procedures used in chloroform treatment were based in part on those of other workers (22,24). The chloroform-treated crude plasminogen solution was activated to crude plasmin solution at 25°C either slowly with no further treatment, or rapidly with addition of a protease activator. Trypsin, chymotrypsin, ficin, pancreatin, papain, and plasmin were tested for their ability to activate the plasminogen solution by adding them in the dry form. In the routine procedure with a protease activator the zero time fibrinolytic activity was measured just after the addition of the protease. This zero time activity, due to the protease activator, was subtracted from the total fibrinolytic activity obtained at later

time intervals as a measure of the plasmin produced. *Plasmin assay.* Assays were conducted by the method of Guest *et al.*(25), who define one fibrinolytic unit as the amount of protease activity which will completely lyse one cc of a standardized fibrin clot in 120 seconds at 28°C(26). *Plasmin purification.* The maximally activated crude plasminogen solutions were partially purified by 40-fold dilution with distilled water and precipitation at pH 5.5 with 0.1 *N* H_2SO_4. The preparation was stored overnight at 5°C and the supernatant decanted. The precipitate was centrifuged and the supernatant rejected. The plasmin precipitate was taken up in 0.066 *M* phosphate buffer, pH 7.5, to approximately one-tenth of the original serum volume and clarified by centrifugation. The procedure of isoelectric precipitation was repeated and the purified plasmin was again taken up in the phosphate buffer, shell frozen and lyophilized. These procedures were modified from those presented by other workers(22,24,27). Portions of the lyophilized plasmins were redissolved in phosphate buffer (10 mg/ml) and, after 20-30 minutes, were assayed for units of plasmin (fibrinolytic) activity. The total fibrinolytic activity of the dry products was assumed to be plasmin activity, since the plasmin was precipitated twice at pH 5.5 and trypsin was very soluble at this pH. Total nitrogen of the samples was determined by the micro-Kjeldahl method(28).

Results. The activation of crude plasminogen to plasmin was followed by the fibrinolytic assay method over a period of time to obtain the maximum increase in activity. This increase was recorded as plasmin activity. Such a method has been used previously by other workers who activated human, dog, and bovine plasminogen with trypsin(8,12,16). Although the fibrinolytic assay method does not distinguish between trypsin and plasmin, the increased fibrinolytic activity observed is most easily explained by attributing it to the trypsin activation of crude plasminogen.

In our activation studies, trypsin caused activation of crude bovine plasminogen as reported previously(12,16). However, data (Table I) show that sufficient trypsin must be present to overcome the trypsin inhibitor in the crude plasminogen, or plasmin will not be produced. Jacobsson(19) observed a similar trypsin inhibitor in his studies with human plasminogen. Our data show that if sufficient trypsin is present to cause maximum activation, increased trypsin decreases the time for the conversion; the data suggest that trypsin functions enzymatically in this conversion. With excessive levels of trypsin, the increase in plasmin activity is not observed (Table I). In these cases the conversion may be so rapid that the zero time activity measurement includes both trypsin and plasmin activity.

TABLE I. Activation of Crude Bovine Plasminogen by Trypsin.

Trypsin conc. (mg/ml crude plasminogen sol.)	Zero time activity† (fibrinolytic units/ml crude plasminogen sol.)	Time to max activation (hr)	Plasmin produced (increased units/ml crude plasminogen sol)
.2	0	—	0 (21 hr)
.4	4.5	2	20
.6	15	1	32
.8	32	½	29
1.0	28	¼	31
1.1	27	¼	28
1.2	45	¼	24
1.4	57	<¼	1 (15 min.)
.05*	0	4½	17
.10*	0.7	4½	21
.15*	5.6	2	24
.2 *	6.2	1¼	24
.3 *	16	½	28
.4 *	11	½	25
.5 *	15	½	23
.6 *	16	¼	22
.8 *	32	¼	25
1.0 *	72	<¼	0 (15 min.)

* After chloroform treatment of crude plasminogen solution.
† Due to trypsin activator.

If the solution of crude plasminogen is first treated with chloroform, the amount of trypsin required for maximum activation is reduced (Table I). Thus, either chloroform or excess trypsin eliminates the blocking effect of trypsin inhibitor in the crude plasminogen. Since trypsin activation was more efficient with the chloroform-treated crude plasminogen solution, this solution was used as the substrate in the testing of other proteases as activators. The proteases tested (chymotrypsin, ficin, pancreatin, papain, and partially purified bovine plasmin) were used in amounts

TABLE II. Activation of Crude Bovine Plasminogen by Various Activators.

Activator†	Conc. (mg/ml crude plasminogen sol.)	Zero time activity‡ (fibrinolytic units/ml crude plasminogen sol.)	Time to max activation (hr)	Plasmin produced (increased units/ml crude plasminogen sol.) (hr)
Chloroform	Saturated	0	100–120	19
Trypsin	1.0	17.3	6	88
Trypsin	.1*	2.5	6	62
Chymotrypsin	.1*	3.0	—	0 (6)
Ficin	1.0*	1.0	—	0 (6)
Pancreatin	5.0*	1.5	4	55
Papain	5.0*	1.0	—	0 (6)
Plasmin (chloroform activated)	25.0*	1.0	48	16

* After chloroform treatment of crude plasminogen solution.

† Source of activator: Chloroform—"Baker Analyzed" Reagent. Trypsin—Armour crystallized trypsin of bovine origin, not more than 50% $MgSO_4$. Chymotrypsin—Crystallized 3 times. We wish to thank Mr. H. N. Wood and Dr. A. K. Balls for this preparation. Ficin—Bios Laboratory, Inc. Pancreatin—U.S.P. Nutritional Biochemicals Corp. Papain—Nutritional Biochemicals Corp. Plasmin—Prepared in the laboratory using chloroform activation.

‡ Due to enzyme activator.

which had approximately the same fibrinolytic activity per ml of chloroform-treated crude plasminogen solution (at zero time) as 0.1 mg of trypsin. When tested over a period of time, fibrinolytic (plasmin) activity increased when trypsin, pancreatin, or partially purified plasmin were used as activators, but did not increase when chymotrypsin, ficin, and papain were used (Table II). Chloroform alone was tested on this lot of crude plasminogen and produced approximately the same increased level of plasmin activity (19 units/ml) as did partially purified bovine plasmin (16 units/ml). Trypsin was the best activator tested.

The data in Tables I and II were obtained by similar methods, but are not directly comparable since different lots of crude plasminogen were used. Separate portions of a third lot of crude bovine plasminogen were activated by trypsin alone, trypsin following chloroform treatment, and chloroform treatment alone. In all cases the activation was followed to maximum activity, at which time the active plasmin was purified by isoelectric precipitation at pH 5.5, and lyophilized as described under *Methods*. The trypsin used for activation of plasminogen was very soluble at pH 5.5.

The lyophilized products were compared on the basis of purity and yield. The data in Table III show that the plasmin activity per mg of nitrogen, and the plasmin yield per liter of serum were higher with both types of trypsin activation than with chloroform activation. It is interesting to note that the differences between chloroform-trypsin and trypsin activation are apparent in the crude solutions (Tables I and II) but are not apparent in the lyophilized plasmins (Table III).

When plasmin was lyophilized from phosphate buffer, pH 7.5, the dry preparations retained all of their activity when stored for 2 weeks in a dessicator *in vacuo* at −20°C. When stored at room temperature for 2 weeks in a screw-top bottle, however, only one-third of the plasmin activity was retained.

Summary. Crude bovine plasminogen was activated to plasmin by trypsin, by chloroform treatment which allowed an autocatalytic type activation, or by chloroform treatment followed by rapid activation with a low level of trypsin. Since the last method was an efficient one, other proteases were tried in the place of trypsin. Pancreatin and partially purified bovine plasmin also activated crude plasminogen but chymotrypsin, ficin, and papain did not. Trypsin was the best activator of the series tested. Partially purified plasmins activated by trypsin, chloroform, or chloroform-trypsin were lyophilized and compared with one another. The plasmins prepared by trypsin activation appeared to be more potent

TABLE III. Comparison of Dry Bovine Plasmins Prepared with Different Activators.

Activator	Trypsin conc. (mg/ml crude plasminogen sol.)	Time to max activation (hr)	Activity of plasmin† (units/mg N)	Yield of plasmin† (units/l serum)
Trypsin	1.0	¼	5.0	1390
	1.0	¼	5.5	1500
	.8	½	5.5	1340
Trypsin*	.4	½	4.5	1510
	.4	½	5.0	1640
	.2	¾	6.0	2310
Chloroform	.0	300	.8	230
	.0	194	.6	225
	.0	194	.9	320

* After chloroform treatment of crude plasminogen solution.
† After separation from trypsin by ppt 2 times at pH 5.5.

than those prepared by chloroform activation alone.

The authors wish to acknowledge the assistance of Mrs. Shirley A. Duffy in conducting these experiments.

1. Delezenne, C., and Pozerski, E., *Compt. Rend. Soc. Biol.*, 1903, v55, 690, 693.
2. Christensen, L. R., *J. Gen. Physiol.*, 1946, v30, 149.
3. Astrup, T., *Acta Physiol. Scand.*, 1951, v24, 267.
4. Christensen, L. R., *J. Gen. Physiol.*, 1945, v28, 363.
5. Lewis, J. H., and Ferguson, J. H., *J. Clin. Invest.*, 1950, v29, 1059.
6. Mullertz, S., and Lassen, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 264.
7. Lewis, J. H., and Ferguson, J. H., *Am. J. Physiol.*, 1951, v166, 594.
8. ———, *ibid.*, 1952, v170, 636.
9. Tagnon, H. J., and Petermann, M. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 359.
10. Astrup, T., and Sterndorff, I., *ibid.*, 1952, v81, 675.
11. Mullertz, S., *ibid.*, 1953, v82, 291.
12. Kocholaty, W., Ellis, W. W., and Jensen, H., *Blood*, 1952, v7, 882.
13. Lewis, J. H., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 677.
14. ———, *ibid.*, 1951, v78, 184.
15. Astrup, T., and Sterndorff, I., *ibid.*, 1953, v84, 605.
16. ———, *Nature*, 1952, v170, 981.
17. Astrup, T., *Biochem. J.*, 1951, v50, 5.
18. Astrup, T., and Stage, A., *Nature*, 1952, v170, 929.
19. Jacobsson, K., *Acta Chem. Scand.*, 1953, v7, 430.
20. Kleinfeld, G., and Habif, D. V., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 432.
21. Seegers, W. H., and Smith, H. P., *J. Biol. Chem.*, 1941, v140, 677.
22. Loomis, E. C., George, C., and Ryder, A., *Arch. Biochem.*, 1947, v12, 1.
23. Mertz, E. T., and Owen, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, v43, 204.
24. Rocha e Silva, M., and Rimington, C., *Biochem. J.*, 1948, v43, 163.
25. Guest, M. M., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, v150, 661.
26. Guest, M. M., Daly, B. M., Ware, A. G., and Seegers, W. H., *J. Clin. Invest.*, 1948, v27, 785.
27. Remmert, L. F., and Cohen, P. P., *J. Biol. Chem.*, 1949, v181, 431.
28. Association of Official Agricultural Chemists, *Methods of Analysis*, 1945, A.O.A.C., Washington, D.C.

Received June 4, 1954. P.S.E.B.M., 1954, v86.