PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 150, 98–103 (1975)

Isolation and Identification of α_1-Antitrypsin as a Component of Normal and Malignant Human Breast and Other Tissues[1] (38981)

SALLY S. TWINING AND ARTHUR S. BRECHER[2]

Department of Chemistry, Bowling Green State University, Bowling Green, Ohio 43403 and the Department of Physiological Chemistry, Ohio State University Medical School, Columbus, Ohio 43210

Proteases and inhibitors of proteolytic activity are believed to be important in the metabolism of malignant cells (1–12). Various reports cite elevated levels of chymotrypsin, trypsin, and cathepsin-like enzymes in transformed cells (1–6). The proteolytic activity is believed to be responsible for several properties of transformed cells, including uncontrolled proliferation and increased migration (3–5). Hydrolases also are believed to play a role in metastases by decreasing the cohesiveness between cells in the primary tumor (9) and by breaking down the intracellular matrix which holds the cells together at the sites of metastases (10). Naturally occurring inhibitors of proteolytic activity are likewise observed in higher amounts in neoplastic tissues (13, 14) and in other conditions (13, 15–17). Naturally occurring and synthetic protease inhibitors have been reported to arrest cell growth of tumor cells and transformed cells (7, 8, 11, 12).

Wasilauskas and Brecher reported the presence of antiproteolytic activity in the 90,000*g* supernatant fraction of glioma, ovarian carcinoma, and normal and malignant breast and colon tissues (18). These fractions contained nondialysable, heat labile inhibitors of tryptic and chymotryptic activity. This communication extends earlier findings (18) and reports the presence of α_1-antitrypsin in the 90,000*g* supernatant fractions of malignant and normal human breast tissue of nonlactating women as well as other tissues by radial immunodiffusion. This inhibitor and several other inhibitors of chymotryptic and tryptic activity have been extensively purified from breast tissue extracts by affinity chromatography on Sepharose–chymotrypsin or Affi-Gel 10-chymotrypsin, representing a new and efficient means of isolating the α_1-antitrypsin. A preliminary report has appeared elsewhere (19).

Materials and methods. Twice crystallized trypsin and three times crystallized α-chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, NJ. Hammersten casein, benzoyl-L-tyrosine ethyl ester (BTEE) and hemoglobin were purchased from Schwarz/Mann Research Laboratories, Orangeburg, NY.

Bovine serum albumin, Cohn Fraction V and heparin containing 158 USP-J-A units/mg were obtained from Sigma Chemical Corporation, St. Louis, MO. Affi-Gel 10 was purchased from BioRad Laboratories, Richmond, CA. Sepharose–chymotrypsin was obtained as a gift from Owens-Illinois, Toledo, OH. *N*, *N*, *N'*, *N'*-Tetramethylethyenediamine, acrylamide and *N*,*N* methylene-*bis*-acrylamide were obtained from Eastman Kodak, Rochester, NY. Basic fuchsin and Coomassie Brilliant Blue R-250 were purchased from Fisher Scientific Company, Fairlawn, NJ and Colab Laboratories, Glenwood, IL, respectively. An α_1-Trypsin Inhibitor Quantitative Kit was purchased from Miles Laboratories, Kankakee, IL.

Samples of normal and/or malignant human breast, colon, ileum, anal, lung, and stomach were obtained after surgery and stored frozen until further use. The tissues were thawed and homogenized 1:9 in either 0.32 *M* sucrose solution or 0.1 *M* Tris buffer, pH 7.6 for 2 min using a Virtis homogenizer. The mixture was centrifuged at 20,000*g* for 30 min using a RC-2 Sorvall centrifuge. The 20,000*g* supernatant fraction was then

[1] Supported, in part, by a grant from the Ohio Division, Inc. of American Cancer Society.

The data presented herein will be presented as a portion of the dissertation to be submitted by S.S.T. in partial fulfillment for the Ph.D. degree.

[2] Supported, in part, by a grant from the Faculty Research Committee of Bowling Green State University.

centrifuged for 75 min at 90,000*g* using an L2-65B Beckman ultracentrifuge. The 90,000-*g* supernatant fraction was stored frozen in aliquots.

Levels of antitryptic and antichymotryptic activity were determined using the modifications of Wasilauskas and Brecher (18) of the caseinolytic assay of Kunitz (20). The protein content was determined by applying the procedure of Lowry *et al.* (21), utilizing bovine serum albumin, Cohn Fraction V as the protein standard.

Sepharose–chymotrypsin was prepared by the procedure of Porath et al. (22). The activity of the active insolubilized chymotrypsin was determined to be 30 μg/ml at pH 7.6 by the casein assay for chymotryptic activity.

Chymotrypsin was coupled to the Affi-Gel 10 by shaking a mixture of 80 ml of 20 mg/ml chymotrypsin in 0.1 *M* phosphate buffer, pH 7.0 with 4 g of Affi-Gel 10 at 4° for 3.5 hr. The mixture was transferred to a 1.5 × 30 cm column and washed sequentially with 3 liters of 0.1 *M* phosphate buffer, pH 7.0, and 500 ml of 0.1 *M* Tris buffer, pH 7.6. The final activity of the column was determined to be 40 μg/ml at pH 7.6 using the casein assay for chymotryptic activity.

Charges of 90,000*g* supernatant fraction of normal and malignant breast extracts were passed through either the Sepharose–chymotrypsin column or the Affi-Gel 10-chymotrypsin column. The columns were eluted sequentially using 0.1 *M* Tris buffer, pH 7.6; a 0.1 *M* to 0.3 *M* NaCl gradient in 0.1 *M* Tris buffer, pH 7.6; 0.1 *M* Tris buffer, pH 7.6; 1 m*M* HCl, and 0.1 *M* Tris buffer, pH 7.6.

Peaks of antitryptic and antichymotryptic activity were pooled and concentrated by ultrafiltration using Amicon Diaflo PM ultrafiltration membranes at 40 psi nitrogen pressure. The 90,000*g* supernatant fractions and the pooled concentrated column fractions were further concentrated for electrophoresis and immunodiffusion experiments using Amicon B-15 miniconcentrators.

The proteins in the 90,000*g* supernatant fractions, the pooled column eluates, concentrated eluates, serum, and chymotrypsin were separated either by using the alternate disc gel electrophoresis method of Davis (23) or the micro disc gel electrophoresis technique of Burr *et al.* (24). In both systems the glycine-Tris buffer, pH 8.3 system of Davis was used in an ISCO Model 1270 electrophoresis apparatus at 4°. The gels were stained for glycoprotein using the periodic acid Schiff's method of either Matthieu and Quarles (25) or Kapitany and Zebrowski (26). Protein was detected with Coomassie Brilliant Blue according to the procedure of Weber *et al.* (27).

Aliquots of 1–10 μl of the pooled inhibitory peaks from the Affi-Gel 10-chymotrypsin column were deposited on cellulose acetate strips and tested for glycoprotein content by application of the periodic acid Schiff's method (28).

Five μl samples of the pooled concentrated peaks from the Affi Gel-10 column and the 90,000*g* supernatant fraction of normal and/or malignant breast, column, anal, ileum, stomach and lung tissue were placed on radial immunodiffusion plates containing human plasma α_1-antitrypsin content of these tissue extracts was determined using the method of Kueppers (29). The amount of blood present was estimated by determining the hemoglobin content in the tissue extracts using a colorimetric oxyhemoglobin method (30).

Results. Figure 1 represents a typical separation of the components found in malignant breast upon affinity chromatography of 300 mg of protein from a 90,000*g* extract on Affi-Gel 10-chymotrypsin. Eleven peaks of antichymotryptic and antitryptic activity were noted. Seven peaks of antichymotryptic activity were found in the NaCl gradient eluate, one peak of antichymotryptic and one peak of antitryptic activity were observed in the subsequent Tris buffer eluate. Two peaks of both antitryptic and antichymotryptic activity were seen in the Tris buffer eluate following the drop in pH. Consistently negligible amounts of protein were detected in the peaks after the initial breakthrough peak, suggesting a very high degree of purification of inhibitors. Very low levels of endogenous activity were seen in these eluates. Peaks 1–8 contained a glycoprotein component as detected with the periodic acid Schiff's method on cellulose acetate. Similar patterns of activity were ob-

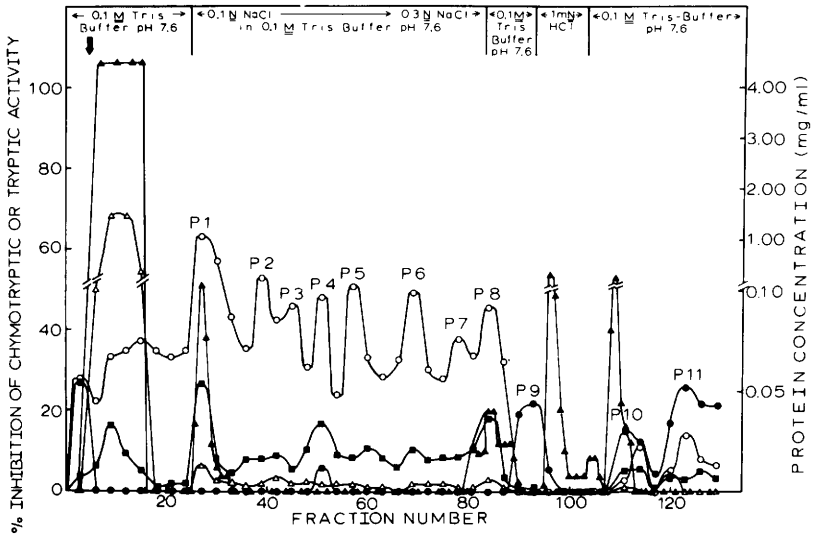

FIG. 1. Separation of chymotrypsin and trypsin inhibitors in the 90,000*g* supernatant fraction of malignant human breast tissue from patient no. 4150 on Affi-Gel 10-chymotrypsin. Affi-Gel 10 was reacted with chymotrypsin to form Affi-Gel 10-chymotrypsin. An aliquot containing 300 mg protein of the 90,000*g* supernatant of malignant breast tissue from patient no. 4150 was applied to the Affi-Gel 10-chymotrypsin column. The column was sequentially eluted with 200 ml of 0.1 *M* Tris buffer, pH 7.6; 1160 ml of a 0.1 *M*–0.3 *M* NaCl gradient in 0.1 *M* Tris buffer, pH 7.6; 220 ml of 0.1 *M* Tris buffer, pH 7.6; 220 ml of 1 m*N* HCl; and 160 ml of 0.1 *M* Tris Buffer, pH 7.6. Antitryptic and antichymotryptic activity were determined by the casein assay (1) using 0.2 ml of the column eluate and 0.5 μg enzyme. ○, Antichymotryptic Activity. ●, Antitryptic activity. △, Protein concentration by method of Lowry *et al.* (21). ▲, Protein concentration at 280 nm. ■, Endogenous caseinolytic Activity. ↓, 90,000*g* supernatant fraction added.

served with Sepharose–chymotrypsin columns. Electrophoresis of the pooled fractions on 15% polyacrylamide micro gels generally showed only one or two protein bands. Similar experiments on pooled peaks (peak 1) with 7% acrylamide gels of Davis (23) exhibited only two detectable protein bands both of which contained glycoproteins (Fig. 2). The major band migrated to the same extent as α_1-antitrypsin of serum. Peak 1 contained antitryptic and antichymotryptic activity. The major component of Peak 1 was determined to be immunologically equivalent to plasma α_1-antitrypsin by immunodiffusion. The 90,000*g* supernatant fraction additionally showed a band of comparable migration to that of α_1-acid glycoprotein of serum.

Table I relates the levels of α_1-antitrypsin found in the 90,000*g* supernatant fractions of normal and/or malignant human tissues. The concentration of α_1-antitrypsin in the tissue extracts from blood (Column 3) was calculated from the hemoglobin content using 0.15 gm/ml and 4.4 mg/ml as the concentration of hemoglobin (31) and α_1-antitrypsin per ml of serum. The value for α_1-antitrypsin was estimated by taking the mean value of α_1-antitrypsin in normal humans (29, 30), which is also the standard value for Miles immunodiffusion plates used in this study, and doubling the value to account for the acute phase increase (13, 33) in α_1-antitrypsin under neoplastic conditions. The α_1-antitrypsin content in the tissue (column 4) was determnied by subtracting the α_1-antitrypsin due to blood from the total amount of α_1-antitrypsin in the tissue extracts.

Discussion. The isolation of chymotrypsin and trypsin inhibitors by affinity chromatography of the 90,000*g* extracts of human breast tissue on Sepharose–chymotrypsin or Affi-Gel 10-chymotrypsin represents a new, simple means of purifying and isolating α_1-antitrypsin almost to electrophoretic homogeneity, in contrast to the multistep procedures (34–36). Ten additional peaks of antiproteolytic activity were obtained subsequent to the elution of the α_1-antitrypsin

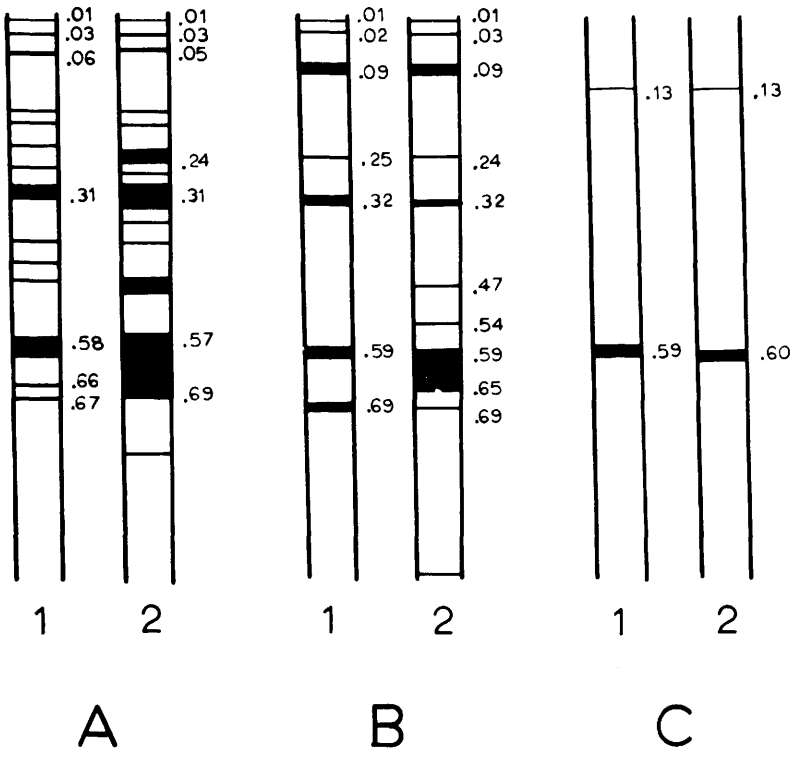

FIG. 2. Polyacrylamide disc gel electrophoresis of the 90,000*g* supernatant fraction of malignant human breast tissue, purified protease inhibitor from Peak 1, serum. Polyacrylamide disc gel electrophoresis was performed by the method of Davis (23). The gels were stained with periodic acid Schiff's reagent for glycoprotein [1] and with Coomassie Brilliant Blue for protein [2]. A. Serum, B. 90,000*g* supernatant fraction from patient no. 4150, C. Peak 1 concentrated eluate from the Affi-Gel 10-chymotrypsin column.

peak in the procedure reported herein. The use of the NaCl salt gradient and the lowering of the pH to three essentially removed all bound inhibitors as the columns were successfully used repeatedly in contrast with Sepharose-trypsin which more tightly binds α_1-antitrypsin (37).

The difference in binding properties of α_1-antitrypsin towards Sepharose-chymotrypsin and Sepharose-trypsin is probably due to the fact that chymotrypsin and trypsin bind to two different sites on the α_1-antitrypsin molecule (38). The inhibitors are highly purified as evidenced by the fact that negligible amounts of protein are associated with the inhibitory peaks, and by the preliminary disc gel electrophoresis experiments on the 15 % acrylamide disc gels and the 7 % Davis gel (23) for peak No. 1.

α_1-Antitrypsin has been reported as a component of mast cells (39) and macrophages (40) which are contained in connective tissue (41). It would therefore be anticipated that α_1-antitrypsin would be widely distributed, as indeed this report suggests and indeed confirms (39, 40, 42).

Protease inhibitors may serve a further role, in addition to the inhibition of normal and neoplastic cell proliferation (4, 7, 8, 11). As result of observations of children after surgery, Lennert *et al.* (43) have suggested that α_1-antitrypsin and α_2-macroglobulin serve a role as wound healers. The increase in α_1-antitrypsin in serum is probably due to an attempt by the body to control proteolytic activity. It is one of the acute phase reactants produced by the liver in response to trauma such as inflammation, injury, surgery, infection, neoplastic growth, pregnancy or administration of hormones (33). It has also been proposed that protease inhibitors in the plant kingdom promote wound healing (44, 45).

Summary. α_1-Antitrypsin has been de-

TABLE I. α_1-ANTITRYPSIN IN HUMAN TISSUE.[a]

Patient number	Tissue	mg α_1-AT/mg protein $\times 10^2$	mg Hb/mg protein $\times 10^1$	mg α_1-AT/mg protein $\times 10^2$ from blood (estimated)	mg α_1-AT/ (corrected) mg protein $\times 10^2$
8217	Malignant breast	3.0	4.7	1.3	1.8
5286	Malignant breast	3.1	1.7	0.5	2.6
8994	Malignant breast	3.2	3.5	1.1	2.1
S73-1335	Malignant breast	2.6	1.2	0.4	2.2
4150	Malignant breast	1.7	3.1	0.9	0.8
S73-2543	Normal breast	1.6	3.9	1.1	0.5
3120	Normal colon	2.5	2.9	0.9	1.6
3120	Malignant colon	1.8	2.0	0.6	1.2
M73-416	Normal colon	3.3	2.1	0.6	2.7
M73-416	Malignant colon	3.6	1.7	0.5	3.1
6161	Normal colon	4.2	2.8	0.8	2.6
6161	Malignant colon	3.1	0.9	0.3	2.8
S73-06098	Normal anal	2.2	2.0	0.6	1.6
S73-06098	Malignant anal	1.5	2.2	0.7	0.8
M7342	Malignant ileum	1.8	2.8	0.8	1.0
113097	Malignant stomach	3.0	0.6	0.2	2.8
73-6430	Malignant lung	4.4	4.6	1.4	3.0

The α_1-antitrypsin concentration in the 90,000*g* supernatant fractions of normal and/or malignant human breast, colon, anal, ileum, lung and stomach was determined using the quantitative radial immunodiffusion technique (29). The hemoglobin content of the tissue extracts was determined by a colormetric hemoglobin determination method (30). The amount of blood in the extracts was calculated using a value of 0.15 g hemoglobin/ml serum (31). From this value the amount of α_1-antitrypsin present due to blood was calculated using 4.4 mg α_1-antitrypsin per ml of serum (29, 32, 13, 33) which is double the mean value for normal serum in order to account for the increased levels in patients with neoplastic diseases.

tected using radial immunodiffusion in the 90,000*g* supernatant fraction of malignant and adjacent normal human breast, colon, and anal tissue, as well as malignant lung, stomach, and ileum. Immobilized chymotrypsin, bound to Sepharose or Affi-Gel 10 has been utilized to separate 11 peaks of antiproteolytic activity by affinity chromatography of normal and malignant human breast tissue extracts. Glycoproteins are associated with eight of the peaks. Peak 1 contains predominantly α_1-antitrypsin in addition to a minor component. The purification of the inhibitors, as judged by disc gel electrophoresis, is extensive. In some peaks, only one or two protein bands are observed, suggesting that affinity chromatography on Sepharose- or Affi-Gel 10-chymotrypsin might be used for the isolation of α_1-antitrypsin and other inhibitors in preparative amounts.

The authors would like to acknowledge the gift of our initial Sepharose–chymotrypsin from Dr. Melvin Keys, Owens Illinois Research Center, Toledo, Ohio. We are grateful to Dr. A. Bennett, Toledo Hospital, Toledo, OH, Drs. L. J. France and D. J. Hansen of Mercy Hospital, Toledo, OH, Dr. A. B. Batista, St. Lukes Hospital, Maumee, OH and Drs. J. R. Janney and B. Tancinco, Wood County Hospital, Bowling Green, OH for supplying the pathological tissue.

1. Blackwood, C. E., Mateyko, G. M., and Mandl, I., Cancer Res. **22**, 993 (1962).
2. Blackwood, C. E., Mandl, I., and Long, M. E., Amer. J. Obstet. Gynecol. **91**, 419 (1965).
3. Koono, M., Ushijima, K., and Hayashi, H., Int. J. Cancer, **13**, 105 (1974).
4. Unkeless, J. C., Tobia, A., Ossowski, L., Quigley, J. P., Rifkin, D. B., and Reich, E., J. Exp. Med. **137**, 85 (1973).
5. Ossowski, L., Quigley, J. P., Kellerman, G. M., and Reich, E., J. Exp. Med. **138**, 1056 (1973).

6. Bosmann, H. B., and Hall, T. C., Proc. Nat. Acad. Sci. U.S.A. **71,** 1833 (1974).
7. Schnebli, H. P., Cold Spring Harbor Conferences on Cell Proliferation **1,** 327 (1974).
8. Chou, I. N., Black, R. H., and Roblin, R., Cold Spring Harbor Conference on Cell Proliferation **1,** 339 (1974).
9. Burger, M. M., Nature (London) **227,** 170 (1970).
10. Marx, J. L., Science **183,** 1279 (1974).
11. Goetz, I. E., Weinstein, C., and Roberts, E., Cancer Res. **32,** 2469 (1972).
12. Ardenne, M. W., and Chaplain, R. A., Experientia **29,** 1271 (1973).
13. Miesch, F., Bieth, J., and Metais, P. Clin. Chim. Acta, **31,** 231 (1971).
14. Worowski, K., and Farbiszewski, R., Pol. Med. J. **11,** 502 (1972).
15. Marasini, B., Agostoni, A., Stabilini, R., and Dioguardi, N., Clin. Chim. Acta **40,** 501 (1972).
16. Nauroy, J., Monteil, R., and Saint-Blancard, J., Ann. Pharm. Fr. **30,** 99 (1972).
17. Kunz, S., Herschlein, J. H., Sterchele, D. F., and Kidess, E., Med. Welt. **24,** 1266 (1973).
18. Wasilauskas, V. P., Jr., and Brecher, A. S., Proc. Soc. Exp. Biol. Med. **130,** 1211 (1969).
19. Twining, S. S., and Brecher, A. S. presented American Chemical Society National Meeting, Atlantic City, New Jersey, Sept. 8–13, Abst. 83 (1974).
20. Kunitz, M., J. Gen. Physiol. **30,** 291 (1974).
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R., J. Biol. Chem. **193,** 265 (1951).
22. Porath, J., Axen, R., and Ernbach, S., Nature (London) **215,** 1491 (1967).
23. Davis, B. J., Ann. New York Acad. Sci. **121,** 404 (1964).
24. Burr, I. M., Balant, L., Stauffacher, W. and Renold, A. E., Anal. Biochem. **35,** 82 (1970).
25. Matthieu, J. M., and Quarles, R. H., Anal. Biochem. **55,** 313 (1973).
26. Kapitany, R. A., and Zebrowski, E. J., Anal. Biochem. **56,** 361 (1973).
27. Weber, K., and Osborn, M., J. Biol. Chem. **244,** 4406, (1969).
28. Advanced Electrophoresis Techniques for Separphore III Cellulose Polyacetate, Gelman Instrument Co., (1966).
29. Kueppers, F., Humanigenetik **5,** 54 (1967).
30. Lynch, M. J., Raphael, S. S., Mellow, L. D., Spare, P. D., and Inwood, M. J. H., Medical Laboratories Technology and Chemical Pathology, p. 671 W. B. Saunders, Philadelphia, PA (1969).
31. Dittmer, D. S., Blood and Other Body Fluids, p. 109 Fed. Amer. Soc. Exp. Biol., Wash. D. C., (1961).
32. Henry, R. J., Cannon, D. C., and Winbelman, J. W., Clinical Chemistry Principles and Technics, p. 487, Harper and Row, New York, N.Y. (1974).
33. Koj, A., Structure Function Plasma Proteins **1,** 72 (1974).
34. Murthy, R. J., and Hercz, A., FEBS Letters, **32,** 243 (1973).
35. Myerowitz, R. L., Handzel, Z. T., and Robbins, J. B., Clin. Chim. Acta **39,** 307 (1972).
36. Pannell, R., Johnson, D., and Travis, J., Biochem. **13,** 5439 (1974).
37. Koh, A., Chudzik, J., Pajdak, W., and Dublin, A., Biochim. Biophys. Acta **268,** 199 (1972).
38. Cohen, A. B., J. Biol. Chem. **248,** 7055 (1973).
39. Benitez-Bribiesca, L., Freyre, R., and Vega, G. D., Life Sci. **13,** 631 (1973).
40. Cohen, A., J. Clin. Invest. **52,** 2793 (1973).
41. Michels, N. A., The Mast Cells, Downey's Handbook of Hematol. **1,** 232 (1938).
42. Schultze, H. E., and Heremans, J. F., Molecular Biology of Human Proteins with Special Reference to Plasma Proteins, Vol. 1, pp. 648, 736, 806, 821, 836, 848, 865, Elsevier Pub. Co., Amsterdam (1966).
43. Lennert, K. A., Kallmar, M., and Hupe, H., Monatsschr. Kinderheilk **121,** 151 (1973).
44. Ryan, C. A., Ann. Rev. Plant Physiol. **24,** 173 (1973).
45. Green, T., and Ryan, C. A., P.ant Physiol. **51,** 19 (1973).

Received April 8, 1975. P.S.E.B.M., 1975, Vol. 150.