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# Assay of Tryptases by Lysis of Fibrinogen.

JOHN H. FERGUSON.

*From the Department of Pharmacology, University of Michigan, Ann Arbor.*

A recent method[1,2] for assay of tryptases by lysis of *fibrin* clot is sufficiently sensitive to give excellent results with normal dog, cat and human plasma tryptases, which, in a limited series of observations to date, fall within the range of 20-200 "units" (*v. infra*), in which the test is accurate to within 5-10%. The principle of the method is the comparison of the "lytic times" of activated tryptase "unknown" with a standard reference series of known trypsin concentrations. A convenient practical *standard* of 100 "units" is defined as the lytic activity of 1 mg per cc of a reliable commercial trypsin* (prepared by dilution of a "stock" 2% solution preserved in glycerol-borate buffer, according to Burdon[3]). Since some species (*e.g.* rabbit[4]) and certain human bloods yield plasma tryptase values under 5 "units" by the above test, a new method has been devised to operate with a sensitivity of ten times that of the original test. It has the same (5-10%) experimental error in the new range of 10-1 "units." Like a number of older attempts (*e.g.*[5]), the new method involves lysis of *fibrinogen*. This is timed by testing the residual clotting activity with a potent thrombin. As in the fibrinolytic method, the assay depends upon comparison of the "unknown" with a series of standard enzyme dilutions.

*Reagents.* 1. *Simplified borate buffer solution.* 2.5% $H_3BO_3$ (45 parts), 0.5% NaCl (45 parts), 4% $Na_3B_4O_7,10H_2O$ (10 parts): pH = 7.7 ± 0.1 (glass electrode). This solution is used as solvent and diluent for fibrinogen and thrombin.

2. *Fibrinogen.* A lyophilized preparation[6] is convenient and an especially pure preparation (about 50% coagulable protein) of human fibrinogen, prepared by Dr. R. M. Ferry and the staff of the Harvard Plasma Fractionation Laboratory and kindly supplied by Dr. E. J. Cohn[7] for this work, is excellent. Essential qualities are (a) stability, denoting freedom from contaminating protease, (b) absence of natural trypsin inhibitors. It is evident that these requirements are fulfilled in the data of Table I. Series B of this table illustrates the clotting-times of this fibrinogen, serially diluted with buffer solution. These values could be used to convert the clotting-times of Series A into terms of residual substrate (a topic not germane to the present discussion). Their routine value, however, is to test the fibrinogen in order to be sure that the initial strength, while giving good solid clots, is such that significant prolongation of clotting-time accompanies reduction of fibrinogen concentration to, say, 25-10% of the original, an obvious requirement for a sensitive lytic test. 1:250 or 1:200 solution of purified human fibrinogen meets these specifications.

3. *Thrombin.* This must be stable in the test solution. An excellent preparation consists of a 1:25 dilution (with borate buffer) of Parfentjev's[8] "hemostatic (clotting) globulin" (Lederle). As now marketed, this material contains preservatives which appear to inhibit proteases, making it unsuitable for our fibrinolytic (original) method. Since the new technic involves addition of the thrombin only *after* the enzyme action, this inhibitor, if anything, is an advantage, in that it tends to arrest the enzyme action at the start of the

1 Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 73.

2 Ferguson, J. H., *Federation Proc.*, 1943, in press.

* Fairchild Bros. and Foster.

3 Burdon, K. L., *Science*, 1941, **93**, 91.

4 Ferguson, J. H., *J. Lab. and Clin. Med.*, 1943, in press.

5 Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 255.

6 Ferguson, J. H., and Erickson, B. N., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 425.

7 Cohn, E. J., *Chem. Rev.*, 1941, **28**, 394.

8 Parfentjev, I. A., *Am. J. Med. Sci.*, 1941, **202**, 578.

TABLE I.
Trypsin Assay by Lysis of Fibrinogen.
Clotting-times (sec) for 0.5 cc thrombin + 1 cc samples of digesting mixtures, *viz.*, 1:250 fibrinogen (10 cc) + enzyme (1 cc).

A. Digestion series:

| Enzyme conc.* | Minutes of incubation time (pH = 7.7, temp. = 25°C) during 5 | 10 | 15 | 20 | 25 | 30 | 60 | 90 | 120 | 150 | 180 min. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 43 | 360 | ∞ | | | | | | | | |
| 9 | 20 | 38 | 85 | 540 | ∞ | | | | | | |
| 8 | 19 | 32 | 64 | 240 | ∞ | | | | | | |
| 7 | 18 | 27 | 40 | 80 | 135 | ∞ | | | | | |
| 6 | 17 | 23 | 31 | 48 | 88 | 180 | ∞ | | | | |
| 5 | 15 | 18 | — | 22 | — | 27 | 48 | 150 | 420 | ∞ | |
| 4 | 15 | 16 | — | 19 | — | 23 | 35 | 55 | 105 | 270 | 600 |
| 3 | 15 | 15 | — | 17 | — | 20 | 29 | 45 | 72 | 155 | 360 |
| 2 | 15 | 15 | — | 16 | — | 17 | 20 | 23 | 25 | 30 | 35 |
| 1 | 15 | 15 | — | 15 | — | 15 | 17 | 18 | 18 | 19 | 22 |
| 0 | 15 | — | — | — | — | — | 15 | — | 15 | — | 15 |

B. Fibrinogen dilution series:

| Fibr. dilu. | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
|---|---|---|---|---|---|---|---|
| Mg per cc | 4 | 2 | 1 | 0.5 | 0.25 | 0.125 | 0.063 |
| Clot-time | 15″ | 16″ | 18″ | 23″ | 45″ | 140″ | trace |

*1 mg per cc of "standard" trypsin (see text) = 100 *units*.

clotting test.

4. *Standard Trypsins.* A series of dilutions varying by 1 unit, between 10 and 1 unit, is made by diluting "stock" trypsin (*v. supra:* 2% solution = 2000 "units" per cc) with physiological saline.

5. *Unknowns.* Some materials, *e.g.*, prostatic fluid, appear to contain the protease in active form, since no significant change in activity is produced by treatment with chloroform. Plasmas, sera, etc., normally contain excess of *trypsin-inhibitor,* which has long been known to be removed (hence "activating" the enzyme) by shaking with $CHCl_3$ (1/5 vol.), removing the latter by centrifuging after 24-48 hours (*cf.*[9]).

*Tests. Digestion of fibrinogen:* 10 cc fibrinogen (*v.* reagents) is incubated with 1 cc enzyme; 1. standard trypsins, 2. unknown(s)—suitably activated and diluted (if indicated, by preliminary test). Arbitrarily selected "standard" conditions include: (a) pH = 7.7 ± 0.1 (*v.* simplified buffer) to control pH and ionic strength, (b) temp. = 25° ± 2°C. Usually, room temperature is sufficiently constant but a thermostat waterbath may be a desirable refinement.

*Assay series.* 1 cc samples of fibrinogen-enzyme mixture are added to 0.5 cc of thrombin (*v.* reagents) in Wassermann tubes and the presence or absence of clotting noted. For a simplified test, the following designations suffice: + : solid clot (tube invertible), ± : incomplete clot, ∞ : no clot or mere trace of flocculation. Meticulous accuracy is obtained by actual timing of the *onset* of fibrin formation, as in Table I. Times of sampling (incubation periods) will be determined by strengths of enzyme under consideration. Comparison of the "lytic time" of unknown with standard enzymes enables assay of the former in terms of the "units" expressed by the latter.

*Data.* Table I illustrates the sensitivity and selectivity of the trypsin standards, 1 "unit" representing the equivalent of 1:100,000 trypsin (0.01 mg per cc of enzyme solution) or somewhat less than 1:1,000,000 trypsin in the final digestion mixture.

Table II illustrates the practical application of the test to the assay of the tryptase content of a sample of dog prostatic fluid, kindly supplied by Dr. C. Huggins (Chicago).[10] The value of 5 units is seen to be obtained both

[9] Tagnon, H. J., *J. Lab. and Clin. Med.*, 1942, **27**, 1119.

[10] Huggins, C., and Neal, W., *J. Exp. Med.*, 1942, **76**, 527.

TABLE II.
Routine Assay of Tryptase in Dog Prostatic Fluid.
A. Untreated; B. $CHCl_3$-treated.

| Enzyme conc. | Incubation period 5′ | 15′ | 20′ | 30′ | 60′ | 75′ | 90′ |
|---|---|---|---|---|---|---|---|
| Sample A | + | + | + | + | + | ± | ∞ |
| Sample B | + | + | + | + | + | ± | ∞ |
| 10 units | + | ∞ | | | | | |
| 5 ″ | + | + | + | + | + | ± | ∞ |
| 2.5 ″ | + | + | + | + | + | + | + |

with untreated and $CHCl_3$-treated fluid.

*Discussion.* Numerous applications of the method to the assay of tryptic enzymes in plasma, serum, cell- and other materials are envisaged. The effects of inhibitors and other conditions modifying the action of known tryptases also lend themselves to quantitative investigation by simple modification of the cited technic. The new method (which may be styled Method B) offers the following advantages over the original[1] assay (Method A): 1. tenfold increase in sensitivity; 2. independence of enzyme-inhibitors in thrombin; 3. much less time-consuming—a matter of minutes or hours (actual test), instead of days, even with very weak enzymes; 4. sharper "end-point," since the enzyme acts in water-clear homogeneous colloidal solution, instead of on the grossly biphasic fibrin gel; 5. no special apparatus (*e.g.*, turbidimeter) required. Within their respective ranges and limitations, both methods are useful practical means of estimating natural trypsin-like proteolytic enzymes. Such enzymes play an important role in blood-coagulation[11] and other functions, to the elucidation of which it is believed that these technics may offer a material contribution. The methods are sufficiently simple to open up new fields as a clinical laboratory test.

[11] Ferguson, J. H., *Science*, 1943, in press.

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## Toxemias of Pregnancy and the Inulin-Diodrast Clearance Tests.

DONALD H. KARIHER AND ROGER H. GEORGE. (Introduced by E. F. Adolph.)

*From the Department of Obstetrics and Gynecology, Strong Memorial and Rochester Municipal Hospitals, University of Rochester School of Medicine and Dentistry.*

The role of the kidneys in the production of toxemia of pregnancy has been studied for many years, and as yet we have only a paucity of information. As one new kidney function test after another has been announced, investigators have applied them to the toxemias of pregnancy and have found them inadequate to detect alterations in the status of the kidneys.

It would seem so even with the newest of function studies, the inulin and diodrast clearance tests. Chesley *et al.*[1] conclude that the renal blood flow is essentially normal in eclampsia, pre-eclampsia, and recurrent toxemia of pregnancy, as measured by the diodrast clearance. Corcoran and Page[2] feel that if the filtration fraction is

[1] Chesley, L. C., Connell, E. J., Chesley, E. R., Katz, J. D., and Glissen, C. S., *J. Clin. Invest.*, 1940, **19**, 219.

[2] Corcoran, A. C., and Page, I. H., *Am. J. Med. Sc.*, 1941, **201** n. s., 385.