meromyosin-end is fragmented in the sonic field. These heavy meromyosin fragments will then be attached to the thinner portion of the L-myosin. In this way the molecule becomes less asymmetric, thus explaining the increase in its sedimentation and the simultaneous decrease in its viscosity.

It is interesting to compare mechanical and chemical degradations. SZENT-GYÖRGYI AND BORBIRO[6] treated the meromyosins with urea and found that light meromyosin depolymerized into small subunits, whereas heavy meromyosin did not undergo such a splitting. On the contrary, in case of ultrasonic treatment, the light meromyosin remained nearly unchanged compared with the pronounced breakdown of heavy meromyosin. These findings clearly indicate that the mechanism of the mechanical and chemical degradations are different.

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[1] E. MIHÀLYI AND A. G. SZENT-GYÖRGYI, *J. Biol. Chem.*, 201 (1953) 189, 197, 211.
[2] W. KIELLEY AND W. F. HARRINGTON, *Biochim. Biophys. Acta*, 41 (1960) 401.
[3] S. LOWEY AND A. HOLTZER, *J. Am. Chem. Soc.*, 81 (1959) 1378.
[4] R. V. RICE, *Abstr. Biophys. Soc.*, 7th Annual Meeting, 1963, New York.
[5] S. LOWEY AND C. COHEN, *J. Mol. Biol.*, 4 (1962) 293.
[6] A. G. SZENT-GYÖRGYI AND M. BORBIRO, *Arch. Biochem. Biophys.*, 60 (1956) 180.

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Quantitative estimation of urokinase and bacterial plasminogen activators using a fibrin substrate formed from plasminogen-free fibrinogen

A quantitative fibrinolytic method for estimating bacterial activators of plasminogen (streptokinase and staphylokinase) has been described using as a source of fibrin commercial bovine fibrinogen[1]. Briefly, the method consists in clotting a mixture of the activator, bovine fibrinogen and human plasminogen with human thrombin. The clots are incubated at 37° for 1 h, activation of plasminogen to plasmin taking place simultaneously with fibrinolysis. Any undigested fibrin is dissolved in strong urea solution thus releasing from within the clot products of plasmin digestion. The fibrin is precipitated with trichloroacetic acid and acid-soluble products of fibrinolysis determined spectrophotometrically in the filtrate.

Although commercial samples of bovine fibrinogen are contaminated with bovine plasminogen this proenzyme is not activated by staphylokinase[2], the resulting fibrinolysis in the fibrinolytic method being entirely due to human plasmin formed from the activation of the added human plasminogen. Streptokinase, however, forms an activator of bovine plasminogen due to combination with proactivator present in the added human plasminogen, while urine activator (urokinase) activates bovine plasminogen directly. Although the use of commercial fibrinogen in the quantitative fibrinolytic method produces useful results in the investigation of streptokinase and urokinase[3], the fact that fibrinolysis is due to the action of both bovine and human plasmin is undesirable since there is no proof that plasmins formed from different species of plasminogens by these activators are identical. Further, commercial samples of bovine fibrinogen vary in their plasminogen content leading to results which may be ambiguous.

For these reasons the quantitative fibrinolytic method has been modified for estimating urokinase, using plasminogen-free fibrinogen as a source of fibrin. Details of the method, which will also estimate bacterial activators, are as follows.

Borate–saline buffer (0.2 M, pH 7.4), glycine buffer (0.1 M, pH 9.5), urea solution (40 %, w/v, in glycine buffer) and trichloroacetic acid (20 %, w/v) were prepared as described previously[1].

One ampoule of bovine thrombin (Parke, Davis and Co., Detroit, Mich., U.S.A.) was dissolved in 10 ml of 50 % (v/v) glycerol in water. This stock solution (500 units/ml) was stored at —20° and diluted with borate–saline buffer to the required concentration immediately before use.

A glycoprotein fraction isolated from rabbit urine by a benzoic acid adsorption method[4,5] was used as a source of urokinase. This preparation was found to contain all the urine activator[6].

Bovine plasma Fraction 1 (Armour Pharmaceutical Co., Ltd.), containing about 40 % sodium citrate and 60 % fibrinogen, was used as a solution (10 %, w/v) in borate–saline buffer as the source of bovine fibrinogen, the fibrinogen concentration being about 6 % (w/v). Plasminogen-free bovine fibrinogen was prepared from commercial bovine fibrinogen by a simple and quick method[7] designed to remove all detectable plasminogen, using the following modifications: All manipulations were carried out below 2°. Commercial bovine fibrinogen (20 g) was dissolved in 0.3 M NaCl (200 ml) and dialyzed exhaustively against 0.3 M NaCl to remove sodium citrate. Fibrinogen was then precipitated at pH 7.0 by addition of ε-aminocaproic acid (to 0.1 M) followed by ethanol (to 7 %, v/v). The precipitate was centrifuged down, re-dissolved in 0.3 M NaCl (200 ml) and the precipitation repeated. The fibrinogen precipitate was dissolved in 0.3 M NaCl, dialyzed against 0.3 M NaCl to remove traces of ε-aminocaproic acid and freeze-dried. This process yielded 10.2 g of solid containing about 32 % NaCl and 68 % fibrinogen. For use in the quantitative fibrinolytic method this preparation was suspended in borate–saline buffer at a concentration of 5 % (w/v), incubated at 37° for 1 h and the small amount of insoluble material centrifuged down. The supernatant fluid, which was used as the source of fibrin, contained 95 % (w/w) of the preparation, the fibrinogen concentration being about 3.2 % (w/v).

Into a series of tubes (T) were measured increasing amounts (0–2 mg) of the rabbit glycoprotein preparation dissolved in borate–saline buffer (0.5 ml). To each tube was added plasminogen-free bovine fibrinogen solution (0.5 ml, fibrinogen concen-

tration 3.2 %), human plasminogen (0.2 ml, $38 \cdot 10^{-3}$ absorbancy units using the unit of activity defined by DAVIDSON[8]) and bovine thrombin (0.1 ml, 2 units). These additions were made in the order given and as rapidly as possible, mixing thoroughly after each addition. A second series of tubes (B) contained the same components except that human plasminogen was replaced by 10 mN HCl (0.2 ml). After incubation of the tubes (37°, 1 h) they were placed in ice-water, chilled urea solution (3 ml) added and any undigested fibrin clot broken up with glass stirrers and allowed to dissolve with occasional agitation (about 1 h). Undigested fibrin was then precipitated with trichloroacetic acid (2 ml) and the mixtures filtered through Whatman No. 1 filter paper (5.5 cm diameter) previously washed with 10 % $HClO_4$ followed by water, and drying. The acid-soluble products of fibrinolysis were then estimated spectrophotometrically in the filtrates at 280 mμ in 1-cm cells using a Unicam S.P. 500 spectrophotometer against a reagent blank consisting of a mixture of 1.3 ml borate–saline buffer, 3 ml urea solution and 2 ml trichloroacetic acid.

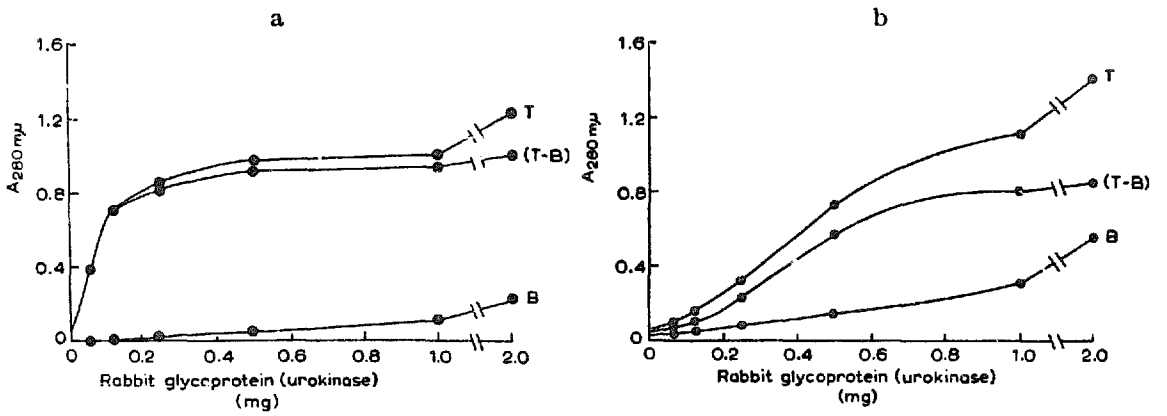

Fig. 1. The estimation of urokinase using, as plasmin substrate, fibrin formed from (a) plasminogen-free bovine fibrinogen and (b) commercial bovine fibrinogen. The curves designated by "T" represent fibrinolysis, expressed as absorbancy values, resulting from the incubation (37°, 1 h) at pH 7.4 of fibrin clots containing increasing amounts of a rabbit-urine glycoprotein preparation and constant amounts of bovine fibrinogen, human plasminogen and bovine thrombin. The curves designated by "B" represent fibrinolysis of clots containing the same constituents as "T" except that human plasminogen was replaced by 10 mN HCl. The total-clot incubation volumes were 1.3 ml. The differences in extinction values of the T and B series, designated by (T — B), represents fibrinolysis resulting from the activation of the added human plasminogen by increasing amounts of urokinase contained in the rabbit-urine glycoprotein preparation.

Fig. 1a shows the extent of fibrinolysis, expressed as absorbancy values as a function of glycoprotein concentration. Fig. 1b shows the results obtained from an identical experiment, using commercial bovine fibrinogen (fibrinogen concentration 6 %, w/v) in place of plasminogen-free bovine fibrinogen. In both figures the differences between Curves T and B, designated by (T — B), represent activation of the added human plasminogen by urokinase components in the rabbit glycoprotein preparation.

A comparison of Curves B shows that removal of contaminated bovine plasminogen resulted in significantly decreased fibrinolysis in the absence of added human plasminogen (Fig. 1a, Curve B), when compared with the corresponding curve (Fig. 1b, Curve B) using commercial fibrinogen. The small amount of fibrinolytic attack using plasminogen-free fibrinogen is probably due to traces of proteolytic enzymes, unrelated to plasmin, in the glycoprotein preparation. From a comparison of the initial slopes of the two curves the removal of plasminogen from fibrinogen resulted in a

fibrin more susceptible to plasmin attack. These differences may be due to the removal of inhibitors to plasmin or plasminogen activators during the purification of the commercial fibrinogen. Alternatively, the bovine fibrinogen may have become slightly denatured during the purification process resulting in a fibrin more sensitive to plasmin digestion.

In addition to the estimation of bacterial activators and crude and purified urokinase preparations, the use of plasminogen-free fibrinogen in the quantitative fibrinolytic method now enables plasmin to be estimated, even though the plasmin preparation may be contaminated with plasminogen activators.

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[1] A. J. ANDERSON, *Biochim. Biophys. Acta*, 57 (1962) 154.
[2] F. M. DAVIDSON, *Nature*, 185 (1960) 626.
[3] A. J. ANDERSON, *Biochem. J.*, 83 (1962) 7P.
[4] A. J. ANDERSON AND N. F. MACLAGAN, *Biochem. J.*, 59 (1955) 638.
[5] B. KICKHÖFEN, F. E. STRUWE, B. BRAMESFELD AND O. WESTPHAL, *Biochem. Z.*, 330 (1958) 467.
[6] A. J. ANDERSON, unpublished results.
[7] M. W. MOSESSON, *Biochim. Biophys. Acta*, 57 (1962) 204.
[8] F. M. DAVIDSON, *Biochem. J.*, 76 (1960) 56.

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Chromatographic separation of a hind-brain inducing substance into mesodermal and neural-inducing subfractions

Starting with 9-day-old chick embryos we isolated protein and nucleoprotein fractions which cause the competent undetermined ectoderm of *Triturus alpestris* gastrulae to form regionally specific organ complexes. One of them induces chiefly spinocaudal structures (notochord, muscle, kidney and neural tube), while others induce preferentially deuterencephalic structures such as hind-brain and ear vesicles[1].

By further purification we obtained from the spinocaudal-inducing fraction a purely "mesodermal"-inducing protein. The inductions evoked by this protein consist of muscle, notochord and pronephros and not any neural tube nor any other neural tissue. It was concluded that spinocaudal inductions arise from cooperation of a mesodermal-inducing factor and a small amount of a neural-inducing factor[2].

The aim of the following investigations was to test whether the deuterencephalic-inducing fractions also consist of more than one factor. In the latter case it should be possible to separate the complex into at least two factors or groups of factors which induce different organs.