of protamine sulfate were found to be ineffective. Fig. 1, curve *D*, is an illustration of the response to 50 mg of protamine sulfate given 8 hours after the administration of paritol.

Comment. Protamine sulfate was found to exert a strong neutralizing effect on paritol activity. There were two substantial differences between its neutralizing effect upon paritol and upon heparin. Parkin and Kvale (3) found that the reaction between heparin and protamine sulfate was completed within 5 minutes. In the case of paritol it required a longer time. The other important difference was the large amount of protamine sulfate required to produce an adequate neutralizing effect upon the activity of paritol (Fig. 1, curves *B* and *C*). The use of such doses may possibly be hazardous because of the well-known toxic effects of protamine sulfate itself (2,6,7).

Two reactions occurred in our studies. Each began within 30 minutes after the injection of protamine sulfate during an attempt to neutralize the anticoagulant activity of paritol which had been given previously. They consisted of chills, fever, nausea, vomiting, burning epigastric pain and aching in the lumbar region of the back. These symptoms began to subside in a few hours and had disappeared completely within 4 days. The one unusual feature that was noted in these 2 cases was that the protamine sulfate had been administered in a more rapid manner than that recommended by other authors. This reaction did not occur when the protamine had been given 4 or 8 hours after the paritol had been given.

Summary. This study indicates that protamine sulfate is an active antagonist of paritol in the human subject. Larger doses than those used in neutralizing the effect of heparin are required to counteract the defect produced by paritol. Large amounts of protamine sulfate may produce toxic symptoms and therefore should be used with caution.

1. Barker, N. W., and Barker, D. N., *Proc. Staff Meet., Mayo Clin.*, 1948, v23, 230.

2. Chargaff, E., and Olson, K. B., *J. Biol. Chem.*, 1937, v122, 153.

3. Parkin, T. W., and Kvale, W. F., *Am. Heart J.*, 1949, v37, 333.

4. Roberts, E. F., personal communication to the authors.

5. Seifter, J., and Begany, A. J., *Am. J. M. Sc.*, 1948, v216, 234.

6. Shelley, W. B., Hodgkins, Myrtle P., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 300.

7. Shelley, W. B., and Tarail, R., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 215.

8. Sorenson, C. W., and Wright, I. S., *Circulation*, 1950, v2, 658.

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A Colorimetric Method for Evaluating Chymotrypsin Inhibitors in Human Serum. (19871)

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(Introduced by Wendell H. Griffith.)

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Elevation of chymotrypsin inhibitor levels occurs in human blood serum with various pathological states(1), with pregnancy(2), and with certain types of mental disorders (3). Determinations of these inhibitor levels by the method of West and Hilliard(4), using homogenized milk as a substrate, can be used to follow the course of diseases such as cancer, and serve as a guide in evaluating various types of therapy(5-7).

Since the determination of chymotrypsin inhibitor levels by the method of West and Hilliard employs a nonsynthetic substrate (milk) and involves subjective end point determinations, it was decided to look for a simple synthetic substrate whose hydrolysis

could be determined colorimetrically. The method presented in this paper is based on the work of Iselin, Huang, and Nieman(8). These workers observed that hydroxamides of aromatic alpha amino acids are hydrolyzed by chymotrypsin and, since these hydroxamides give colored solutions in the presence of ferric chloride, that their hydrolysis by chymotrypsin may be quantitated by colorimetric analysis.

Materials and methods. Substrate. The synthetic substrate, l-tyrosine-hydroxamide, is prepared by the method of Cunningham, *et al.*(9) and is buffered to pH 7 with tris (hydroxymethyl) aminomethane hydrochloride acid buffer.* In a 100 ml volumetric flask, dissolve 100 mg of l-tyrosinehydroxamide in 0.1 M buffer with gentle heating. Cool, and bring to volume with buffer. This solution is stable. A mixture containing 0.5 ml of buffered substrate, 0.5 ml buffer, and 5.0 ml methanol-$FeCl_3$ solution gives a Klett reading† of approximately 250 (green filter No. 540, reference at 0 with distilled water). *0.1 M Buffer.* To 12.11 g of purified tris (hydroxymethyl)aminomethane and 22 ml of 4 N HCl in a 1000 ml volumetric flask, add approximately 900 ml distilled water. Adjust to pH 7.0 with 1 N HCl and bring up to volume. Filter this solution just before using. *Chymotrypsin.* Dissolve 20 mg crystalline chymotrypsin‡ in 20 ml of 0.1 M buffer. Shake the solution well to insure proper mixing and refrigerate for an hour before using. This enzyme solution must be prepared freshly and standardized daily. The activity of different lots of chymotrypsin will vary and it will decrease gradually even when stored at refrigeration temperatures. *Stock Ferric Chloride Solution.* Dissolve 7.0 g $FeCl_3 \cdot 6H_2O$ in 200 ml of 1 N HCl and bring up to a 250 ml volume with absolute methanol and filter. *Methanol-Ferric Chloride Color Reagent.* To 50 ml of stock ferric chloride solution add 250 ml absolute methanol. For rapidity in the procedure and to eliminate toxic effects of pipetting methanol, this solution should be added to the test mixtures from a 25 ml burette. *Blood Serum.* Tests must be made on fresh blood serum or on serum which has been refrigerated not longer than 24 hours. Hemolysis interferes with the accuracy of the test. Fasting blood samples are preferable.

Standardization of the chymotrypsin control. To each of 6 test tubes (for convenience in mixing, the tubes should be approximately 13 x 120 mm) add 0.5 ml substrate and then 0.5 ml of freshly prepared chymotrypsin solution. Mix quickly with a rotary motion and place in a 37°C water bath; agitate every 20 minutes. Incubate the first 2 tubes for exactly 1½ hours, and stop the reaction by adding 5 ml methanol-ferric chloride reagent. Mix and determine the density in a Klett colorimeter (filter 540, reference with distilled H_2O). The color develops immediately and is stable for several hours. If these first two enzyme control tubes check each other and give a Klett reading between 90 and 95, the 1½-hour incubation period is used for the test and the remaining four enzyme control tubes may be discarded. If the reading exceeds 95, the remaining tubes are allowed to incubate for longer periods in order to bring their colorimetric readings within the proper range. The increment of time beyond 1½ hours may be estimated by referring to Fig. 1 which shows the change in the density readings of the *chymotrypsin control* with time. The usual period of incubation is 2 hours.

Determination of chymotrypsin inhibitor in serum. Pipette 0.1 ml serum directly into the bottom of each of 4 test tubes. Add 0.5 ml substrate to each. To tubes 1 and 2, which will be the *serum-substrate controls,* add 0.5 ml buffer. To tubes 3 and 4, the *serum assay tubes,* add 0.5 ml standardized chymotrypsin solution. Immediately after adding the enzyme, mix the contents of all 4 tubes with a rotary motion, place in a 37°C water bath and record the exact time. These tubes should be swirled about every 20 minutes dur-

* Purified tris (hydroxymethyl) aminomethane may be purchased from G. Frederick Smith Chemical Co., Columbus, O., or the impure product obtained from Commercial Solvents, New York, may be prepared for use by twice crystallizing from absolute ethanol.

† The optical density is equal to the Klett reading multiplied by 0.002.

‡Crystalline chymotrypsin may be obtained from Spicer-Gerhart, Sunland, Calif., or from Armour Co., Chicago, Ill.

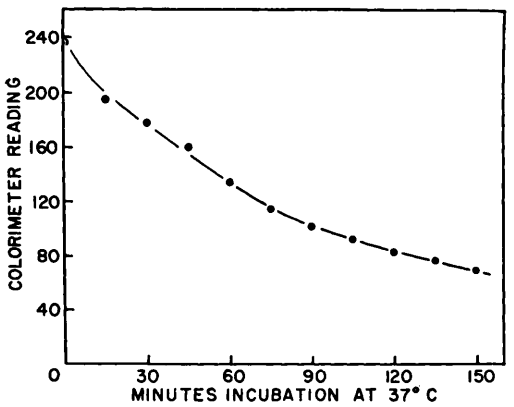

FIG. 1. Change in density readings of the chymotrypsin control with min of incubation in a 37°C water bath.

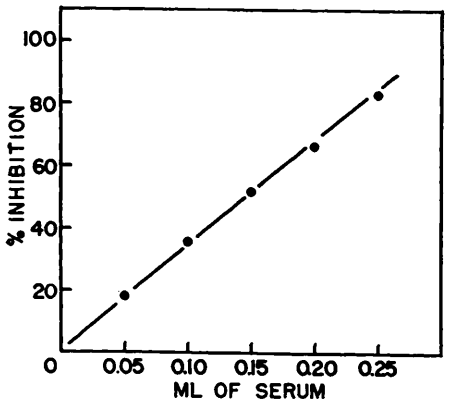

FIG. 2. Inhibition of enzyme activity with increasing amounts of serum.

ing the incubation period which has been determined by the chymotrypsin control as described above. At the end of this period, 5.0 ml of methanol-ferric chloride solution is added to all 4 tubes. This stops the reaction in tubes 3 and 4. *Density readings on all serum containing tubes must be made immediately, since a fine protein precipitate will form within a few minutes after the methanol-ferric chloride color reagent is added.* Therefore, when testing more than one serum a five minute interval should elapse between the addition of enzyme to each succeeding set of serum tubes, thus allowing time for colorimetric readings to be made on these 4 tubes before the incubation period has elapsed for the following set.

Calculations. The serum chymotrypsin inhibitor values, expressed in terms of per cent inhibition, are calculated as follows: % inhibition $= A - C/S - C \times 100$. A—Colorimeter readings for serum assay tubes; C—Colorimeter readings for chymotrypsin control; S—Colorimeter readings for serum-substrate control.

Discussion. Fig. 2 shows the linear relationship between enzyme inhibition and the amount of serum used in the assay. Under the experimental conditions described above, the sera from normal individuals give inhibitions ranging from 15 to 30%. The empirically determined curve in Fig. 3, based on data from 400 determinations,§ shows the relationship between units of chymotrypsin inhibitor as determined by the milk method (4) and inhibition of tyrosinehydroxamide hydrolysis. Tables I and II show the effect of varying either the enzyme or the substrate level in the test.

Summary. A colorimetric method of determining serum chymotrypsin inhibitor levels has been described. This method has the following advantages over the original milk method described by West and Hilliard: 1. It is based on the hydrolysis of a stable, synthetic substrate. 2. Colorimetric determina-

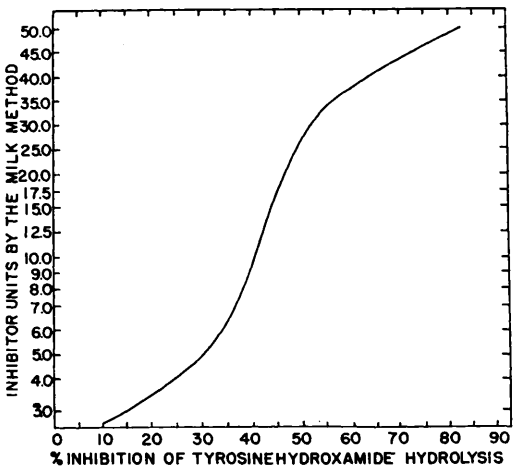

FIG. 3. Relationship between units of chymotrypsin inhibitor as determined by the milk method and the % inhibition of tyrosinehydroxamide.

§ The authors are indebted to Dr. Philip M. West and his laboratory staff at the Long Beach Veterans Administration Hospital for the data from which the graph in Fig. III was derived.

TABLE I. Effect of Varying the Concentration of Chymotrypsin Solution on Analysis of Serum Chymotrypsin Inhibitors.

Chymotrypsin sol'n, mg/ml	Incubation time, min	Klett readings: Serum substrate control (S)	Klett readings: Enzyme control (C)	Klett readings: Serum assay tube (A)	% inhibition
.25	120	248	180	244	94.1
.50	"	"	135	208	64.6
.75	"	"	98	161	41.5
1	"	"	83	129	27.9
1.50	"	"	52	90	19.4
2	"	"	40	63	11.1

TABLE II. Effect of Varying the Concentration of Substrate Solution on Analysis of Serum Chymotrypsin Inhibitors.

Substrate* sol'n, mg/ml	Incubation time, min	Klett readings: Serum substrate control (S)	Klett readings: Enzyme control (C)	Klett readings: Serum assay tube (A)	% inhibition
.25	105	86	32	49	27.5
.50	"	137	54	77	27.7
1	"	250	83	145	37.1
1.50	"	362	120	203	34.1
2	"	490	165	264	30.5

* l-tyrosinehydroxamide.

tions on timed reactions have obviated the need for subjective end point determinations.

1. West, P. M., and Hilliard, J., *Ann. West. Med. Surg.*, 1949, v3, 227.

2. West, P. M., Hilliard, J., and Mietus, A. C., *Surg. Gyn. Obst.*, 1951, v72, 209.

3. Jacobs, J. S. L., West, P. M., and Tempereau, C. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 410.

4. West, P. M., and Hilliard, J., PROC. SOC. EXP. BIOL. AND MED., 1940, v71, 169.

5. West, P. M., Rapaport, S. I., and Tempereau, C. E., *Cancer*, 1951, v4, 177.

6. Ellis, F. W., and West, P. M., *J. Clin. Invest.*, 1951, v30, 547.

7. Szujewski, H. A., *J. Am. Med. Assoc.*, 1952, v148, 929.

8. Iselin, B. M., Huang, H. T., and Niemann, C., *J. Biol. Chem.*, 1950, v183, 403.

9. Cunningham, K. G., Newbold, G. T., Spring, F. S., and Stark, J., *J. Chem. Soc.*, 1949, 2091.

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Inhibition of Rapid Production of Antibody by Cortisone. Study of Secondary Response.* (19872)

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The administration of cortisone or ACTH to rabbits results in a diminution of circulating antibody, as has been demonstrated by independent quantitative immunochemical studies(1,2). The diminished level of antibody resulted if the hormone was given during the period of primary immunization, or after antibody production was well established. Since the disappearance rate of rabbit antibody pas-

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