SCIENTIFIC APPARATUS AND LABORATORY METHODS

A STABLE TRYPSIN SOLUTION

IN connection with a new procedure developed by the writer for the quantitative titration of antitrypsin an extract of Fairchild's trypsin is used which retains its original activity for many months. We have found no mention in the literature of a solution of active trypsin having comparable stability, and therefore believe a description of this preparation may be of general interest.

The solution takes advantage of the stabilizing effect of *glycerin* upon enzyme preparations[1,2] and of the bacteriostatic action of this substance, and, in addition, controls the hydrogen-ion concentration by a suitable buffer.

The stock buffer solution is a borax-boric acid mixture, consisting of nine parts of M/5 boric acid, M/20 sodium chloride solution (12.4 grams H_3BO_3 and 2.9 grams NaCl in one liter of distilled water) and one part of M/20 borax solution (19.1 grams $Na_2B_4O_7 \cdot 10 H_2O$ in one liter of distilled water), having a final pH of 7.3.[3] To make the standard trypsin preparation, this buffer solution is mixed with an equal volume of neutral CP glycerin. Powdered trypsin (Fairchild Bros. and Foster) in the proportion of 2 per cent. by weight is added to the glycerin-buffer solution a little at a time, dusting it on the surface of the liquid and stirring with a glass rod until all lumps are broken up. The mixture is then allowed to stand at room temperature with frequent shaking of the flask for 15 minutes. It is then filtered through S and S No. 588 filter paper into a sterile screw-top bottle.

Glycerin-buffer extracts prepared in this way at different times from several different lots of Fairchild's trypsin have exhibited an almost identical original activity. The remarkable stabilizing effect of the glycerin in the solution, and other advantages of a glycerin-buffer extract over one prepared in the same way with the plain buffer solution (without glycerin) have been clearly demonstrated by the results of repeated tests during the past four years. The glycerinated extracts have invariably shown (1) a slightly greater initial activity, (2) freedom from bacterial contamination, even though kept at 37° C. for several weeks, whereas plain buffer solutions, unless a preservative was added, always became contaminated within a few days, and (3) a far-greater stability.

[1] Ernst Waldschmidt-Leitz (translated by R. P. Walton), "Enzyme Actions and Properties," John Wiley and Sons, Inc., New York, 1929.

[2] R. P. Walton, *Jour. Pharm. and Exp. Therap.*, 40: 403, 1930.

[3] Prepared according to the directions of Palitzsch, as given by W. M. Clark, "The Determination of Hydrogen Ions," pp. 115, 117. Baltimore: Williams and Wilkins Company, 1923.

Titrations of tryptic activity have been carried out by the author's film-disc method, which will be described in detail in a separate paper. As now conducted the method is a greatly improved and simplified form of a test originally developed in 1936.[4] It depends upon the fact that when properly prepared photographic film is exposed to a series of dilutions of active trypsin the gelatin layer is digested in proportion to the activity of the enzyme, releasing the silver, so that when digestion is complete the originally black film becomes completely transparent. The titer of tryptic activity is expressed in terms of the milligrams of trypsin per cc contained in the lowest dilution which causes complete clearing of a standard-sized disc of film after incubation at 40° C. for from 8 to 10 minutes. Not all lots of film digest at exactly the same rate, but for the freshly made and fully active stock trypsin solution prepared as above the titer is very readily established at 1.0 for any one lot of film by adjusting the incubation time. Usually 10 minutes is the required time.

Assuming that a trypsin solution having a titer of 1.0 mg per cc in 10 minutes at 40° C. shows 100 per cent. activity, and one with an end-point at 3.5 mg per cc has zero activity (since so high a titer represents the limit of the useful range of end-points in this method of titration), the percentage activity lost with the passage of time by trypsin solutions made or stored in different ways may be stated. The results of strictly comparable tests are summarized in Table I.

TABLE I

STABILITY OF TRYPSIN IN GLYCERIN-BUFFER SOLUTION AND IN PLAIN BUFFER SOLUTION AT PH 7.3

Trypsin solution			Average activity loss (per cent.)*	
Lots	Stored		Plain buffer	Glycerin buffer
	Temp.	Time		
I, II, V	37° C.	1 day	80	0
		3 days	90	15
		7 days		20
V	25° C.	1 day	50	0
		3 days	80	0
		6 days		0
		10 days	100	5
V	10° C.	2 days	10	0
		6 days	20	0
		10 days	30	0
		11 mos.	70	0
II		3 mos.		0
VI		9 mos.		0
IV		1 yr.		20
I		2 yrs.		10
II, III		3 yrs.		25

* Based on titration by the film-disc method, considering an end-point of 1.0 mg of trypsin per cc as 100 per cent. activity and an end-point of 3.5 mg per cc as zero activity.

[4] K. L. Burdon and C. Lafferty, *Proc. Soc. Exp. Biol. and Med.*, 34: 787, 1936.

It will be seen that the glycerin-buffer trypsin solutions stored in the icebox are stable over long periods. By slightly increasing the incubation time for digestion (at the most by two or three minutes), the identical end-point may be obtained for an indefinite period (three years at least) with a single standard solution of trypsin.

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PURIFICATION OF DIPHTHERIA ANTI-TOXIN

A PROTEIN has been obtained from equine antidiphtheria plasma which is highly antitoxic and which satisfies the criteria for a pure protein. The preparation contains about 700,000 to 1,000,000 anti-toxin units per gram protein nitrogen by the Ramon flocculation test and about 700,000 anti-toxin units per gram by guinea pig protection test. This is about 20 times the activity of the original antisera and agrees approximately with that reported by Pappenheimer and Robinson[1] and by Pope[2] for anti-toxin prepared by a different method. The material is completely precipitated by diphtheria toxin.

Dr. Alexandre Rothen has examined a sample of the protein in the ultra-centrifuge and in the Tiselius electrophoresis cell. Both methods show the presence of only one protein at pH 7.3. A slow migration to the anode is observed at this pH ($\mu \backsimeq 4 \times 10^{-6}$ cm^2/sec. volt). The rate of sedimentation ($S^{26} = 5.77 \times 10^{-13}$) indicates a figure of about 80,000 for the molecular weight. At pH 8.0 the protein migrates also slowly to the anode and is homogeneous. At this pH the material shows some aggregation in the ultra-centrifuge. Salt precipitation shows that some denaturation occurs at pH 8.0 and becomes marked in slightly more alkaline solution.

The solubility of this sample varied slightly, however, with different quantities of solid indicating the presence of more than one protein. A second more highly purified preparation showed constant solubility, independent of the quantity of solid present. The protein therefore satisfies the criteria for a pure protein.

Several samples have been obtained in the form of fine, poorly formed needles and thin plates. The protein has been prepared from three different lots of plasma precipitated with four different lots of toxin.

The method of preparation consists in precipitating the toxin anti-toxin complex. This precipitate is dissolved by the addition of acid and the toxin destroyed by digestion with trypsin at about pH 3.7. The anti-toxin is then purified by fractionation with ammonium sulfate at pH 7.2. The purified antibody is soluble in 0.50 saturated ammonium sulfate pH 7.4 and insoluble in 0.65 saturated ammonium sulfate.

Twenty-five to 50 per cent. of the original antibody may be recovered after the removal of the toxin. This crude antibody has a titre of from 300,000 to 500,000 units per gram protein nitrogen. It exhibits only one moving boundary in the electrophoresis cell, but there is marked spreading and the solubility test shows this crude preparation to be far from homogeneous.

The toxin and anti-toxin were furnished by the Biological Laboratories of E. R. Squibb and Sons. The animal protection tests were carried out by Dr. W. E. Bunney, at E. R. Squibb and Sons, New Brunswick, N. J.

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[1] A. M. Pappenheimer, Jr., and E. S. Robinson, *Jour. Immunol.*, 32: 291, 1937.
[2] C. G. Pope, *Brit. Jour. Exp. Path.*, 19: 245, 1938.

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