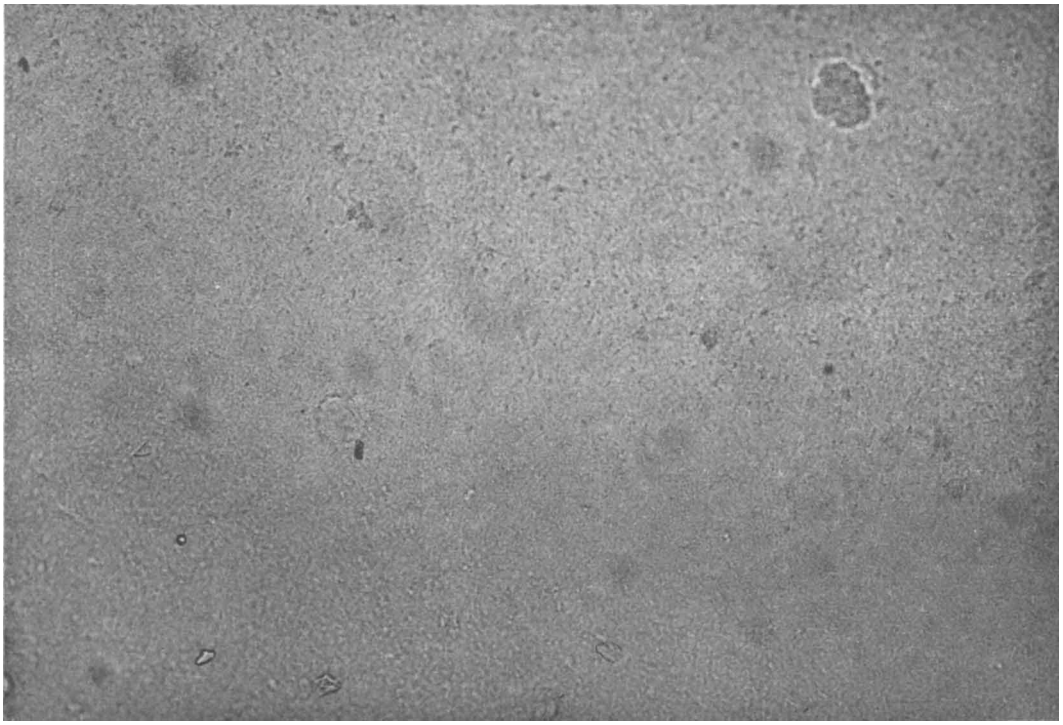

FIG. 2.
PPLO colonies, strain No. 36 from prostatic secretion, growing under *Proteus vulgaris*. When an agar block containing the growth of the two types of organisms was stained as described for Fig. 1 the PPLO colonies, with this strain, appeared as small darkly stained areas.

group of microorganisms.

Summary. Nine of 14 strains of PPLO of human origin and one strain of rat origin were observed to grow on beef heart infusion agar without ascitic fluid or serum only in symbiosis with *Staphylococcus albus* and all 15 strains grew in symbiosis with *Proteus vulgaris*.

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A Simple, Quantitative Method for Titration of Trypsin and Trypsin-Inhibitors.* (17424)

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In recent years the interest of an increasing number of investigators in the mechanism of fibrinolysis[1] and related phenomena, and especially in the role played by the activators and inhibitors of natural plasma proteases in the complicated process of blood coagulation,[2-5] has led to improved procedures for

* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

1 Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

2 Ferguson, J. H., *Science*, 1943, **97**, 139.

3 Tagnon, H. J., *J. Lab. and Clin. Med.*, 1942-43, **27**, 1119.

studying tryptase-antitryptase systems. The fibrinogenolytic method for measuring blood proteases and protease inhibitors, as first suggested by Ferguson,[6] or in somewhat modified form,[7-9] has proven especially useful. These procedures, however, leave something to be desired for routine tests on a large scale.

The need for a truly simple, quantitative technic for accurate titration of the changes in the protease inhibitor titers of body fluids associated with various physiologic and pathologic states, and particularly in connection with allergic phenomena, has been obvious for a long time. The concept that hypersensitivity reactions are caused primarily by toxic products formed, or released, when the natural proteases are suddenly activated following antigen-antibody union, was originally expressed many years ago, as was also the correlated idea that allergic reactions are controlled by the action of protease inhibitors.[10-12] Until recently very little precise data in direct support of this view has been published, however. The importance of supplying such data on an adequate scale led one of us some years ago to attempt development of a quantitative procedure for antitrypsin titration especially suitable for use by immunologists.[13-15] The method was based upon the fact that when properly prepared photographic film is brought into contact with solutions of active trypsin the gelatin is digested, releasing the silver and clearing the film to a degree proportional to the activity of the trypsin. When digestion is complete the originally opaque, black film becomes completely transparent.

The present paper outlines the vastly improved technic for the performance of this film-gelatin-digestion method developed during the past two years.

Reagents. 1. *Stock buffer solution pH 7.5* is made by mixing 1.3 parts of M/20 sodium borate (19.1 grams $Na_2B_4O_7$:10 H_2O per liter of distilled water) with 8.7 parts of M/5 boric acid-salt solution (12.4 grams H_3BO_3 and 2.9 g NaCl per liter of distilled water). This reagent is used for all dilutions of trypsin and of sera or other fluids under test.

2. *M/5 sodium borate solution* (76.3 grams Na_2B_4:10 H_2O per liter of distilled water) is kept in a sealed bottle in a 37°C incubator.

3. *Stock crystalline trypsin solution* (*CT*) is prepared by dissolving 0.3 g of commercial crystalline trypsin† in 100 ml of a glycerin-buffer mixture consisting of equal volumes of stock buffer solution and C.P. glycerine; the pH is about 4.4. It is stored in a sterile, chemically clean, screw-top bottle at 4-10°C. Freshly-made solutions are allowed to stand undisturbed in the refrigerator 24 hours before use. (A precipitate appearing at this time is removed by filtering through paper.) The effect of the glycerine and the low pH is to stabilize the enzyme[16] so that the proteolytic activity of the solution remains unchanged for many months.

4. *Working trypsin solution* (*WT*) is made by mixing one part of stock trypsin solution with two parts of the M/5 sodium borate solution (Reagent 2). This reduces the concentration of trypsin to 0.1% (1 mg/ml) and the pH is brought to 6.8. It is practicable to make up this reagent in a volume of 100 ml or more. Freshly made solutions are allowed to stand undisturbed in the refrigerator for 24 hours before use. When stored in

4 Glazko, A. S., *J. Clin. Invest.*, 1947, **26**, 364.

5 Milstone, J. H., *Science*, 1947, **106**, 546.

6 Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

7 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285.

8 Christensen, L. R., *J. Clin. Invest.*, 1949, **28**, 163.

9 Downie, G. R., and Cliffton, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 138.

10 Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1915-16, **13**, 42.

11 Bronfenbrenner, J., and Schlesinger, M. J., *J. Immunol.*, 1918, **3**, 321.

12 Rusznyak, S., *Deutsch. med. Wchnschr.*, 1912, **38**, 168.

13 Burdon, K. L., and Lafferty, C., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 787.

14 Burdon, K. L., *J. Bact.*, 1941, **41**, 62.

15 Burdon, K. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 24.

† Worthington Biochemical Laboratory, Freehold, N. J., or Armour Laboratories, Chicago, Ill.

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

TABLE I.
Scheme for Dilution of Working Trypsin Solution.

Dilution No.	Working trypsin, ml*	Buffer, ml*	Trypsin conc., mg/ml		Tryptic units (TU)
			In dilution	Final†	
1	.4	7.6	.05	.025	.5
2	.5	7.5	.063	.031	.62
3	.6	7.4	.075	.037	.75
4	.7	7.3	.087	.044	.87
5	.8	7.2	.1	.05	1.0
6	.9	7.1	.113	.056	1.12
7	1.0	7.0	.125	.062	1.25
8	1.1	6.9	.138	.069	1.37
9	1.2	6.8	.15	.075	1.5
10	1.3	6.7	.163	.081	1.62
11	1.4	6.6	.175	.087	1.75
12	1.5	6.5	.188	.094	1.87
13	1.6	6.4	.2	.1	2.0
14	1.7	6.3	.213	.106	2.12
15	1.8	6.2	.225	.112	2.25
16	1.9	6.1	.238	.119	2.37
17	2.0	6.0	.25	.125	2.5
18	2.1	5.9	.263	.131	2.62
19	2.2	5.8	.275	.137	2.75
20	2.3	5.7	.288	.144	2.87
21	2.4	5.6	.3	.15	3.0

* The amounts required to make up a total volume of 8.0 ml are shown because this is often a convenient quantity for routine tests.

† After mixture with equal volume of buffer, or diluted specimen, as in the test proper.

sterile, chemically clean screw-top bottles at 4-10°C the tryptic activity remains constant for at least 6 months.

5. *The fixing solution* consists of 95% ethyl alcohol to which 10% by volume of 28% glacial acetic acid is added.

6. *The filmstrips* are cut from the special film prepared to our specifications by the Eastman Kodak Company.‡ The film is a duplitized, 35 mm ciné-film, flashed on both sides to a density of 1.0, so that it has a total density of about 2.0. Both the density and the hardness of the emulsion are uniform throughout. The reels of stock film are kept at all times in tightly sealed cans in the refrigerator. A supply of strips is made, when needed, by cutting the film crosswise into pieces ¼ inch wide with a small paper cutter. The width of the strips need not be exact. About ¼ inch from the end each strip is bent upward at nearly a right angle to make it easier to grasp with forceps. In handling the film it is important to avoid finger marks, and exposure to heat or dust. The newly-cut strips are transferred at once to clean, screw-top jars or tubes, and thus kept carefully sealed in the refrigerator until actually used in a test.

I. *Trypsin Dilution Method.* 1. *Initial dilution of the serum or other fluid to be tested.* The majority of specimens of normal human, rabbit, or guinea pig sera will give end-points in a convenient range when they are diluted originally with stock buffer 1:50. For some high-titer sera, or other fluids, it may be found desirable to use a different initial dilution (*e.g.* 1:100). In any series of comparative tests all specimens should be titrated at the same initial dilution.

2. *Dilutions of the working trypsin solution.* For the *trypsin control* tubes in routine tests it is necessary to prepare only dilutions 3, 4, and 5 (Table I). For the *test mixtures* a series of six or eight additional dilutions are usually made through a range likely to give end-points in the presence of the serum or

‡ We wish to express our sincere appreciation to Dr. Burt H. Carroll of the Research Laboratories, and Mr. W. F. Swann of the Industrial Photographic Sales Division for their generous cooperation in the development and production of this essential material.

other fluid under test.

All dilutions are made with the stock buffer solution in chemically clean glassware, using plugged, 1 ml serological pipettes. The trypsin control dilutions have a pH of 7.5 and succeeding tubes in any part of the test series differ in hydrogen ion concentration by only about 0.01 pH.

These dilutions gradually lose activity at room temperatures, especially at temperatures over 85° F., but the same set of solutions may be used throughout a working day if the tubes are kept in a pan of ice water, or if they are placed in the refrigerator except when actually being used.§

3. *Preliminary adjustment of the incubation time.* A new working trypsin solution is first titrated in dilutions 1 to 7 under the standardized conditions of the test proper, as outlined below, except that the digestion incubation time in the 40°C water bath is varied in successive runs until the time required to give the end-point regularly with dilution No. 5 (Table I) is determined. The tube made up with dilution No. 5, plus an equal volume of stock buffer, will thus have, as intended, just *one unit of tryptic activity, i.e.* it will contain the amount of WT just sufficient to digest all the gelatin (clear the film completely) in a certain time at 40°C. It will be seen (Table I) that the final concentration of crystalline trypsin in this end-point tube is 0.05 mg/ml.‖ The time required with the present lot of film used by the writers is 6½ minutes.

First tests are commonly run at times differing by half-minute time intervals, then by quarter-minutes and finally by one-eighth minutes. The incubation time, once chosen on the basis of these tests, is kept constant throughout all future tests with that lot of working trypsin solution, and indeed, rarely requires any adjustment so long as the same *stock* trypsin solution is used. Thus, strictly comparable titrations can be obtained in separate runs carried out weeks or months apart.

4. *The test proper. a.* Ordinary serological tubes (12 x 75 mm) are arranged in a water bath rack. Using a separate, plugged 1.0 ml pipette for each dilution, 0.4 ml of dilutions 3, 4, and 5 of the working trypsin solution is introduced into a series of 3 tubes that are to serve as trypsin controls, and 0.4 ml of other appropriate dilutions is placed in a further series of 6 or 8 additional tubes. Now, 0.4 ml of the stock buffer solution is added to each of the control tubes, and 0.4 ml of the diluted serum, or other fluid under test, to each of the remaining tubes. The rack is now shaken, as in a Kahn test, for one-half minute, then placed in a 40°C water bath¶ with a timer set for 10 minutes.

b. The filmstrips are now obtained from their jar in the refrigerator and arranged in parallel rows on some convenient holder (such as a small slide-box lid) so that they can be more readily picked up one by one with forceps.

c. At the end of the warming period the interval timer is carefully reset for the test incubation time, *e.g.* 6½ minutes. The timer is started and the introduction of the filmstrips into the test tubes (still in the water bath) is begun immediately. The strips are added one by one in a definite order, and following a rhythmic time pattern which must be perfected by practice.

d. The fixing solution and equipment are now obtained from the refrigerator. Some of the solution is poured into a 600 ml beaker and heavy-walled capillary pipettes with large rubber bulbs attached are filled with it.

e. When the timer rings the rack is removed immediately from the water bath, and then, without delay, the cold fixing solution is added to each tube in the same order, and following the same time pattern in which the filmstrips were originally introduced. The fixing solu-

§ The stability of the reagents and the effects of various other factors upon the validity of the tests have been experimentally studied, and findings will be reported in a separate communication.

‖ The fact that the powdered commercial crystalline trypsin contains approximately 50% $MgSO_4$ is disregarded.

¶ Best results have been obtained by the use of an Army-type electric water bath (Chicago Surgical and Electrical Co.) with separate gable cover over each rack.

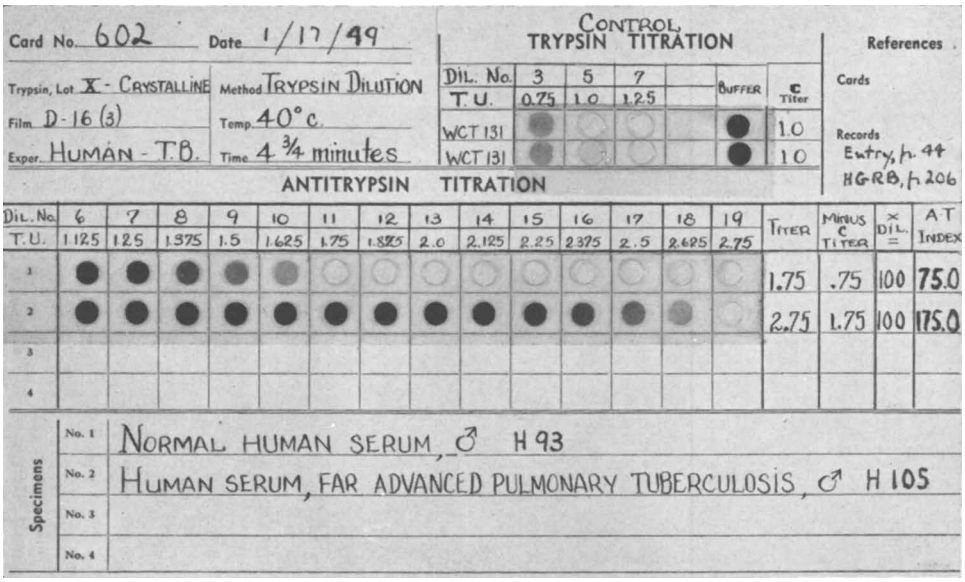

FIG. 1.

tion is added with sufficient force to cause the strips to float upward somewhat, thus bringing the fixative into contact with the film throughout its length. The rack is now placed in the refrigerator for 5 minutes to assure the full hardening of any of the gelatin emulsion remaining undigested.

f. The filmstrips are now removed from the tubes with forceps, rinsed briefly in fresh, cold fixing solution, and touched, at the edges only, to blotting paper. They are then inverted and set upright in a bed of plasticine so that they will drain and dry uniformly.

5. *Reading of the test; preparation of the permanent record card.* By simple inspection of the dried strips when held to the light, the end-points (the first completely cleared films) in the trypsin control, and in the test series, are determined and recorded. From each strip a disc 5 mm in diameter is then cut from the digested portion with an ordinary paper punch. These discs are mounted, with the aid of Scotch tape, upon a white card which bears the desired descriptive data. Thus a permanent record card for filing may be made of every test. (Fig. 1, 2).

6. *Expression of the results.* The *titer of proteolytic activity* may be expressed most conveniently in terms of tryptic units (TU). By preliminary adjustment of the incubation time, as described above, the titer in the trypsin control tubes (in all properly performed tests) is always *1.0 TU.* The *difference* between this titer and that obtained in the test series of tubes in the presence of a specimen of serum or other inhibiting material represents the antitryptic power of that sample. A final figure to represent the *antitryptic index* is obtained by multiplying this difference by the final dilution (*i.e.* double the initial dilution) of the specimen. For example, in the presence of specimen H-93 (Fig. 1) the titer is 1.75 TU, a difference of 0.75 over the control titer of 1.0 TU. This difference, times the final dilution, gives the antitryptic index: 0.75 x 100 = 75.0.

The antiprotease action may also be expressed more directly in terms of the amount of trypsin inhibited. Thus specimen H-93, in final dilution 1:100, (Fig. 1) may be said to have prevented the activity of 0.0375 mg/ml of trypsin, the difference between the 0.0875 mg/ml in the first tube showing 100% digestion (No. 11) and the 0.05 mg/ml present in the control end-point tube (No. 5) Each milliliter of *undiluted* H-93 has the apparent capacity to inhibit the action of 3.75 mg/ml crystalline trypsin. The correspond-

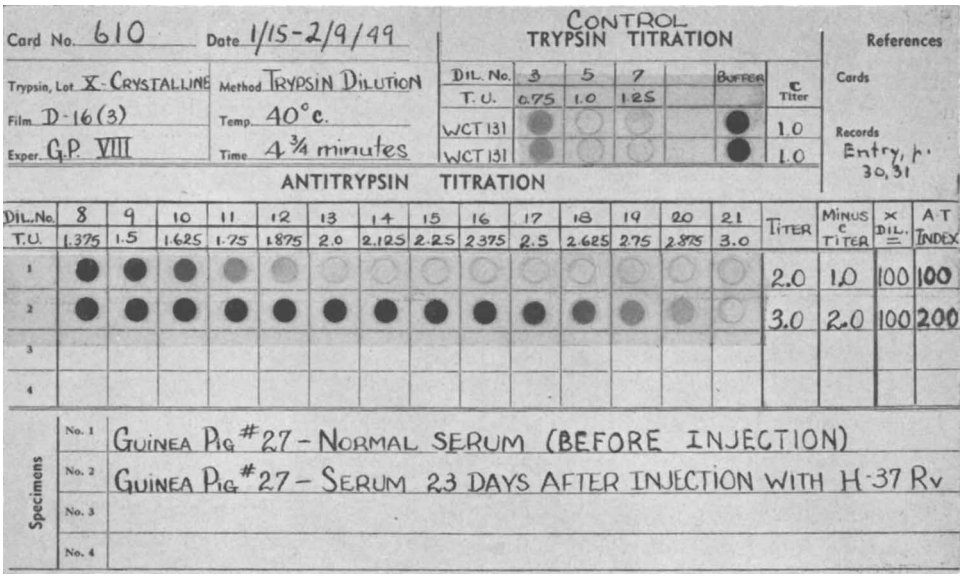

FIG. 2.

ing figures for undiluted specimens H-105, G.P. No. 27 normal, and tuberculous (Fig. 2) are 8.75, 5.0, and 10 mg/ml respectively.

Again, the antitryptic effect may be compared with that exhibited by a chemically purified material, such as commercial crystalline soybean trypsin inhibitor (CSBI).† We have found that one TU (*i.e.* a working trypsin solution which in final dilution contains 0.05 mg/ml CT) is *completely* inhibited by only 0.0167 mg/ml CSBI, or a proportion of inhibitor to trypsin of 1:3, and that *partial* inhibition continues so long as the final concentration of CSBI is not less than one part to 5.6 parts of trypsin. In the presence of 6 parts of trypsin the film is completely digested. Each milliliter of *undiluted* specimen H-93 (Fig. 1) may thus be calculated to have shown an inhibiting power equivalent to that of 0.625 mg/ml of CSBI,—0.0875 mg/ml (the amount of trypsin present in the end-point tube) minus 0.05, divided by 6 = 0.00625 mg/ml x the final serum dilution (1:100) = 0.625 mg/ml. For H-105, G.P. No. 27 normal, and tuberculous, the figures are 1.46, 0.833 and 1.66 mg/ml respectively.

Finally, advantage may be taken of the fact that the relative amount of clearing of the filmstrips in the several tubes just preceding the end-point tube is as reproducible as the end-point itself, and these strips may be read in terms of *percentage of digestion,* thus furnishing in some instances a distinction between two specimens, both of which have shown the same end-points. In other words, antitryptic indexes based on end-points do not always express all the degrees of tryptase-inhibiting activity possessed by different sera, even when the differences between the succeeding trypsin dilutions used is as small as is practicable, *i.e.* only 0.125 tryptic units, as in Table I. The estimate of the percentage digestion is made by close comparison under uniform illumination of the film discs in question with a standard set of discs showing 20 graduations from zero to 100% digestion. These readings are valuable in reinforcing the information obtained by comparison of end-points, and could be utilized for further statistical analysis of the findings.

II. *Specimen Dilution Procedure.* The purpose in this form of the test is to determine the highest dilution of the serum or other fluid under test that will inhibit in any degree the complete clearing of the film in the presence of one TU of trypsin at the standard time and temperature.

1. *Preliminary steps.* A sufficient volume

of the working trypsin solution is made up in dilution No. 5 (Table I) to supply the 0.4 ml required for a test with each of 8 or 10 specimen dilutions, plus a control. Small amounts of dilutions 3, 4, and 5 are also made up separately to be run as independent trypsin controls. The specimen is diluted with stock buffer by the two-fold dilution procedure generally used in agglutination titrations, or by some other convenient scheme. The great majority of serum specimens from human beings, guinea pigs, or rabbits will give titers within the range 1:64 to 1:2500.

2. *The test proper.* To 0.4 ml of each of the specimen dilutions is added 0.4 ml of WT, dilution No. 5. Control tubes containing 0.4 ml of this identical trypsin solution + 0.4 ml of buffer, as well as the separately-made trypsin controls, are set up. The remaining steps of the test are now carried out as in the Trypsin Dilution Method outlined above.

3. *Expression of the results.* The titer of antitryptic activity here exhibited is ordinarily stated in terms of the highest final dilution of the specimen that inhibits in any degree the clearing of the film. The controls must show that the trypsin solution used in all the test mixtures contained, as intended, one TU. The results may also be stated in terms of the amount of trypsin inhibited, or they may be interpreted in comparison with the action of known amounts of crystalline soy bean inhibitor. A reading of the percentage of digestion at the titer dilution may also be made, differing degrees of inhibiting action may thus be revealed in different sera having the same dilution titer.

Use of crude trypsin. The film-gelatin digestion method was originally developed before crystalline trypsin preparations became available commercially, and the procedures outlined above may still be used without essential change for tests with extracts of powdered crude trypsins. The stock trypsin solutions are made by weighing out 6 g of Fairchild's trypsin,** or 3 g of other commercial preparations†† and placing the powder in a mortar. One hundred milliliters of glycerine-buffer solution (equal parts of C.P. glycerine and stock buffer) are added gradually, stirring in a small amount at first to make a paste. The mixture is transferred to a stoppered flask and allowed to stand at room temperature for about 2 hours, with occasional stirring, then overnight in the refrigerator. A crystal clear extract (pH about 5.8) is finally obtained by filtering through S and S No. 588 paper, or preferably through a Seitz filter after adding one percent of filter-aid.‡‡ These glycerin-buffer extracts are stable over long periods of time[16] when stored in a chemically-clean, screw-top bottle in the refrigerator.

Comparative activity of crude and crystalline trypsin solutions; differences in reaction with trypsin inhibitors. Crystalline trypsin solutions were found to have almost exactly the same activity in the filmstrip tests as the extracts obtained from *10 times* the weight of the currently available crude powdered trypsins. Hence, with the crude trypsin extracts made as described above end-points are obtained in a similar range of dilutions as with crystalline trypsin, and the same scheme of tryptic units may be used to express the titers obtained.

However, the amount of trypsin *inhibitor* revealed in serum and other materials is much greater in tests with crystalline trypsin than in parallel titrations with the less pure enzyme preparations. Thus, the antitryptic indexes of various sera tested with the newer powdered trypsins were found to be about twice those obtained in titrations with the older, relatively impure, Fairchild's product. Crystalline trypsin again regularly reveals more than twice as much inhibitor as is shown in comparable tests with any of the currently available crude trypsins. Thus, the average antitryptic index of 23 human sera, (initial dilution 1:50), tested in parallel, was 51.6 with one of the powdered trypsins and 114.1 with crystalline trypsin. The difference,

** Fairchild Bros. and Foster, N. Y. ("Trypsin 1:70"). This material is no longer being manufactured.

†† ("Trypsin 1:300"), Nutritional Biochemicals Corporation, Cleveland, Ohio, or "Pancreatic Trypsin," Bios Laboratories, N. Y.

‡‡ Hyflo Super-Cel, Fisher Scientific Co., Pittsburgh, Pa.

moreover, is greatest when the inhibitor content of the specimen is especially high. Evidently the impurities in crude trypsin solutions prevent effective contact or combination of a considerable proportion of the antitryptic substances with the active trypsin molecules.

Despite these limitations, antitrypsin titrations with crude trypsin extracts have revealed data of apparent significance in support of the autodigestion theory of allergy.[13,17] For critical studies, however, only tests with crystalline trypsin can now be recommended.

Comment. It is important to realize that all of the procedures outlined above require meticulous attention to detail, and can only be carried out successfully by a conscientious technician, since the slightest deviation from the required technic shows up inexorably. Nevertheless, the tests are readily adaptable to routine use and an average of 20 separate specimens a day may be titrated by a single, practiced worker.

The method makes it feasible to add to the data ordinarily obtained in experimental and clinical studies of allergy precise information on the protease-inhibiting power of the sera of patients and experimental animals, or of the reagents used. The titrations should aid materially in appraising the significance of the previously-neglected antitryptic effect *in vivo* or *in vitro* of the materials and manipulations employed in any experiment.

The basic film-gelatin-digestion technic should prove readily adaptable also for the titration of proteases other than trypsin, for experiments on the kinetics of these enzyme reactions and for the study of important physiologic and pathologic phenomena, other than hypersensitivity, in which natural proteases, their activators and inhibitors, appear to play so vital a part.

Summary. A technically simple, rapid method for the quantitative titration of trypsin or similar proteolytic enzymes, and of protease inhibitors, suitable for critical tests on a large scale is described. The procedure utilizes technics familiar to serologists, rather than to skilled biochemists only, and gives definitive, reproducible results that can be expressed in simple figures. A feature is the provision for the making of a permanent record of each titration. Since the reagents are stable, and readily standardized, comparable findings can be obtained in separate experiments conducted in the same or in different laboratories at any time.

The assistance of Mrs. David A. Cook with records and statistics is gratefully acknowledged.

[17] Burdon, K. L., and Mudd, R. P., PROC. SOC. EXP. BIOL. AND MED., 1949, in press.

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Quantitative Paper Chromatography: A Simplified Procedure. (17425)

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The now classical paper chromatographic method of Consden, Gordon and Martin[1] has been applied to the separation of many types of organic and inorganic substances. Although the method as originally described was only qualitative or roughly quantitative (± 50%), a number of quantitative adaptations have been published. These adaptations may be classified into several general categories: (A) the paper chromatogram is cut so that each substance separated may be extracted with the appropriate solvent and then the concentration of the material in the extract is determined by conventional colorimetry or other means (*cf.*[2]); (B) the materials on the chromatogram are revealed by their own color,

[1] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.