A Rapid, Simple, Sensitive Method for Measuring Fibrinolytic Split Products in Human Serum*
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A variety of procedures have been developed to demonstrate and/or measure fibrinolytic split products in human serum. These include immunoelectrophoresis (1), immunodiffusion (2), and the tanned red-cell hemagglutination inhibition immunoassay (TRCHII) (3, 4) which is the most sensitive by far. The original TRCHII utilized formalinized, tanned, and sensitized sheep red cells though unformalinized cells could also be used. Formalinized red cells can be stored for many months and thus kept constantly available, whereas unformalinized cells are not suitable for use after 7–14 days. Tanning and sensitization require 2–3 hr, and the cells may react poorly after 21 days. Optimal sensitivity is reached in 12–18 hr though results can be read after 2 hr. A rapid method (1–2 hr) was described utilizing formalinized sheep cells which had been previously sensitized (5); however, the sensitivity is only 4–8 μg/ml.

The modified TRCHII method described herein, using tanned but not formalinized human, group O blood cells, is carried out on a blood grouping plate. The sensitized cells can be stored for 3 weeks (or more) at 4° prior to use; when stored in glycerol at —20°, they remain sensitive for at least 4 months. The test can be completed within 1 hr, even including removal of glycerol if the cells were frozen. It is very sensitive, detecting 1.0 μg/ml of fibrinogen or split products in human serum.

Materials. Human group O cells, preferably from blood collected in ACD solution (20 ml of blood + 3 ml of ACD); blood collected in sodium citrate or EDTA is also suitable. The cells can be stored for 2–3 days at 4° in ACD solution before sensitization.

Buffers (4): (a) phosphate–saline buffer, pH 6.4: 1 vol of 0.15 *M* phosphate buffer + 1 vol of normal saline. (b) phosphate–citrate buffer, pH 6.4: 1 vol of 0.15 *M* phosphate buffer + 1 vol of 0.1 *M* sodium citrate. The citrate is added to inhibit clotting of fibrinogen.

ACD solution, formula A.

Glycerol–citrate: Glycerol USP diluted to appropriate concentration with trisodium citrate 5%.

Antiserum to human fibrinogen (4), diluted in phosphate–citrate buffer.

Bovine serum albumin[1] or human serum albumin, USP.

Standard normal plasma: pooled normal citrated plasma containing 50 units of Trasylol[2]/ml, stored in aliquots at —20°.

Blood grouping plates: transparent plates with rounded wells 1.6 cm in diameter and 1.6 cm deep.

Microtiter,[3] Autotiter,[4] or another similar system, including centrifuge carrier for titration plates with appropriate centrifuge head, if many samples are to be tested.

Methods. Sensitization of cells. 1. Wash and centrifuge cells (1000*g*) 3× in 20× the volume of phosphate–saline buffer to remove plasma.

2. Mix equal volumes of 2% cell suspen-

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[1] New England Reagent Laboratory, Riverside, Rhode Island.

[2] Preparation A 128 (proteinase inactivator), Farbenfabriken, Bayer A. G., Leverkusen, Germany, U. S. Distributor; F. B. A. Pharmaceuticals Inc., New York.

[3] Cooke Engineering Co., Alexandria, Va.

[4] Astec Inc., Orange, Conn.

sion in phosphate–saline buffer and 1:20,000 dilution of freshly prepared tannic acid in the same buffer.

3. Incubate 60 min at room temperature, gently mixing with magnetic stirrer.

4. Wash and centrifuge cells 3× in 20× the volume of phosphate–citrate buffer (to remove tannic acid) and resuspend at 4% concentration in the same buffer.

5. Divide the sample of cells: one fraction (±90%) is incubated at 37° for 1 hr (with occasional mixing) with an equal volume of a 1:250 dilution of normal citrated plasma in phosphate–citrate buffer. The remainder of the sample is similarly incubated with a 1:250 dilution of thrombin-treated normal serum. In this step the tanned red cells become coated with fibrinogen (and other plasma proteins) when incubated with plasma, and with serum proteins (but not fibrinogen) when incubated with serum. The serum-treated cells provide an important control since they should not be agglutinated by the specific antifibrin serum and thus are able to detect nonspecific agglutination, *e.g.*, the presence of a panagglutinin.

6. Wash and centrifuge cells 3× in 20× the volume of phosphate–citrate buffer to remove plasma or serum.

7. Before use, suspend cells at 4% concentration in phosphate–citrate buffer.

8. To prepare cells for storage at 4°, suspend them at 4% concentration in phosphate–citrate buffer containing 0.25% bovine serum albumin and 0.1% sodium azide. Before use, wash cells once and resuspend them in phosphate–citrate buffer.

9. To prepare cells for storage at −20° (6), centrifuge at 1000*g* 2.0 ml of a 6.5% suspension of cells in phosphate–citrate buffer and discard supernatant. At 37°, add glycerol citrate 40% (4 vol of glycerol, 6 vol of citrate) by drops to the red cells (a total of 0.5 ml for each 2 ml of original suspension), shake fluid after each drop is added. When required for use, the glycerol is removed by adding glycerol citrate at 37° (30, 20, 10, 5, and 2%) at 5-min intervals, without centrifuging, in amounts equal to the total volume at that time. Centrifuge the final mixture, discard the supernatant, and resuspend the cells at 4% concentration in phosphate–citrate buffer.

Measurement of fibrinolytic split products.

1. Samples are diluted to ensure a relatively constant protein concentration. Phosphate–citrate buffer containing 2% bovine serum albumin is used for all dilutions except the initial 2 (doubling) dilutions of undiluted serum when citrate–phosphate buffer is used without additional albumin.

2. Determine concentration of antiserum to be used. Prepare doubling dilutions of antiserum (0.1-ml volume) in citrate buffer containing 2% bovine serum albumin on the blood grouping plate, add 0.1 ml of buffer and 1 drop (0.025 ml) of plasma-treated cells to each well. The antiserum concentration used in the test should be 1–2 doubling dilutions less than the greatest dilution of antiserum which gives good agglutination in 15 min, *i.e.*, if a 1:20,000 dilution causes good agglutination, use 1:5000 in the test.

3. Prepare dilutions of standard normal plasma in the wells of the blood grouping plate; doubling dilutions (0.1-ml volume) are prepared with an automatic pipette. The dilutions should range from 1:50 to about 1:25,000.

4. Prepare similar dilutions of the test sample, the starting dilutions depending on the anticipated antigen concentration.

5. Add 1 vol of antiserum to each well; antiserum diluted 1:2000 was used in these studies.

6. Allow antibody–antigen mixture to stand 30 min at 4°. Add 1 drop (0.025 ml) of plasma-treated cells to each well; mix by gently rotating the blood grouping plate, and let it stand at room temperature.

7. Read results in 30 min. The end point is determined by the last tube in which agglutination has been inhibited, on macroscopic examination. The inhibition produced by dilution of the unknown sample is compared directly to that of plasma, giving the result immediately.

Results. 1. The optimal preincubation time for the antigen–antibody reaction and the optimal time for reading test results are shown in the Table I. The antigen–antibody

TABLE I. Optimal Time for Preincubating Antigen–Antibody Mixture and for Reading Test Results.[a]

Antigen–antibody incubation (min):	5							15							30						
Plasma dilution (×100):	4	8	16	32	64	128	256	512	1024	4	8	16	32	64	128	256	512	1024	4	8	16	32	64	128	256	512	1024
Time after addition of cells (min)																											
1	—	—	—	—	—	—	T	T		—	—	—	—	—	—	—	T	T	—	—	—	—	—	—	—	—	—
5	—	—	—	T	1	1	1	1		—	—	—	—	T	1	1	1	1	—	—	—	—	—	—	—	—	—
15	—	—	—	T	2	2	2	2		—	—	—	T	1	2	2	2	3	—	—	—	—	—	—	—	—	—
30	—	—	—	2	3	3	3	3		—	—	—	1	2	3	3	3	3	—	—	—	T	1	2	2	3	3
60[b]	—	—	—	2	3	4	4	4		—	—	—	1	2	3	4	4	4	—	—	—	T	2	3	4	4	4

[a] T = Trace agglutination; 1, 2, 3, 4 = degrees of agglutination from weak (1) to 4+ (4).
[b] Similar results were obtained after 2, 4, and 24 hr.

reaction is relatively stable after 30 min preincubation, though less time may be required. Results can be read 15–30 min after the cells are added. If the standard curve and the unknown sample are set up at the same time, results are directly comparable 5 or 10 min after addition of the red cells. Changes are negligible after 30 min.

2. The standard normal plasma in a 1:3200 dilution usually inhibits agglutination. If the plasma contains 320 mg/100 ml of fibrinogen, the dilution contains:

$$320 \times \frac{1000}{100} \times \frac{1}{3200}\ \mu\text{g/ml, or } 1.0\ \mu\text{g/ml.}$$

If an unknown sample in a 1:25 dilution caused similar inhibition, it would be regarded as containing 25 μg/ml of fibrinogen or split products. Results with a number of samples of normal plasma containing known amounts of fibrinogen indicate a sensitivity of approximately 1.0 μg/ml of fibrinogen. Cells stored at —20° for 3–4 months give essentially similar results.

The above method is suitable for testing a small number of samples, but for assaying many samples a Microtiter or Autotiter system which gives comparable results is preferred. The cell concentration is reduced to 0.5%. Serial dilutions of antigen (0.025-ml volume) and 0.025 ml of antiserum are mixed and left at 4° for 30 min; 0.025 ml of cold plasma-treated cells is added and the well contents are mixed for 5–10 min by rotation on an automatic rotator.[5] The results can then be read immediately. Alternatively the plates may be centrifuged at 1500 rpm for 90 sec in the special centrifuge carrier (7). The well contents are then agitated by a jet of air and the presence or absence of agglutination in each well can be determined.

Discussion. The present test has a number of advantages over the original TRCHII and other similar tests; (i) human red cells are usually easier to obtain than sheep cells; (ii) in occasional instances either sheep or human cells react poorly in this system; the use of human donors allows the immediate avail-

[5] Rotating apparatus no. 3623, Arthur H. Thomas, Co., Philadelphia, Pa.

ability of known sensitive cells; (iii) sensitized cells store well for months and are thus conveniently available; (iv) the test is very sensitive and can be completed within 1 hr. This is true even when cells frozen in glycerol are used since the glycerol is removed during the time required for preincubation of antigen and antibody; (v) the agglutination reaction is very strong and the end point easily observed, thus diminishing observor error.

The assumption on which this technique is based—that fibrinolytic split products can be quantitated by determining their immunologic reactivity as compared to that of fibrinogen—may not be completely valid for several reasons:

1. Prolonged or intensive digestion of purified fibrinogen by plasmin, more extensive than usually found in clinical disease, results in a number of fragments of different molecular size, (8, 9) some of which do not react to antifibrinogen serum and are therefore not detectable by the assay.

2. The number of exposed immunologically reactive binding sites increases two- to threefold in the early stages of *in vitro* digestion of fibrinogen by plasmin, then decreases slowly and progressively with extensive degradation (4). Thus, an immunoassay may exaggerate the amount of material present in the early stages of digestion and underestimate the amount present in the late stages. However, validity of the assay is supported by qualitative studies (immunoelectrophoresis and immunodiffusion in agar and other gels) on the plasma of patients with various diseases, which seem to indicate that the split products are mainly in the early stages of digestion.

3. Variable amounts of digestion products from fibrinogenolytic states may be incorporated in the fibrin clot formed *in vitro* during clotting of patient plasma sample which would then not be detectable in the assay. On the other hand, fibrin digestion products in a patient with defibrination syndrome are less likely to be incorporated in the fibrin and may therefore be measured relatively quantitatively.

4. In the early stages of fibrinogen digestion the split products are clottable by thrombin, albeit at a slower rate than undigested fibrinogen. These early split products are usually removed by thrombin and tend not to appear in the serum. A method has been described for measuring the early and late derivatives of fibrinogen digestion in plasma by small-pore acrylamide gels (11), but it is not very sensitive.

5. Nonspecific inhibition of agglutination by proteins other than fibrinogen (or its derivatives) is conceivable but has not been recognized to date.

Despite these difficulties in quantitation, the assay appears to have practical clinical value. The level of split products in normal serum, when assayed by such a procedure, does not usually exceed 8 μg/ml, so that elevated levels are considered abnormal. Furthermore, the high levels found in many patients severely ill with defibrination syndrome decrease as they improve clinically. Although an abnormal level is probably not present in the sera of all patients with fibrinogen lysis, this finding appears to be very helpful diagnostically since it suggests either fibrin or fibrinogen lysis. Because the serum sample is prepared by clotting patient's plasma with an excess of thrombin, the test is more sensitive to fibrin lysis than to fibrinogen lysis.

Summary and Conclusions. A simplified procedure for recognizing and quantitating fibrinolytic split products has been described. It utilizes human group O cells which can be sensitized and stored at 4° for 3 weeks and at —20° for many months prior to use. It is very sensitive, detecting 1.0 μg/ml of fibrinogen or split products. Application of the test to the Microtiter and Autotiter systems permits assay of many samples at once. Only 1 hr is required to complete the test and read the results. The interpretation of the quantitative assay result should be carefully made since, while fibrin split products are not absorbed from the sera during the preparation of the sample and are relatively accurately measured, fibrinogen split products tend to be absorbed during sample preparation and are thus less accurately measured. The test would appear to be more useful with fibrin

lysis than with fibrinogen lysis and to be especially applicable in the diagnosis of defibrination syndrome.

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Purification of Bovine Thyroid-Stimulating Hormone by Solubility Fractionation with Ammonium Sulfate*

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Ammonium sulfate fractionation has been a useful method for purifying proteins but has achieved only limited success when applied to TSH. Fraenkel-Conrat and co-workers (1) as well as Albert (2) incorporated ammonium sulfate precipitation of TSH in early procedures which provided low yields of activity. Bergman and co-workers (3, 4) also employed ammonium sulfate for the recovery of TSH from solution. However, none of these techniques provided a preparation suitably high in biological activity. Heideman's introduction of ion exchange chromatography for TSH purification (5) has led to preparations by Bates and Condliffe (6) and by Pierce and Carsten (7) with potencies of 20–60 U/mg of protein. Since substantial losses of biological activity were encountered during ion exchange chromatography, these investigators utilized simplified methods for preliminary purification of TSH. Bates and Condliffe used the alcohol percolation technique developed by Bates *et al.* (8) while Pierce and Carsten (7) employed an interphase precipitation technique. The most spectacular achievement with respect to TSH purification was recently reported by Condliffe and Jakoby (9) who crystallized bovine TSH by extracting a highly purified preparation with decreasing concentrations of ammonium sulfate. This suggested to us that the use of ammonium sulfate techniques might also be valuable in the preparation of highly purified TSH. The present paper gives the results of a study conducted on solubility fractionation of TSH with ammonium sulfate as a means of purifying the hormone.

Materials and Methods. General. Two batches of crude bovine TSH (0.49 and 0.84 μ/mg), prepared by the method of Ciereszko (10), were purchased from Princeton Laboratory Products Company, Princeton, New

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