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17120. Proteolytic Enzyme Inhibitors of Human Serum in Health and Disease.*†

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The antiproteolytic power of blood serum has been described in the reports of Flexner *et al.*,[1] Sure,[2] Weil and Russell[3] and Clark *et al.*[4] Following purification of several proteolytic enzymes it became possible to determine the probable nature of this enzyme antagonism. Crystalline pepsin inhibitor isolated by Herriott[5] and trypsin inhibitor by

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1 Flexner, L. B., Berkson, J., Winters, H., and Wolman, I., PROC. SOC. EXP. BIOL. AND MED., 1929, **26**, 592.

2 Sure, B., *Biochem. J.*, 1935, **29**, 1508.

3 Weil, L., and Russell, M. A., *J. Biol. Chem.*, 1938, **126**, 245.

4 Clark, D. G. C., Clifton, E. E., and Newton, B. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 276.

5 Herriott, R. M., *J. Gen. Physiol.*, 1941, **24**, 325.

Kunitz and Northrop[6] have been shown to be highly specific, reacting to form inert dissociable complexes with the respective proteinase. As a sensitive means of studying protein metabolism in various pathological states, an investigation of the role of certain of the circulating proteolytic enzyme inhibitors appears to offer a new approach of considerable promise. This paper presents a method for quantitative estimation of both rennin and chymotrypsin inhibitors of serum, together with results obtained in a survey of 2000 patients.

Materials and methods. The method is based on the milk coagulating property of both chymotrypsin and rennin. Determinations are made on fresh serum or serum stored at ice-box temperature not over 24 hours. Fasting blood is not required. 1.*Enzyme Standardization.* (a) *Chymotrypsin.* Two to 3 mg crystalline chymotrypsin (supplied through courtesy of Spicer-Gerhart Company, Pasadena, California) are dissolved in 10 ml of distilled water. 0.2 ml of solution is pipetted into two 13 x 120 mm tubes containing 2.0 ml of fresh homogenized milk (pH 6.6) which is at ice-box temperature. A stop watch is started, contents of tubes mixed quickly by rotation, and placed immediately in a water bath at 37° ± 0.1°. If coagulation has not occurred in 9 minutes tubes are shaken gently every 15 seconds. The time at which the first granular precipitation of calcium paracaseinate appears in the film on the wall of the tube is the end point. Dilution of enzyme solution is continued until the end point occurs at 10 minutes 45 seconds to 10 minutes 50 seconds. This precise time is selected to provide reasonable stability of the enzyme as well as optimum conditions for measurement of inhibitor. At 10 minutes, variation in inhibitor levels is barely detectable, and over 11 minutes, inhibition is too great for accurate measurement and the enzyme is unstable. It is essential that the enzyme activity fall exactly within the 5 second time limitation. The enzyme will retain this activity for about 3 hours. Stability is not improved by buffering. No variation attributable to natural homogenized milk as a substrate has been encountered in over a year of continuous use of the method. Powdered and condensed varieties are unsuited to accurate detection of the end point. Both milk and enzyme are kept in the ice-box except when actually in use. (b) *Rennin.* One rennet enzyme tablet ("Junket" brand, Chr. Hansen's Laboratory Inc., Little Falls, N. Y.) is dissolved in 80 ml of distilled water, and filtered after 10 minutes. Activity is adjusted as for chymotrypsin to an end point of 10′ 55″ to 11′ 0″. The difference of 10 seconds between the two enzymes allows measurement of inhibitors over a comparable range. 2. *Determination of Inhibitor.* (a) *Chymotrypsin inhibitor.* Two control tubes and 6 others representing 3 duplicate serum determinations are set up together. Dilute serum 1:10 with distilled water and pipette 0.04 ml into each tube. 2 ml of milk is added and the contents mixed. Finally, 0.2 ml of standardized chymotrypsin solution is added quickly and accurately, a stop watch started, the eight tubes thoroughly mixed in 20 seconds or less, and placed together in the water bath. At 10′45″ when controls reach the end point the watch is reset. Units of inhibitor are the number of additional minutes required for the serum-containing tubes to reach the end point. Results must agree within 30 seconds. Failure to reach the end point 30 minutes beyond the control is equivalent to complete inactivation of enzyme and is therefore the maximum reading. Time units are not related arithmetically to actual units but this correction is not applied. (b) *Rennin inhibitor.* Proceed as above except that 0.05 ml of undiluted serum is required in this reaction and the maximum value obtainable is 50 units. 3. *Interpretation.* (a) *Chymotrypsin inhibitor*: normal range 5-10 units; 13-30 units abnormal. (b) *Rennin inhibitor*: normal ranges 5-10 (70%) and 20-50 units (30%); 13-18 units abnormal.

Results. In this preliminary survey, 2000 random blood specimens representing all available types of pathology were studied. Six to 8 weeks later, pertinent clinical and laboratory findings that had been collected on each case were assembled with special reference to the

[6] Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

TABLE I.
Occurrence of Abnormal Levels of Chymotrypsin and Rennin Inhibitors in Various Groups of Patients.

	No. of cases	Abnormal chymo and/or rennin	
		No.	%
1. Pregnancy (all stages)	103	88	85.3
2. Malignancy (entire group)	220	130	59.0
" (active, untreated)	133	118	88.7
" (treated)	87	12	13.7
3. Allergy	33	9	27.2
4. Acute infections, active phase (bacterial and virus)	176	45	25.5
5. Benign breast lesions	33	8	24.2
6. Cardiovascular (entire group)	101	20	19.8
" (decompensation)	23	12	52.1
" (hypertension)	22	8	36.3
7. Chronic infections (tuberculosis, syphilis, coccidiomycosis, bronchiectasis, etc.)	140	26	18.5
8. Psychoneuroses and psychoses	55	10	18.1
9. Traumatic damage and surgical procedures (within 1 week)	280	45	16.0
10. Peptic ulcer, ileitis, colitis	83	6	7.2
11. Metabolic (diabetes, cirrhosis, nephritis, arthritis, hyperthyroidism)	137	7	5.1
12. Benign tumors (polyps, papilloma, fibromyoma, fibroma, lipoma, adenoma)	75	4	5.3
13. Unclassified	346	13	3.7
14. Healthy subjects (including routine preoperative hernia, hemorrhoids, varicose veins, etc.)	218	0	0.0
Total	2000	411	20.5

TABLE II.
Pathological and Physiological States Influencing Chymotrypsin and Rennin Inhibitors.

A. Pathology influencing both chymotrypsin and rennin inhibitors with equal frequency.	
1. Pregnancy.	
2. Malignancy	
3. Trauma and surgery	
4. Cardiac decompensation	
5. Chronic infections	
B. Pathology influencing chymotrypsin inhibitor predominantly.	
1. Acute infections (bacterial and virus)	24% of cases
C. Pathology influencing rennin inhibitor predominantly.	
1. Hypertension	31.8%
2. Benign breast lesions	24.2
3. Allergy	21.2
4. Psychoneuroses	18.1
5. Fractures (healing stage)	17.5

exact condition of the patient at the time the inhibitor determination was made. Results were correlated with these facts and the conclusions are summarized in Tables I and II.

Discussion. From a survey of data presented, it seems probable that rennin and chymotrypsin inhibitors reflect different phases of metabolism, since the concentration of one is seen to vary independently of the other. However, in disturbances of fluid balance, both may follow the same trend. With diminished fluid output in persistent vomiting, burns, hemorrhage, shock, high fever or congestive failure, both values frequently increase together. That the full explanation may be more complex is suggested by the fact that most of the above conditions are characterized by increased excretion of adrenal cortical ster-

oids having a profound influence on protein metabolism. Factors which influence the rennin inhibitor predominantly further implicate the steroid hormones. The association of estrogens with certain benign breast hyperplasias is well known and the significance of stimulated estrogenic or androgenic activity in healing fractures has been demonstrated by Albright and co-workers.[7] Rennin inhibitor may increase also in many of the psychoses which have been shown by Pincus and Elmadjian[8] to exhibit diminished adrenal response to stress. The unexpected finding that a high percentage of the hypertensive and allergic groups have abnormal rennin inhibitor levels is of interest but difficult to interpret. Studies to determine the direct effects of steroid hormone administration on the proteolytic enzyme inhibitors are being undertaken.

Pregnancy is usually accompanied by a pronounced elevation of both inhibitors in the mother's circulation. In contrast, the cord blood of the newborn is high only in rennin inhibitor, while the other is very low.

A study of malignancy by this method reveals a characteristic but fluctuating picture, the host doing well clinically when the rennin inhibitor only is elevated, as occurs early, or at intervals in the course of the disease, but failing rapidly when the chymotrypsin inhibitor rises very high and rennin inhibitor drops to low levels (unpublished data). Obviously, it would be impossible to diagnose malignancy by these methods since an increase in inhibitor of either proteolytic enzyme is not specific for cancer. The inhibitor concentrations are profoundly influenced by radiation therapy and surgical excision, and thus a low percentage of abnormal inhibitor levels was noted in treated cases. Repeated observations on the serum inhibitors in cases with neoplasm may prove to be of considerable prognostic value and may indicate when further treatment is required as well as the effectiveness of the treatment given. This appears to us to be the chief value of the method as applied to the cancer patient. This aspect of the problem is being extended at the present time.

Summary. Methods are described for the determination of chymotrypsin and rennin inhibitors in serum. The many physiological and pathological conditions which influence their concentration are discussed.

[7] Albright, F., Bloomberg, E., and Smith, P. H., *Trans. Assn. Am. Phys.*, 1940, **55**, 298.

[8] Pincus, G., and Elmadjian, F., *J. Clin. Endocrinol.*, 1946, **6**, 285.

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17121. A Comparison of Total Body Water as Determined by Antipyrine and Desiccation in Rabbits.

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A method for determining total body water which employs the volume of uniform distribution of antipyrine, an analgesic drug, has been described recently.[1] Further support for the antipyrine method is presented in the present paper which records the values for total body water estimated by the antipyrine procedure and the values obtained by actual desiccation of the experimental animals.

Experimental. Four rabbits were injected intravenously with 60-240 mg of antipyrine according to body weight (approximately 100 mg/kg). Blood samples were drawn from each animal at 50, 75 and 100 minutes following the administration of the drug. The zero time plasma concentration (the concentration at the time of injection if uniform distribution

[1] Soberman, R. J., Brodie, B., Hollander, V., Levy, B., Axelrod, J., and Steele, J. M., *J. Biol. Chem.*, 1949, **179**, 31.