in the same range. This is in accordance with the fact that, in both cases, one deals with the same bond located in different substrates. If the representation of Schechter & Berger (1976) is taken for the amino acid residues surrounding the cleaved bond, that is, NH_2-...-P_4-P_3-P_2-P_1-P'_1-P'_2-P'_3-...-COOH, it is seen that only residues located between P_3 and P'_3 play a major role in the enzymic reaction. This is in accordance with the results obtained by Antonov (1977) on pepsin, an enzyme that is quite similar to chymosin. This author showed that site P_1 is the most important and that sites P_2, P_3, P'_1, and P'_2 have an appreciable influence on the catalysis. Similar conclusions were obtained by Visser (1981) for chymosin.

Acknowledgments

We thank Dr. J. C. Collin, I.N.R.A., Poligny, for the determination of chymosin activity and for checking the absence of pepsin in the chymosin preparation.

Supplementary Material Available

Additional information regarding the principle of calculation used for the determination of k_1–k_4, the apparent rate constants of the studied enzymic reactions (2 pages). Ordering information is given on any current masthead page.

Registry No. Chymosin, 9001-98-3.

References

Antonov, V. K. (1977) in *Acid Proteases: Structure, Function and Biology* (Tang, J., Ed.) pp 179–198, Plenum Press, New York and London.

Berry, G. P., & Creamer, L. K. (1975) *Biochemistry 14*, 3542–3545.

Collin, J. C., Musset de Retta, G., & Martin, P. (1982) *J. Dairy Res. 49*, 221–230.

Creamer, L. K. (1976) *N.Z. J. Dairy Sci. Technol. 11*, 30–39.

Creamer, L. K., Mills, O. E., & Richards, E. L. (1971) *J. Dairy Res. 38*, 269–280.

Delfour, A., Jollès, J., Alais, C., & Jollès, P. (1965) *Biochem. Biophys. Res. Commun. 19*, 452–455.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.

Foltmann, B., Bardholt-pedersen, V., Kaufman, D., & Wybrandt, G. (1979) *J. Biol. Chem. 254*, 8447–8456.

Gripon, J. C., Desmazeaud, M. J., Le Bars, D., & Bergère, J. L. (1975) *Lait 55*, 502–516.

Martin, P., Collin, J. C., Garnot, P., Ribadeau-Dumas, B., & Mocquot, G. (1981) *J. Dairy Res. 48*, 447–456.

Mercier, J. C., Maubois, J. L., Poznanski, S., & Ribadeau-Dumas, B. (1968) *Bull. Soc. Chim. Biol. 50*, 521–530.

Pelissier, J. P., Mercier, J. C., & Ribadeau-Dumas, B. (1974) *Ann. Biol. Anim., Biochim., Biophys. 14*, 343–362.

Ribadeau-Dumas, B., Brignon, G., Grosclaude, F., & Mercier, J. C. (1972) *Eur. J. Biochem. 25*, 505–514.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Schmidt, D. G. (1982) *Dev. Dairy Chem. 1*, 61–85.

Swaisgood, H. E. (1982) *Dev. Dairy Chem. 1*, 1–59.

Thompson, M. P., Kalan, E. B., & Greenberg, R. (1967) *J. Dairy Sci. 50*, 767–769.

Uriel, J. (1966) *Bull. Soc. Chim. Biol. 48*, 969–982.

Visser, S. (1981) *Neth. Milk Dairy J. 35*, 65–88.

Visser, S., & Slangen, K. J. (1977) *Neth. Milk Dairy J. 31*, 16–30.

Activation of Transglutaminase during Embryonic Development†

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ABSTRACT: Incorporation of [^{3}H]putrescine into proteins was shown to increase markedly in sea urchin eggs upon fertilization. Emetine, an inhibitor of protein synthesis, had no effect on the rate of protein labeling. However, the reaction could be prevented by the addition of 2-[3-(diallylamino)-propionyl]benzothiophene, a noncompetitive inhibitor of transglutaminase, and also by dansylcadaverine, which is a substrate for transglutaminase. The inert N^{α}-dimethyl analogue of dansylcadaverine had no influence. Considering the complexity of the incorporation of the [^{3}H]putrescine tracer in this system, it was deemed essential to prove by rigorous analytical methods that the reaction was, indeed, consistent with a transglutaminase mechanism. γ-Glutamyl[^{3}H]putrescine could be recovered in 80–90% yield from the proteolytic digest of proteins from the 20-min fertilized cell. Another sign of the in vivo activity of transglutaminase was the isolation of substantial amounts of ϵ-(γ-glutamyl)lysine from proteins of sea urchin embryo, yielding a frequency value for this cross-link as high as 1 mol/400 000 g of protein in the 32-cell-stage material.

The elevation of cytoplasmic concentration of Ca^{2+} ions seems to play a central role in embryogenesis. In sea urchin, the rise in Ca^{2+} concentration occurs whithin a few seconds after fertilization. The wave of free Ca^{2+} is thought to be essential for triggering further steps in development including the cortical reaction, phosphorylation of nicotinamide adenine dinucleotide, production of hydrogen peroxide, activation of lipoxygenase, polymerization of actin, and, perhaps most significantly, promotion of a Na^+–H^+ exchange. Uptake of Na^+ and expulsion of H^+ ions, with the concomitant increase of intracellular pH, seem to be responsible for the increase in DNA and protein synthesis [for a review, see Epel (1980); also see Hamaguchi & Hiramoto (1981) and Schmidt et al. (1982)].

In the present paper, evidence is provided for the activation of transglutaminase, yet another Ca^{2+}-dependent enzyme [for

† From the Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, Illinois 60201. *Received May 14, 1984.* This work was aided by a U.S. PHS Research Career Award (5 KO6 HL-03512) and by a grant from the National Institutes of Health (HL-02212).

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0006-2960/84/0423-6843$01.50/0

a recent review, see Lorand & Conrad (1984)], soon after fertilization in *Arbacia*. Activity of the enzyme was recognized by the covalent incorporation of a labeled polyamine, 1,4-diaminobutane, i.e., [^{3}H]putrescine,[1] into γ-glutamine side chains of proteins of the fertilized egg. Another sign of the in vivo functioning of transglutaminase was the finding of substantial amounts of ϵ-(γ-glutamyl)lysine peptides in embryonic proteins.

Materials and Methods

Arbacia punctulata was collected in the waters near Woods Hole, MA, during the months of July and August, and *Arbacia lixula* in the bay of Naples between January and April. For a typical experiment, eggs were removed from the body cavity of females and were washed 3 times in filtered seawater (800 mL for eggs from three females), each time passing them through four layers of cheesecloth. Before the eggs were allowed to settle in the final washing fluid, an aliquot was placed on a 25-mm HA 0.45-μm Millipore filter for cell counting. For collecting sperm, a male specimen was opened, and the sperms were left in the body cavity until needed, when a drop of the concentrated seminal fluid was diluted about 100-fold with seawater. After a sample of eggs was mixed with sperm, synchrony of fertilization was checked by observing time of elevation of fertilization membrane as well as the first cell division.

[1,4(N)-^{3}H]Putrescine dihydrochloride, with specific activities of 19 and 31 Ci/mmol, was purchased from Amersham Corp. in aqueous solution. Dansylcadaverine (Lorand et al., 1968) and N$^{\alpha}$-dimethylated dansylcadaverine (Lunden et al., 1972) were prepared as the hemifumarate salts, and stock solutions of 2 mM were made up in filtered seawater, buffered at pH 8.3 with 10 mM Tris. 2-[3-(Diallylamino)propionyl]-benzothiophene or 2-(diallylamino)ethyl 2-benzothienyl ketone was obtained as the citrate salt from M. Maamer, and a 1 mM aqueous stock solution was prepared. Methylglyoxal bis(guanylhydrazone) dihydrochloride hydrate was a product of Aldrich Chemical Co. and emetine dihydrochloride tetrahydrate of British Drug Houses. All other chemicals were of the highest purity available, and unless otherwise noted, all compounds used for the treatment of sea urchin eggs were dissolved directly in seawater.

Incorporation of [^{3}H]putrescine was terminated by rapidly sedimenting the treated eggs or embryos from 15 mL of suspensions (with 40 turns of a hand-driven centrifuge; Fisher Scientific Co.), decanting the supernatant, and mixing the cells with a 4-fold volume of a solubilizing medium containing 2% sodium dodecyl sulfate ($NaDodSO_4$; Bio-Rad), 1% 2-mercaptoethanol (Aldrich), 10 mM putrescine (Sigma), 10 mM ethylenediaminetetraacetate (EDTA; Sigma), and 0.025 M tris(hydroxymethyl)aminomethane (Tris)–0.2 M glycine buffer of pH 8.3 (Sigma). The cells were allowed to lyse for about 1 h at room temperature (ca. 18 °C), and the debris was removed by centrifuging at 12000 rpm in a Sorvall SS34 rotor for 60 min. A portion of the supernatant was precipitated with 1 N perchloric acid (British Drug Houses); the precipitate was dissolved in 1 mL of 0.1 N NaOH, and a sample was taken for protein determination (Lowry et al., 1951). Other aliquots were used for measuring protein-bound isotope. For this purpose, 1 mL of the supernatant was diluted with 5 mL of cold water, and 3 mL of 21% cold trichloroacetic acid (Cl_3CCOOH; Sigma) was added dropwise followed by another 3 mL of 7% cold Cl_3CCOOH. The mixture was placed in ice and was allowed to equilibrate overnight. The protein precipitate was removed by centrifugation (International clinical centrifuge, maximum speed) and was washed with 10 changes (12 mL each time) of 7% cold Cl_3CCOOH. To ensure removal of all free radioactivity, the final acid treatment was extended overnight. The white protein pellet was then washed with two changes of 12 mL of 95% ethanol and was taken up in 2 mL of 0.1 N NaOH by boiling for 3 min. Samples of 25–100 μL, containing up to 1 mg of protein, were used for isotope counting either with 10 mL of Aquasol (New England Nuclear) or Insta-Gel (Packard Instrument Co.), in a Beckman scintillation counter (Model 330 or LS7500). Preparations of in vivo labeled proteins on a larger scale were obtained by drying the ethanol-washed Cl_3CCOOH precipitates.

Electrophoretic analysis was carried out in a Bio-Rad Protean 16 CM apparatus using polyacrylamide gradient gels (5–12%; Laemmli, 1970). A current of 25 mA was applied for about 90 min to allow samples [50–500 μg of protein taken up in 0.125 M Tris-HCl of pH 6.8, 4% $NaDodSO_4$, 20% glycerol (v/v), and 10% 2-mercaptoethanol (v/v)] to pass through the stacking gel, and it was augmented to 40 mA for 90–120 min to achieve separation. Staining with Coomassie Brilliant Blue R (Sigma) was performed according to Weber & Osborn (1969); destaining was promoted by adding a few grains of Dowex-1 resin in the OH form. For fluorography, polyacrylamide gels were placed in EN^{3}Hance (New England Nuclear) for 1 h and, following impregnation, were kept in water for 1 h under gentle agitation and then dried in a Bio-Rad gel dryer. Kodak X-Omat AR film was used (exposure 31 days at −70 °C) for autoradiography. Molecular weight standards were obtained from Bio-Rad and were comprised of myosin (M_r 200 000), β-galactosidase (M_r 116 000), phoshorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200), and ovalbumin (M_r 45 000).

Following protein staining, some gels were sliced into 2-mm segments (using a Bio-Rad Model 190 gel slicer), which were treated with 30% H_2O_2 (0.4 mL) at 40 °C overnight (Young <<map Fulhorst, 1965) and mixed with 10 mL of Ready-Solv MP (Beckman) prior to counting of radioactivity.

Acid hydrolysis of lyophilized, [^{3}H]putrescine-labeled proteins (2–4 mg) was carried out with 1 mL of constant boiling HCl (Pierce) at 108 °C in evacuated and sealed vials for 1, 3, ,6, 8, 16, 24, 32, 48, and 72 h. After the acid was evaporated (SpeedVac, Savant), samples were taken up in H_2O and dried again. The evaporation procedure was repeated 4 times before preparing solutions of approximately 10 mg of protein equivalent/mL of H_2O.

Prior to *digestion with proteolytic enzymes*, the lyophilized proteins (5 mg) were taken up in 0.1 N NaOH (0.5 mL), dialyzed for 24 h at 4 °C against 4 × 500 mL of 0.1 M NH_4HCO_3 at pH 8, and made up to 1 mL with the bicarbonate solution. Subtilisin (protease type VIII; Sigma) was dissolved in the NH_4HCO_3 to a concentration of 2 mg/mL, and 50 μL of this stock solution was added. After 12 h at 34 °C, a second addition of 50 μL of subtilisin (stored at −20 °C) was made and incubation continued for another 12 h. Then 50 μL of a 2 mg/mL solution of Pronase-CB (Calbiochem-Behring) in NH_4HCO_3 was added for 12 more h. The samples were heated for 10 min in a boiling water bath and allowed

[1] Abbreviations: dansylcadaverine, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; putrescine, 1,4-diaminobutane; DAPBT, 2-[3-(diallylamino)propionyl]benzothiophene or 2-(diallylamino)ethyl 2-benzothienyl ketone; MGBG, methylglyoxal bis(guanylhydrazone); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Cl_3CCOOH, trichloroacetic acid; HPLC, high-pressure liquid chromatography; OPA, *o*-phthalaldehyde; THF, tetrahydrofuran.

to cool to room temperature. An additional 50 μL of the Pronase solution (stored at −20 °C) was added for a further 12 h followed by the heat treatment. After the solution was cooled, the pH was lowered to pH 6.7 (with HCl). A stock solution of carboxypeptidase Y (Sigma; stored at −20 °C as 10 mg of protein/mL of H_2O) was diluted to 2 mg/mL, and 50 μL was added to the samples at 31 °C for 12 h. At the end of this treatment, the pH was raised to pH 8 (with NH_4OH), and 5 μL of 1.2 M $MgCl_2$ was added. Preactivations of leucine aminopeptidase (type III-CP; Sigma) and prolidase (Sigma) were carried out during the last 3 h of the carboxypeptidase Y incubation in the following manner: leucine aminopeptidase was incubated at a concentration of 1.1 mg/mL in 4 mM Tris-HCl, pH 8.0, with 2.5 mM $MnCl_2$ at 37 °C; prolidase was incubated at 1.0 mg/mL in 4 mM Tris-HCl, pH 8.0, with 5 mM $MnCl_2$, also at 37 °C. Following preactivation, 34 μL of the leucine aminopeptidase and 38 μL of the prolidase were added to the samples and incubated for 10 h at 33 °C. A final addition of 34 μL of a freshly preactivated (for 3 h at 37 °C) leucine aminopeptidase solution was made for another 10-h period, also at 33 °C. At the end of digestion, the samples were dried and dissolved in 0.5 mL of H_2O for analysis.

Two independent chromatographic techniques were employed, patterned on the work of Griffin et al. (1982), for purposes of identifying putrescine in the acid hydrolysates and also γ-glutamylputrescine and ε-(γ-glutamyl)lysine in the enzymatic digests. One was based on a reverse-phase HPLC procedure following precolumn derivatization with *o*-phthaladehyde (Fluoropa, Pierce), and the other was an ion-exchange separation method using ninhydrin as reagent.

HPLC. The Water Associates HPLC apparatus consisted of three M6000A pumps, a 720 System Controller, a U6K injector, a WISP 710B Sample Processor and a 420AC fluorescence detector fitted with a 334-nm excitation filter and a 425-nm emission filter. A 730 Data Module was used for plotting and integrating chromatograms. A Zorbax C-8 column (Du Pont) was employed for all separations.

Precolumn derivatizations of the acid-hydrolyzed samples for [^{3}H]putrescine measurement and of the enzymatically digested samples for γ-glutamyl[^{3}H]putrescine measurement were performed manually in the following manner: 75 μL (about 900 μg) of acid hydrolyzed, or 50 μL (about 500 μg) of enzymatically digested materials, was treated with 150 μL of methanol and 50 μL triethylamine and then dried under a stream of N_2 in order to lower NH_3 content (Tarr, 1982). When dry, the residues were dissolved in 588 μL of freshly prepared OPA reagent. For [^{3}H]putrescine, the OPA reagent was prepared by mixing 400 μL of 0.4 M potassium borate, pH 10.4, 400 μL of OPA (100 mg/mL in methanol), and 140 μL of 2-mercaptoethanol. For γ-glutamyl[^{3}H]putrescine, the OPA reagent was prepared by mixing 400 μL of 0.4 M potassium borate, pH 10.4, 200 μL of OPA (100 mg/mL in methanol), and 70 μL of 2-mercaptoethanol. After vigorous mixing of OPA reagent with the samples for 30 s (Vortex, Scientific Products), they were allowed to stand at room temperature for 1.5 min, and volumes of 500 μL were injected onto the column. Fractions of 1 mL were collected at 30-s intervals, and 0.2-mL aliquots were added to 10 mL of Ready-Solv MP (Beckman) for measuring radioactivity. Just prior to chromatography, the isotope contents of the applied samples were also analyzed on small aliquots (25–50 μL) taken from the OPA-derivatized materials by using the same procedure. Authentic putrescine dihydrochloride (Sigma), spermidine trihydrochloride (Sigma), [1,4-^{14}C]putrescine dihydrochloride (122 mCi/mmol; Amersham Corp.), and γ-glutamylputrescine (Vega) were added, as needed for reference, prior to treatment with triethylamine.

Precolumn derivatization of enzymatic digests for ε-(γ-glutamyl)lysine determination was performed by a modification of the Waters AUTO-TAG OPA precolumn derivatization technique (Waters Technical Bulletin), utilizing the WISP attachment. With no flow, the following injections were made: 25 μL of 0.4 M potassium borate, pH 10.4 (T. E. Wheat, personal communication), 25 μL of OPA reagent (prepared by dissolving 20 mg of OPA in 1.0 mL of methanol and then adding 1.0 mL of 0.4 M potassium borate, pH 10.4, and 20 μL of 2-mercaptoethanol) and, lastly, 10 μL of sample (diluted with water to contain 5 μg of protein). Flow was started immediately at a rate of 0.1 mL/min and maintained for 1 min. The starting solvent composition is described below. A 2.5-ft length (0.03 in i.d.) of tubing was used between the WISP and guard column to allow mixing of sample and OPA reagent before reaching the column. After the 1-min mixing period, the flow was increased linearly over 1 min to a rate of 1.8 mL/min.

Gradients were formed with three M6000A pumps by using the following solutions: solution A, 40 mM potassium acetate, pH 5.5, and 1% (v/v) tetrahydrofuran (THF); solution B, methanol (99% v/v) and THF (1%); solution C, water and THF (1%). As the methanol concentration increased in the gradient, the concentrations of solutions A and C decreased at equal rates. The starting solvent composition for all gradients, and for the OPA derivatization procedure, was formed in the following manner: solution A was pumped at 40%, solution B at 20%, and solution C at 40% of the total flow rate. All changes in gradient conditions are given in terms of the percentage of solution B (i.e., methanol).

The elution of [^{3}H]putrescine was performed by using a linear gradient from 20% solution B to 95% over 20 min at 2.0 mL/min (Griffin et al., 1982): procedure A. For separating γ-glutamyl[^{3}H]putrescine, the linear gradient was altered to include an isocratic hold of 2.25-min duration with 38% solution B commencing at 4.8 min and a second isocratic hold of 2.75-min duration with 54% solution B, commencing at 11.3 min: procedure B.

When ε-(γ-glutamyl)lysine was measured, after the flow rate reached 1.8 mL/min at 2 min, the linear rate of pumping of solution B was increased to raise methanol from 20% to 36% by 6.6 min. By 15 min, methanol was increased to 40%, and by 18 min it reached 53%. Between 18 and 20 min, the flow rate was augmented to 2.0 mL/min with solution B remaining at 53%. From 20 to 23.5 min, solution B was raised to 54%, and at 23.5 min the flow rate was lowered to 1.8 mL/min. Thereafter, solution B was raised to 95% by 36 min: procedure C.

At completion of the HPLC runs, the concentration of solution B was increased to 100% within 30 s, and the column was washed for 2 min. This was followed by a reverse gradient back to 20% of solution B over 6 min and a 10-min reequilibration period before the next injection.

Ion-exchange chromatography of the enzymatically digested samples was performed on the Glenco Model MM amino acid analyzer with lithium citrate buffers (Pierce Pico-Buffer System IV), Dionex DC-4A resin, and ninhydrin as detecting reagent as described previously (Griffin et al., 1982). Elution (0.2 mL/min) was carried out according to the following program: buffer A (pH 2.90) was pumped for 30 min (40 °C), buffer B (pH 3.04) for 22 min (40 °C), buffer C (pH 2.95) for 43 min (40 °C), buffer D (pH 3.34) for 25 min (40 °C),

buffer D (pH 3.34) for 5 min during which column temperature was raised to 65 °C, and buffer E (pH 4.24) for 60 min (65 °C). Ninhydrin flow rate was 0.1 mL/min, with the reaction chamber at 115 °C.

This program with minor changes was used to separate [^{3}H]putrescine in acid hydrolyzates; the pH of buffer D was raised to pH 5.24 with solid lithium hydroxide. Buffer E was diluted 1:3 (instead of 1:4) with H_2O, and its pH was raised to pH 5.50 with solid lithium hydroxide.

At the completion of runs, the column was washed for 30 min with 0.3 M lithium hydroxide containing 5 mM EDTA at a column temperature of 65 °C. The column was then reequilibrated for at least 60 min in buffer A, with the column temperature returning to 40 °C.

For measurement of [^{3}H]putrescine and γ-glutamyl[^{3}H]-putrescine, fractions were collected every 3 min (0.9 mL) after reaction with ninhydrin. Aliquots of 0.2 mL were taken for measurement of radioactivity. To calculate percentage recovery, 10-μL aliquots of the samples before injection were also measured for radioactivity.

Results

Amine Incorporation following Fertilization. Washed *Arbacia* eggs were suspended to a concentration of 1.5×10^5 eggs/mL of seawater (75–100 mL) with 0.7 μM [^{3}H]putrescine (to yield about 13 μCi/mL) and were incubated for 30 min at room temperature in 500-mL beakers so that the depth of the medium was less than 1 cm. For analyzing unfertilized eggs, 15-mL aliquots were withdrawn, and the cells were sedimented by centrifugation and then solubilized by addition of a mixture of $NaDodSO_4$, 2-mercaptoethanol, putrescine, and EDTA as described under Materials and Methods. The cold putrescine and EDTA were included in the solution to minimize the possibility of the continued Ca^{2+}-dependent incorporation of [^{3}H]putrescine into proteins during and following cell lysis.

The remainder (60–85 mL) of the [^{3}H]putrescine-loaded eggs were fertilized by adding 10 drops of diluted sperm and were allowed to develop up to 20 min. Aliquots (15 mL) were withdrawn at various times, and the cells were sedimented by centrifugation and lysed in the solubilizing medium exactly as with unfertilized eggs. After cell debris which was pelleted by centrifugation, was discarded, the Cl_3CCOOH-precipitable, protein-bound radioactivity was measured according to the procedure given under Materials and Methods.

Time courses for the incorporation of [^{3}H]putrescine are presented in Figure 1 for the standard condition described above, as well as for experiments in which either *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide or dansylcadaverine (1 mM), N^{α}-dimethylated dansylcadaverine (1 mM), methylglyoxal bis(guanylhydrazone) or MGBG (0.1 mM), 2-[3-(diallylamino)propionyl]benzothiophene or DAPBT (0.05 mM), or emetine (0.1 mM) were also present during the entire period of incubation. Incorporation into unfertilized eggs amounted to about 4×10^{-6} mol of [^{3}H]putrescine equivalent/10^5 g of protein, except when dansylcadaverine or DAPBT was present. With these inhibitors, incorporation dropped to approximately half this value, and after fertilization, little or no time-dependent increase was observed. By contrast, in all the other experiments there was a significant and progressive rise in protein labeling following fertilization.

We considered the possibility that the observed enhancement of incorporation of [^{3}H]putrescine might be due to the greater permeability of the fertilized cell to the polyamine and, therefore, carried out experiments by fertilizing eggs which

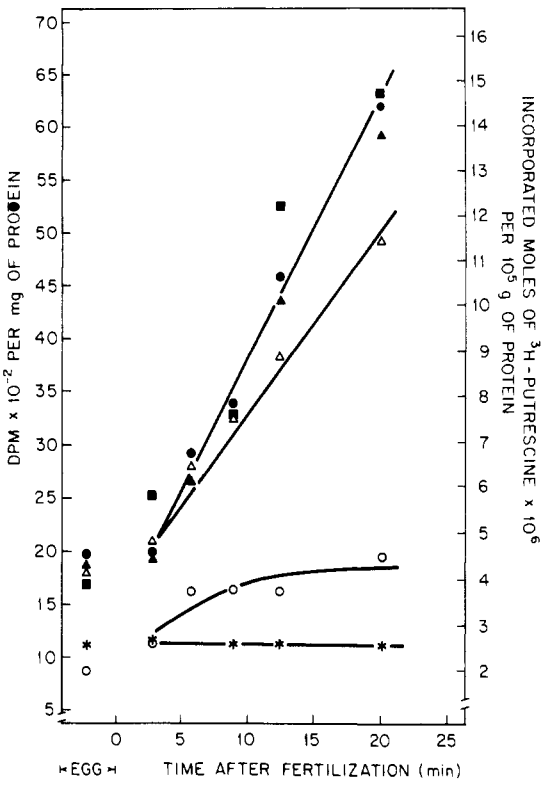


FIGURE 1: Time course for the in vivo incorporation of [^{3}H]putrescine (0.7 μM; 19 Ci/mmol) into proteins of *A. punctulata* eggs (1.5×10^5 per mL of seawater) following fertilization. Abscissa: time after fertilization. Data for the corresponding unfertilized eggs are given in the space between the arrows. Ordinates denote dpm per milligram of protein (left) and moles of [^{3}H]putrescine equivalents incorporated per 100 000 g of protein (right). Incorporation under standard conditions, as described under Materials and Methods, (●) and with either 1 mM dansylcadaverine (○) or 0.05 mM DAPBT (*), 0.1 mM MGBG (■), 0.1 mM emetine (▲), or 1 mM N^{α}-dimethylated dansylcadaverine (△) also present during incubation with [^{3}H]putrescine.

were washed twice in 75 mL of seawater following the preliminary 30-min incubation with [^{3}H]putrescine. However, reducing the concentration of the labeled amine just prior to fertilization did not change the time course of the labeling of embryonic proteins. In subsequent experiments no effort was made to remove [^{3}H]putrescine before fertilization.

All the analytical work with [^{3}H]putrescine-labeled proteins pertains to 20-min time points after fertilization, and the extent of labeling for three different preparations varied only from 17.4×10^{-6} to 19.6×10^{-6} mol of [^{3}H]putrescine equivalents/10^5 g of protein.

By gel electrophoresis of the Cl_3CCOOH-precipitated protein material, appreciable amounts of radioactivity could be found only in the high molecular weight ($>10^6$) region either by counting of gel slices or by autoradiography.

Recovery of [^{3}H]Putrescine from the Labeled Embryonic Proteins. In order to prove that the [^{3}H]putrescine was not oxidized to the mono- or dialdehyde or to γ-aminobutyric acid and was not metabolically converted to some other polyamines (e.g. spermidine, hypusine) prior to or following reaction with the proteins in the cell, it was deemed important to be able to recover the labeled amine from the protein conjugate. First, it was shown that hydrolysis with 5.7 N HCl for 8 h at 108 °C was required to release all the bound radioactivity from the labeled proteins (Figure 2). Then, examination of the 72-h acid hydrolysate by ion-exchange chromatography was found to yield only a single radioactive peak emerging at 224–225 min, with a full recovery (91–105%) of the ^{3}H isotope applied. Cochromatography with added [^{14}C]putrescine (Figure 3) or

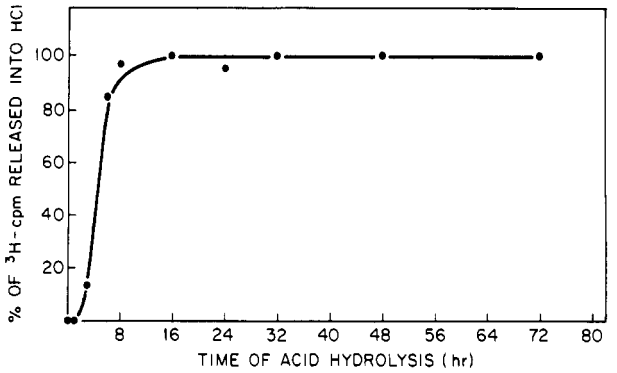


FIGURE 2: Release of [^{3}H]putrescine-derived radioactivity by acid hydrolysis from proteins labeled in fertilized *A. lixula* eggs. Abcissa denotes time of hydrolysis in 5.7 N HCl at 108 °C. Ordinate shows the percentage of radioactivity recovered from the acid phase after different times of hydrolysis of protein samples. The labeled *A. lixula* material contained 18.2 μmol of [^{3}H]putrescine equivalents/100 000 g of protein. Samples of 2–4 mg of protein (15 300–30 500 cpm) were hydrolyzed.

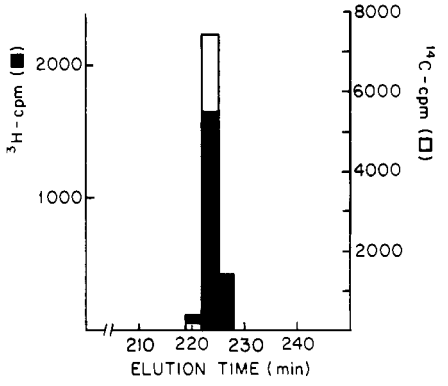


FIGURE 3: Radioactivity recovered from the acid hydrolysate of labeled *A. lixula* proteins by ion-exchange chromatography using Glenco analyzer. The injected sample was the 72-h acid hydrolysate of 0.25 mg of protein (1900 cpm of ^{3}H) described in Figure 2, with 32.5 pmol of the reference [^{14}C]putrescine (8740 cpm of ^{14}C) added as a marker. Solid bars denote radioactive counts for ^{3}H and open bars for ^{14}C in the fractions (0.9 mL) collected. Recovery was 105% for ^{3}H and 86% for ^{14}C.

with cold putrescine showed this elution time to be characteristic for the diamine.

Analysis of the same 72-h acid hydrolysate of labeled proteins in the high-pressure liquid chromatographic (HPLC) system (procedure A) yielded the profile shown in Figure 4 where the peak at 19.55–20.25 min contained 89% of the ^{3}H isotope applied. The slight ^{3}H-containing shoulder preceding the main peak, amounting to 5% of the applied radioactivity, was also seen with the [^{14}C]putrescine control where, of the applied ^{14}C, 11% was recovered in the shoulder region and 81% in the main peak.

Examination of the γ-Glutamyl[^{3}H]putrescine Content of Labeled Proteins. After the demonstration that [^{3}H]putrescine could be reclaimed from the labeled proteins by acid hydrolysis in good yield, the same protein samples were digested by a series of proteolytic enzymes so as to be able to analyze for γ-glutamyl[^{3}H]putrescine. Again, two independent chromatographic procedures were employed. Authentic γ-glutamylputrescine was shown to elute from the ion-exchange column at 180.75–182.50 min, between the amino acids lysine and histidine. When this position was marked, by mixing 15 nmol of the authentic substance with 0.3 mg of the total enzyme digest of labeled proteins, a single ^{3}H peak (representing 91% recovery of the applied radioactivity) was seen to emerge at the same place (Figure 5). Similar experiments

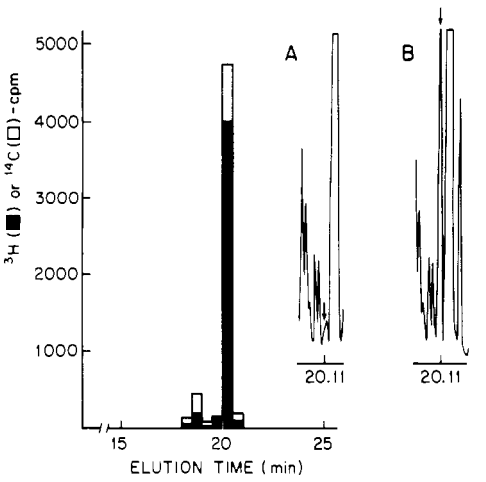


FIGURE 4: HPLC analysis of the acid-hydrolyzed, labeled, *A. lixula* proteins. The sample, corresponding to 0.67 mg of protein and 5100 cpm, was the same 72-h hydrolysate used in the experiment for Figure 3, mixed with 23 pmol of reference [^{14}C]putrescine (6250 cpm). Solid bars show the ^{3}H and open bars the ^{14}C counts in 1-mL fractions. The inserts show the corresponding fluorescent analytical profile for the hydrolysate above (A) and for the same with 2.1 nmol of cold putrescine added as marker (B). The position of putrescine is shown by the arrow.

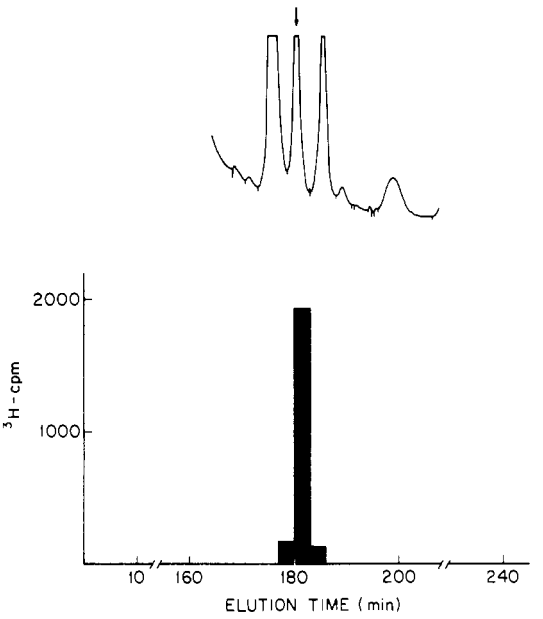


FIGURE 5: Ion-exchange chromatography of the enzymatic digest of [^{3}H]putrescine-derivatized proteins from fertilized *A. lixula* eggs. The labeled material contained 19.6 μmol of [^{3}H]putrescine/100 000 g of protein, and 0.3 mg (2460 cpm) was applied to the column together with 15 nmol of authentic γ-glutamylputrescine. The bar diagram shows the recovery of ^{3}H in the 0.9-mL fractions. The elution pattern on top shows the ninhydrin color profile from the Glenco analyzer with the position of γ-glutamylputrescine marked by the arrow.

were carried out on the HPLC system (procedure B), and here too, it could be shown that authentic γ-glutamylputrescine coeluted at 20.25–20.75 min with most (78%) of the applied ^{3}H radioactivity. A small fraction, estimated as 16% of the applied isotope, emerged as a broad band around 15 min, representing some unknown derivative (Figure 6).

Demonstration of ε-(γ-Glutamyl)lysine Cross-Links in the Normal Embryo. Inasmuch as experiments regarding the incorporation of [^{3}H]putrescine into the γ-position of protein glutamines in the fertilized egg suggested the involvement of transglutaminase, a direct examination of embryonic proteins for ε-(γ-glutamyl)lysine was initiated. *A. lixula* eggs were fertilized and were allowed to multiply to the 32-cell stage

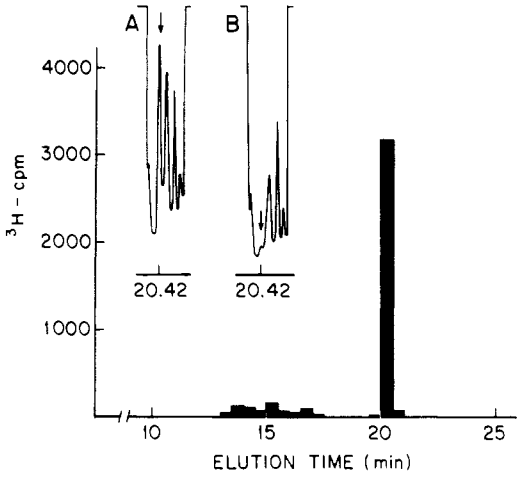


FIGURE 6: HPLC analysis of the enzymatic digest of [^{3}H]putrescine-labeled proteins from fertilized *A. lixula* eggs. The digested material contained 18 μmol of [^{3}H]putrescine/100 000 g of protein, and 0.55 mg of sample (4200 cpm) was injected together with 1.3 nmol of authentic γ-glutamylputrescine added as marker. The bar diagram shows the ^{3}H radioactive counts in 1-mL fractions collected, and insert A is the fluorescent analytical elution profile. A profile for the material with no addition of γ-glutamylputrescine is presented in insert B. The position of γ-glutamylputrescine is marked by the arrow.

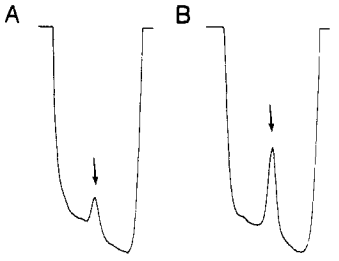


FIGURE 7: Ion-exchange chromatography of the proteolytic digest of 32-cell-stage *A. lixula* proteins. A sample of 0.52 mg of protein was applied for the experiment in panel A; panel B shows the same but with 2 nmol of authentic ε-(γ-glutamyl)lysine added as the marker; the arrow indicates the 136-min elution time for this compound.

when they were harvested by centrifugation (12 000 rpm, Sorvall SS 34 rotor, 20 min) and lyophilized. The freeze-dried cells (500 mg) were solubilized by suspending them in 20 mL of Tris (0.025 M)–glycine (0.2 M) buffer of pH 8.3 containing 2% $NaDodSO_4$–1% 2-mercaptoethanol–10 mM EDTA, for a period of 1 h at room temperature. Insoluble material was removed by centrifugation (Sorvall, 1 h). Supernatant proteins were precipitated by Cl_3CCOOH, as described under Materials and Methods, washed with ethanol, and dried. Digestion with enzymes was carried out as described, and the digests were analyzed for ε-(γ-glutamyl)lysine by ion-exchange chrogomatography as well as by HPLC (procedure C). The results are shown in Figures 7 and 8, respectively, and indicate an average presence of about 1 mol of the dipeptide in 400 000 g of protein material at this stage of development.

Discussion

It was known for some time that homogenates of sea urchin eggs and embryos contained high, Ca^{2+}-dependent transglutaminase activities (Campbell-Wilkes, 1973; Lorand & Stenberg, 1976). By use of fluorescent dansylcadaverine for the staining of enzyme activity (Lorand et al., 1979a), on the basis of electrophoretic mobilities three forms of the enzyme could be identified in *S. purparatus*, one of which was recently purified (Takeuchi et al., 1982). It remained to be shown, however, that transglutaminase could become functional in vivo. The present paper addresses itself to this question, focusing, in particular, on the finding that a significant increase of enzyme activity occurred following fertilization. The incorporation of [^{3}H]putrescine into proteins before and after fertilization of *A. punctulata* and *A. lixula* eggs was used as a biological probe for monitoring the activity of the intracellular enzyme.

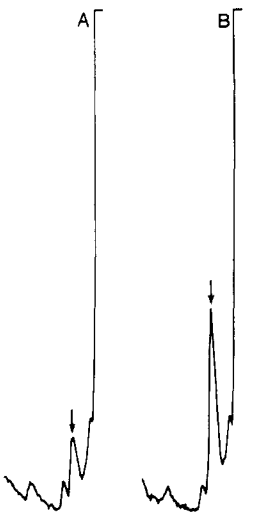


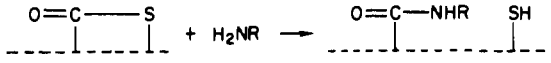
FIGURE 8: HPLC analysis of the digest of the 32-cell-stage *A. lixula* material. Panel A shows the profile obtained with 5 μg of proteins injected. For the experiment in panel B, authentic ε-(γ-glutamyl)lysine (30 pmol) was included, in addition. Retention time (20.0 min) of this compound is indicated by the arrow.

Inasmuch as the labeled polyamine might become metabolically altered prior to or after incorporation into proteins, it was essential to show by rigorous analytical criteria that the mode of attachment of the radioactive probe was consistent with a direct, transglutaminase-mediated mechanism. With experiments of this type, it is important to recall that oxidative reactions, catalyzed by diamine oxidases (Zeller, 1963) or by myeloperoxidase (Thomas et al., 1982), could give rise to spurious incorporation of the putrescine-derived label into proteins through aldehyde or chloramine intermediates. Moreover, conversion of the putrescine marker to spermidine and spermine (see Cohen, 1971; Bachrach, 1973) could not be excluded, and the higher polyamines could have been the substrates for transglutaminase (Clark et al., 1959; Lorand et al., 1979b). Though MGBG, an inhibitor of both *S*-adenosylmethionine decarboxylase (Corti et al., 1974) and diamine oxidase (Porter et al., 1981), had no demonstrable effect on the incorporation of putrescine, it may not have penetrated into the egg. In addition, putrescine can act as a precursor of protein-bound hypusine (Park et al., 1981). For such reasons [see Lorand & Conrad (1984)], one of the critical issues was to examine whether [^{3}H]putrescine could be recovered in good yield upon acid hydrolysis of the biologically labeled proteins. Working with the 20-min fertilized samples of *A. lixula* eggs, it was shown by two independent chromatographic procedures that virtually all of the radioactivity which became attached to proteins could be reisolated in the form of putrescine.

Next, it had to be proven that the [^{3}H]putrescine was attached to γ-glutamines in proteins. Since the γ-glutamylamide of putrescine is at least as acid labile as the α-amides of proteins, we had to resort to the sequential digestion of the peptide backbone by a series of proteolytic enzymes which would spare the side-chain adduct. Direct isolation of the ^{3}H-labeled, γ-glutamylputrescine, $H_2NCH(COOH)CH_2CH_2CONHCH_2CH_2CH_2CH_2NH_2$, from the enzymatic digest

by ion-exchange chromatography gave a yield of 91%, and precolumn derivatization with *o*-phthaladehyde (OPA) followed by reverse-phase HPLC allowed a recovery of 78%. This indicated that the bulk, if not all, of the polyamine probe added to the cell suspension could be accounted for through modification of γ-glutamyl residues.

There are a few proteins (the C1 and C3 components of complement and α_2-macroglobulin) that react with amine nucleophiles even in the absence of transglutaminase. Moreover, incorporation occurs at γ-glutamine sites because of the presumed existence of an internal γ-glutamyl-*S*-cysteinyl thio ester that can be readily opened up by amines:



It should be observed, however, that the reports thus far relate only to the reactions of the cyclic thio ester with ammonia, hydroxylamine, and methylamine, i.e., small amines [see Tack et al. (1981), Barrett (1981), and Mortensen et al. (1981)]. Nevertheless, there is at least a theoretical possibility that putrescine could react likewise. Thus, even though the above examples all relate to plasma proteins found in the extracellular environment, it cannot be excluded a priori that sea urchin eggs could not contain proteins with similarly activated γ-glutamyl residues. The demonstration, however, that 2-[3-(diallylamino)propionyl]benzothiophene, a potent noncompetitive inhibitor of transglutaminase (Lorand & Conrad, 1984), prevented the enhanced incorporation of the [^{3}H]putrescine probe into proteins of fertilized *A. lixula* egg is a strong argument against the nonenzymatic pathway of amine incorporation and suggests a transglutaminase mechanism. The idea of activating a preexisting, latent form of this enzyme is supported by the finding that the addition of emetine, a known inhibitor of protein synthesis during embryogenesis in sea urchin (Hogan & Gross, 1971), had no effect on putrescine incorporation.

Though the influx of Ca^{2+} from the surrounding seawater is not considered an essential element for fertilization (Schmidt et al., 1982), the freeing and redistribution of this Ca^{2+} from intracellular compartments is thought to be a key feature for development [see Epel (1980) and Hamaguchi & Hiramoto (1982)]. Transglutaminase may be one of the enzymes that becomes activated by the rise in free Ca^{2+} concentration. The experimentally measured incorporation of the [^{3}H]putrescine probe into proteins may actually underestimate the true activity of this enzyme for a number of reasons. Putrescine is synthesized in fertilized eggs in significant and increasing amounts (Kusunoki & Yasumasu, 1976; Brachet et al., 1978); thus, the added [^{3}H]putrescine would undergo progressive isotope dilution in the cell. Once the eggs were loaded with [^{3}H]-putrescine, it made virtually no difference if the labeled polyamine was removed from the incubation medium just before fertilization. Hence, from a practical point of view, [^{3}H]putrescine was used in our experiments as a pulse label.

The incorporation of [^{3}H]putrescine into embryonic proteins (P) is a reflection on only one of several possible manifestations of the functioning of transglutaminase: $P{\sim}^{\gamma}CONH_2 + H_2N(CH_2)_4NH_2 \rightarrow P{\sim}CONH(CH_2)_4NH_2 + NH_3$ (reaction 1). As described by Waelsch and collaborators [Clark et al., 1959; see also Lorand & Conrad (1984)], the hydrolytic deamidation of γ-glutamines and the formation of ε-(γ-glutamyl)lysine bridges between proteins (P and P′) would offer two possibilities: $P{\sim}^{\gamma}CONH_2 + H_2O \rightarrow P{\sim}COOH + NH_3$ (reaction 2), $P{\sim}^{\gamma}CONH_2 + H_2N^{\epsilon}{\sim}P' \rightarrow P{\sim}CONH{\sim}P' + NH_3$ (reaction 3). Investigation of reaction 2 in a cellular setting would be a forbidding analytical task at present. Nevertheless, its possible significance, together with the more readily measurable amine incorporation reaction illustrated in reaction 1, can best be appreciated by reference to the drastically altered macromolecular behaviors of hemoglobin variants with single amino acid mutations regarding sickling or tetramer–dimer ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$) dissociations [see Klotz et al. (1975)]. In reaction 1, the neutral glutaminyl residue of the target protein would be replaced by a positively charged γ-glutamylputrescine moiety and, in reaction 2, by a negatively charged glutamyl side chain. It is impossible to predict what effects such replacements might have if the target protein was an enzyme, an activator, or an inhibitor of a cellular reaction. It would be equally difficult to estimate how the leaving group generated in reactions 1–3 might contribute to the accumulation of the ammonia pool which seems to be of importance for the alkaline shift of pH and the ordering of further steps during embryogenesis [see Epel (1980)].

Current analytical methods (Griffin et al., 1982), however, were found to be sensitive enough to prove the presence of ε-(γ-glutamyl)lysine isodipeptide in proteins of *A. lixula* embryos, yielding values for the average frequency of this cross-link as high as 1 mol/400 000 g of protein for the 32-cell-stage material. Thus, covalent polymeric assembly of the extracellular embryonic matrix and/or intracellular proteins seems at least to be one of the functions of transglutaminase in development. Regarding extracellular structures, it should be mentioned that fibronectin, which is known to serve as a substrate for transglutaminase (Mosher, 1976), is present in the sea urchin embryo (Spiegel et al., 1980). Concerning intracellular proteins, transglutaminase-mediated cross-linking seems to be a common feature in the biological models of aging and terminal differentiation, readily induced in as varied cell types as human erythrocytes (Lorand et al., 1976, 1978) and platelets (Bruner-Lorand et al., 1982), tissue culture keratinocytes (Rice & Green, 1977), and rabbit lens (Conrad et al., 1983, 1984).

Stabilizing the intracellular organization of structural components in the fertilized egg might be due to a similar cross-linking reaction. Future efforts must be aimed at delineating the location of polymeric products within the embryo and at determining its constituent protein units by immunological methods similar to those previously employed in connection with the human erythrocyte system (Bjerrum et al., 1981).

Acknowledgments

L.C. thanks the hospitality of Dr. P. Gross at the Marine Biological Laboratory in Woods Hole, MA.

Registry No. Transglutaminase, 80146-85-6; ε-(γ-glutamyl)lysine, 17105-15-6; γ-glutamylputrescine, 58316-51-1.

References

Bachrach, U. (1973) *Function of Naturally Occurring Polyamines*, Academic Press, New York.

Barrett, A. J. (1981) *Methods Enzymol. 80*, 737–754.

Bjerrum, O. J., Hawkins, M., Swanson, P., Griffin, M., & Lorand, L. (1981) *J. Supramol. Struct. 16*, 289–301.

Brachet, J., Mamont, P., Boloukhere, M., Baltus, E., & Hanocq-Quertier, J. (1978) *C.R. Hebd. Seances Acad. Sci., Ser. D 287*, 1289–1292.

Bruner-Lorand, J., Bjerrum, O. J., & Lorand, L. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1118 (Abstract 4959).

Campbell-Wilkes, L. K. (1973) Doctoral dissertation, Northwestern University.

Clarke, D. D., Mycek, M., Neidle, A., & Waelsch, H. (1959) *Arch. Biochem. Biophys. 79*, 338–354.

Cohen, S. S. (1971) *Introduction to the Polyamines*. Prentice-Hall, Englewood Cliffs, NJ.

Conrad, S. M., Velasco, P. T., Michalska, M., Hsu, L., & Lorand, L. (1983) *Invest. Ophthalmol. Visual Sci., Suppl. 3 24*, 157 (Abstract 38).

Conrad, S. M., Velasco, P. T., & Lorand, L. (1984) *Invest. Ophthalmol. Visual Sci., Suppl. 3 25*, 141 (Abstract 64).

Corti, A., Dave, C., Williams-Ashman, H. G., Mihich, E., & Schenone, A. (1974) *Biochem. J. 139*, 351–357.

Epel, D. (1980) *Cell Surf. Dev.* [*Symp.*], *38th*, 169–185.

Griffin, M., Wilson, J., & Lorand, L. (1982) *Anal. Biochem. 124*, 406–413.

Hamaguchi, Y., & Hiramoto, Y. (1982) *Exp. Cell Res. 134*, 171–179.

Hogan, D., & Gross, P. R. (1971) *J. Cell Biol. 49*, 692–701.

Klotz, I. M., Darnall, D. W., & Langerman, N. R. (1975) *Proteins (3rd Ed.) 1*, 293–411.

Kusunoki, S., & Yasumasu, I. (1976) *Biochem. Biophys. Res. Commun. 68*, 881–885.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lorand, L. & Stenberg, P. (1976) *Handb. Biochem. Mol. Biol., 3rd Ed. 2*, 669–685.

Lorand, L., & Conrad, S. (1984) *Mol. Cell. Biochem. 58*, 9–35.

Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, N., & Bruner-Lorand, J. (1968) *Biochemistry 7*, 1214–1223.

Lorand, L., Weissman, L. B., Epel, D. L., & Bruner-Lorand, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4479–4481.

Lorand, L., Siefring, G. E., Jr., & Lowe-Krentz, L. (1978) *J. Supramol. Struct. 9*, 427–440.

Lorand, L., Siefring, G. E., Jr., Tong, Y. S., Bruner-Lorand, J., & Gray, A. J., Jr. (1979a) *Anal. Biochem. 93*, 453–458.

Lorand, L., Parameswaran, K. N., Stenberg, P., Tong, Y. S., Velasco, P. T., Jonsson, N. A., Mikiver, L., & Moses, P. (1979b) *Biochemistry 18*, 1756–1765.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.

Lunden, R., Hartkoorn, A., Nilsson, J. L. G., Stenberg, P., Bruner-Lorand, J., & Lorand, L. (1972) *J. Med. Chem. 15*, 98–99.

Mortensen, S., Sottrup-Jensen, L., Hansen, H. F., Rider, D., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 129*, 314–317.

Mosher, D. F. (1976) *J. Biol. Chem. 251*, 1639–1645.

Park, M. H., Cooper, H. L., & Folk, J. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2869–2873.

Porter, C. W., Dave, C., & Mihich, E. (1981) In *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 407–436, Marcel Dekker, New York.

Rice, R. H., & Green, H. (1977) *Cell (Cambridge, Mass.) 11*, 417–422.

Schmidt, T., Patton, C., & Epel, D. (1982) *Dev. Biol. 90*, 284–290.

Spiegel, E., Burger, M., & Spiegel, M. (1980) *J. Cell Biol. 87*, 309–313.

Tack, B. F., Janatova, J., Thomas, M. L., Harrison, R. A., & Hammer, C. H. (1981) *Methods Enzymol. 80*, 64–101.

Takeuchi, K., Cariello, L., & Lorand, L. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1453 (Abstract 6913).

Tarr, G. E. (1982) In *Methods in Protein Sequence Analysis* (Elzinga, M., Ed.) pp 223–232, Humana Press, Clifton, NJ.

Thomas, E. L., Jefferson, M. M., & Grisham, M. B. (1982) *Biochemistry 21*, 6299–6308.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Young, R. W., & Fulhorst, H. W. (1965) *Anal. Biochem. 11*, 389–391.

Zeller, E. A. (1963) *Enzymes, 2nd Ed. 8*, 313–335.