7. Kunkel, H. G., Zone Electrophoresis in Methods of Biochemical Analysis, D. Glick, Ed., N. Y. Interscience Publishers, 1954, v1, 141.

8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. S., *J. Biol. Chem.*, 1951, v193, 265.

9. Kunkel, H. G., Franklin, E. C., Müller Eberhard, H. S., *J. Clin. Invest.*, 1959, v38, 424.

10. Oreskes, I., Singer, J. M., *J. Immunol.*, 1961, v86, 338.

11. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry*, Charles C Thomas, Springfield, Ill., 1948.

12. Grubb, R., Swahn, B., *Acta Path. & Microbiol. Scand.*, 1958, v43, 305.

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A Spectrophotometric Method for Determination of Elastase and Serum Elastase Inhibitor.* (27196)

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The presence of an elastolytic enzyme in pancreatic extracts and the inhibiting effect of human and various animal sera on this enzyme were described by Balo and Banga(1, 2). Methods for the assay of both elastase and its serum inhibitor have included gravimetric(3), Kjeldahl(1), agar plate(4) and nephelometric(5) technics. Sachar *et al.*(6) measured with a spectrophotometric technic the amount of dye released into solution from orcein-impregnated elastin by enzymatic digestion. In attempting to adapt this method to measurement of the serum elastase inhibitor it was found that opalescent sera produced significant errors in the optical density readings. To obviate this the above method was modified by extracting the liberated orcein dye from solution with n-butyl alcohol prior to the spectrophotometric determination.

Materials. Substrate. Purified elastin-orcein powder.‡ Buffers: Primary standard grade Tris buffer [Tris (hydroxymethyl) aminomethane] 1/5 M, pH 8.8. Reagent grade phosphate buffer, 1/15 M, pH 6.0. Enzyme: Crystalline pancreatic elastase.§ A unit of activity as defined by Sachar *et al.* (6) is that amount of enzyme which will solubilize 1 mg of elastin-orcein in 20 minutes under the prescribed conditions. Each sample of enzyme is tested for activity and standardized accordingly. Acid for protein precipitation: Reagent grade 40% trichloroacetic acid (TCA). Extracting medium: Reagent grade n-butyl alcohol. Serum: Venous blood samples obtained prior to the morning meal from 17 patients on the Gynecology Service of the Sloane Hospital for Women, Columbia-Presbyterian Medical Center. The serum was harvested from clotted blood in the customary manner and immediately frozen. Samples were assayed for elastase inhibitor within 2 weeks from date of collection. Samples of sera which were stored at minus 20°C, at 4°C and at room temperature (22°C) showed no change in inhibitor activity at the end of 2 weeks. Hemolyzed sera were not used.

Method for measurement of elastase. Duplicate 25 mg samples of elastin-orcein are placed in 1.5 × 10 cm tissue culture tubes fitted with plastic lined screw caps. Two ml of Tris buffer are added to the substrate in each tube. A 1 ml suspension of elastase in Tris buffer containing 30 units of elastase activity per ml of suspension is added to the reaction mixture in each tube. The screw caps are secured on the tubes and the tubes

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‡ Obtainable from Sigma Chemical Co., St. Louis, Mo.

§ Obtainable from Sigma Chemical Co., St. Louis, Mo., and from Worthington Biochemical Corp., Freehold, N. J.

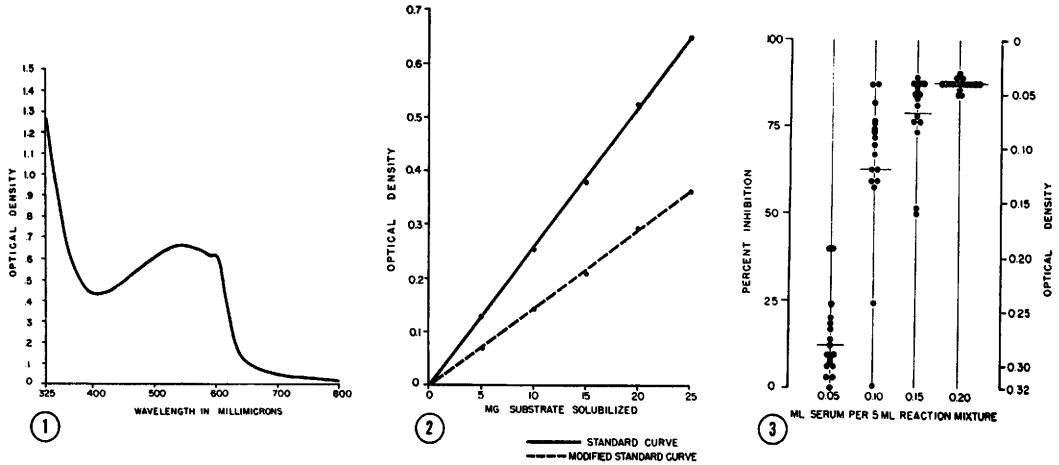

FIG. 1. Absorption spectrum for 25 mg elastin-orcein solubilized by elastase and extracted with 2 ml n-butyl alcohol.

FIG. 2. Relationship between mg elastin-orcein solubilized and optical density of orcein in solution at 540 mμ as described in text.

FIG. 3. Relationship between percentage inhibition of elastase activity and amount of serum present in 5 ml reaction mixture.

are immediately placed obliquely in a test tube rack in a water bath at 37°C. The test tube rack is attached to a shaker‖ to provide uniform agitation at 300 oscillations per minute during the one hour incubation. At the end of this period, since excess elastase is provided, all the substrate is completely solubilized and all the orcein is in solution. The tubes are removed from the water bath and 2 ml phosphate buffer and 1 ml 40% TCA are added in order, following which the color of the mixture changes from blue to pink-violet with the formation of a protein precipitate. Since removal of the protein precipitate by centrifugation results in the loss of much orcein from solution by occlusion, the orcein is extracted with n-butyl alcohol prior to centrifugation. For this process 2 ml of n-butyl alcohol are added to each tube and the screw tops fastened tightly. The tubes are vigorously shaken for 10 seconds, then centrifuged at 2000 rpm for 5 minutes. A white precipitate is seen at the interphase, and the aqueous phase is cloudy but colorless. The alcohol phase containing the dye is pipetted into test tubes and re-centrifuged for 30 minutes. During this second centrifugation, the water retained in the sample accumulates at the bottom of the tube and the sample remains clear thereafter even when stored at 4°C.

To determine the wave length at which maximal absorption occurs by orcein in n-butyl alcohol the samples initially were scanned in a Cary model 11 double beam recording spectrophotometer¶ using a microcuvette having a volume of 0.6 ml and a light path of 10 mm.** With n-butyl alcohol as the blank, each sample was scanned in the visible light region from 325 to 800 mμ. The absorption curve of orcein in n-butyl alcohol is shown in Fig. 1, and the absorption peak is seen to be between 535 and 545 mμ. The optical density of all subsequent samples was then recorded at 540 mμ.

Standard curve. Five mg increments of substrate from 5 to 25 mg are placed in 5 tissue culture tubes. Two ml of Tris buffer and 1 ml of elastase solution (containing 30 units of elastase per ml) are then added to each tube. The tubes are shaken for one hour in the water bath at 37°C. The remainder of the procedure for extracting the dye and recording the optical density is exactly

‖ Burrell wrist action shaker suspended over a constant temperature water bath was used in these experiments.

¶ Manufactured by Applied Physics Corp., Monrovia, Calif.

** Obtainable from Pyrocell Mfg. Co., New York City.

as described before. The results are shown in Fig. 2, with optical density plotted against mg of substrate. Each point represents 7 determinations in duplicate.

Modification of the standard curve. The above method for measurement of elastase activity utilizing protein precipitation and subsequent extraction with butyl alcohol is valid only in cases where all the substrate has undergone enzymatic digestion and has been entirely solubilized prior to addition of TCA. We have found, however, that 40% TCA is capable of removing orcein from elastin even when enzymatic digestion has not occurred. Since the aim is to measure only the dye which has been released by enzymatic action, it is necessary to remove the intact substrate and measure only the orcein which has been solubilized by the elastase. The procedure undertaken is similar to that described above up to the end of the incubation period and the addition of phosphate buffer. Instead of extracting the color from the entire reaction mixture, the mixture is centrifuged for 5 minutes and the supernatant filtered. A 3 ml aliquot of each filtrate is removed. The protein is precipitated, the color extracted with n-butyl alcohol, and the optical density recorded. The resulting modification of the standard curve is shown in Fig. 2.

Measurement of serum elastase inhibitor. Into each of 5 tissue culture tubes are placed 25 mg of substrate, and the following volumes of Tris buffer are added respectively: 1.95 ml, 1.90 ml, 1.85 ml, 1.80 ml. Serum is then added to the tubes in the following amounts respectively: 0.05 ml, 0.1 ml, 0.15 ml, 0.2 ml, so that each of the 4 tubes contains a total of 2 ml of Tris buffer and serum. Into the fifth tube, 2.0 ml of Tris buffer are added without serum and this is the substrate control tube. One ml of elastase solution, containing only 20 units of elastase per ml, is then added to each tube. Screw caps are fastened and the tubes are placed in the water bath for 30 minutes at 37°C, shaking uniformly at about 300 oscillations per minute. Following incubation, 2 ml of phosphate buffer are added immediately to each tube and the tubes centrifuged for 5 minutes. The solutions are filtered and 3 ml aliquots of filtrate are transferred to tissue culture tubes into which one ml 40% TCA and 2 ml butyl alcohol are added. The tubes are shaken vigorously for 10 seconds, then centrifuged for 5 minutes. The butyl alcohol is separated and recentrifuged for 30 minutes and the optical density is then recorded at 540 mμ against a butyl alcohol blank. Control blanks containing serum and Tris buffer are prepared by diluting serum aliquots of 0.05 ml, 0.1 ml, 0.15 ml, 0.2 ml, up to 5 ml with Tris buffer. Three ml aliquots of each blank solution are placed in test tubes and extracted using TCA and butyl alcohol as before and are also read in the spectrophotometer at 540 mμ.

Results. The results obtained from assays of elastase inhibitor in 17 samples of female sera are shown in Fig. 3. The ages of the patients ranged from 20 to 71. Fifteen of the patients were being evaluated for minor gynecological conditions but none were pregnant. Two patients suffered from pelvic carcinoma with intra-abdominal metastases. The values obtained for elastase inhibition with the latter, however, were not different from the majority of the other values at each concentration of serum used, and so have been incorporated in the diagram shown in Fig. 3. The results for each concentration of serum are plotted as percentage of inhibition compared with the substrate control sample, and in optical density units, as shown. Mean values for the 17 points at each concentration of serum are represented by horizontal lines drawn through the respective ordinates. The blanks containing serum and Tris buffer without enzyme or substrate showed no significant optical density using this technic, the highest optical density reading obtained being less than 0.02. Additional controls containing substrate and Tris buffer without enzyme showed no spontaneous hydrolysis following 30 minutes incubation. Hemolyzed sera do show considerable optical activity, with readings up to 0.2.

Discussion. Since the discovery of the inhibitory effect of human, rabbit and cattle serum on elastase by Balo and Banga(2), a number of serum components have been found to exercise a similar inhibitory effect.

Robert and Samuel(7) found the alpha lipoprotein fraction of serum to possess strong inhibitory activity. Tolnay and Bagdy(8), working with human, bovine, rabbit, and guinea pig serum showed the presence of macromolecular inhibitors of elastase which were not dialyzable after 72 hours of dialysis against saline and which were not de-activated by heating to 56°C for 30 minutes. In bovine serum the strongest inhibitors were associated with pseudoglobulin and albumin-type proteins. The work of Walford and Schneider(3), utilizing starch-block electrophoresis of human serum, has indicated the presence of an elastase inhibitor at the junction of the alpha-1 globulin and albumin. Others(8,9) have shown that NaCl, KCl, $(NH_4)_2SO_4$, and Cu^{++} ions all possess inhibitory activity for elastase. The method we have described may be applied to measurement of any elastase inhibitor whether it be serum or any fraction isolated from serum. The stability of the orcein dye in n-butyl alcohol permits storage of samples without fear of change in optical density.

From the points shown in Fig. 3, and the mean values shown for each concentration of serum, it is clear that there is sudden increase in percentage inhibition, as the concentration of serum present is increased from 0.05 to 0.1 ml per 5 ml of reaction mixture. Robert and Samuel(7) also, using a spectrophotometric technic, but with a different substrate and different enzyme preparation, have made a similar observation on pooled human serum. We have found that for any given sample of enzyme and substrate, the results obtained with this technic from repeated assays of a single serum sample are very consistent.

Summary. A spectrophotometric method for determination of elastase and elastase-inhibitory activity has been presented, incorporating Sachar's method of enzymatic digestion of an elastin-orcein substrate with subsequent extraction of the orcein dye from solution with n-butyl alcohol. The inhibitory activity of 15 samples of normal female serum and 2 samples of serum obtained from patients with metastatic pelvic carcinoma was assayed.

1. Balo, J. K., Banga, I., *Schweiz. Z. allg. Path.*, 1949, v12, 350.

2. ———, *Nature, London,* 1949, v164, 491.

3. Walford, R. L., Schneider, R. B., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 31.

4. Sbarra, A. J., Gilfillan, R. F., Bardavil, W. A., *Nature, London,* 1960, v188, 322.

5. Gilfillan, R. F., Sbarra, A. J., Bardavil, W. A., *Fed. Proc.,* 1960, v19, 144.

6. Sachar, L. A., Winter, K. K., Sicher, N., Frankel, S., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 323.

7. Robert, L., Samuel, P., *Ann. biol. clin.,* Paris, 1957, v15, 453.

8. Tolnay, P., Bagdy, D., *Biochim. et biophys. acta,* 1959, v31, 566.

9. Lewis, U. J., Williams, D. E., Brink, N. G., *J. Biol. Chem.,* 1956, v222, 705.

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Lipid Content of Chick Erythrocytes and Plasma. (27197)

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The present concern with regard to lipid metabolism and its relationship to health has resulted in a growing interest in the lipids of red blood cells. Mancini and Keys(1) have reported values for cholesterol content of human erythrocytes; Vacca *et al.*(2) have presented glyceride values for human and canine red cells and James *et al.*(3) have demonstrated a high rate of lipid synthesis in human erythrocytes. Since the majority of the studies reported on erythrocyte lipids have been conducted with mammals, comparison of values obtained for the lipid content of avian red cells is of interest since the chicken, in contrast to most mammals, has nucleated erythrocytes.