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PARTIAL PURIFICATION AND CHARACTERIZATION OF AN OVARIAN TRIPEPTIDYL PEPTIDASE: A LYSOSOMAL EXOPEPTIDASE THAT SEQUENTIALLY RELEASES COLLAGEN-RELATED (Gly-Pro-X) TRIPLETS

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SUMMARY: The pregnant hog ovary is a rich source of a novel exopeptidase that catalyzes the release, at pH 5.0, of collagen-related (P_3-P_2-P_1) "triplets" such as Gly-Pro-Met, Gly-Pro-Arg, and Gly-Pro-Ala from arylamide derivatives, provided the N termini are unsubstituted. Corresponding derivatives of related (P_2-P_1) dipeptides (Pro-Met, Pro-Ala) or (P_1) amino acids (Met, Arg, Ala) are not attacked. The enzyme was purified 58-fold from a detergent extract of a water-extracted tissue residue. Activity was determined on Gly-Pro-Met-2-naphthylamide at pH 4.5 and 37°C (K_m 0.45 mM; V_{max} 722 nmoles/min/mg protein). The responsible M_r 55,000 exopeptidase, termed "tripeptidyl peptidase", forms high-M_r aggregates, belongs to the serine catalytic class, and has a lysosomal localization. Gly-Pro-Ala triplets were released sequentially at pH 5.0 from a M_r 14,000 polypeptide, poly(Gly-Pro-Ala-). When this reaction was coupled to that of homologous dipeptidyl peptidase II, the liberated tripeptides were reduced to dipeptides and free amino acids: $(Gly\text{-}Pro\text{-}Ala)_n \longrightarrow n(Gly\text{-}Pro\text{-}Ala) \longrightarrow n(Gly\text{-}Pro) + n(Ala)$.

Two lysosomal dipeptidyl peptidases have thus far been reported (1,2). Whereas dipeptidyl peptidase I possesses a broad specificity and is capable of releasing dipeptides successively from the N termini of polypeptides of virtually any chain length (3), dipeptidyl peptidase II, on the other hand, acts almost exclusively on tripeptides (2,4) and is uniquely capable of cleaving prolyl bonds in tripeptides possessing the Gly-Pro-X sequence (5). Because collagen contains a high proportion of such "triplets", dipeptidyl peptidase II was seen as having the potential for making a contribution to the intracellular (lysosomal) degradation of collagen fibrils, provided a tripeptide-releasing activity exists within lysosomes that is capable of liberating the collagen triplets.

Based on a report by Doebber et al. (6), which described a pituitary "tripeptidyl aminopeptidase" capable of releasing tripeptides sequentially from the N terminus of bovine growth hormone, we attempted to locate an N-terminal exopeptidase capable of

Abbreviations: TPP, tripeptidyl peptidase; DPP, dipeptidyl peptidase; Suc(MeO)-methoxysuccinyl-; Boc-, t-butyloxycarbonyl-; Bz-, benzoyl-; Z-, benzyloxycarbonyl-; -NNap, -2-naphthylamide; -NMec, -7-(4-methyl)coumarylamide; $-NPhNO_2$, -p-nitroanilide.

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depolymerizing a collagen-like sequence represented by $(Gly\text{-}Pro\text{-}X)_n$. Because Gly-Pro-Met and Gly-Pro-Arg occur in both the α1 and α2 chains as the first and third triplets in the helical region of Type I collagen (7), fluorogenic derivatives of these tripeptides were designed as probes with which to search for proteases capable of releasing such tripeptides.

The porcine ovary was found to be a rich source, especially during pregnancy and proestrus (8), of an enzyme capable of hydrolyzing tripeptidyl arylamides and depolymerizing poly(Gly-Pro-Ala-) at acidic pH. This report describes initial efforts to characterize the responsible "tripeptidyl peptidase". A preliminary account has already been presented (9).

MATERIALS AND METHODS

Materials: Peptidyl arylamide substrates were custom preparations. The methylcoumarylamides were prepared by Enzyme Systems Products (Livermore, California) and the 2-naphthylamides and p-nitroanilides by Bachem Inc. (Torrance, California). Poly(Gly-L-Pro-L-Ala-) was a gift from Professor E.R. Blout (Harvard University Medical School). The method (10) used to synthesize the polymer yields a molecular weight of 14,000 ± 500 (11). The Gly-Pro-Ala polymer was also prepared commercially by Bachem Inc. Amino acid analysis revealed equimolar amounts of glycine, proline, and alanine. The presence of a free N terminus and a sequence comprised of repeating Gly-Pro-Ala "triplets" were confirmed by several automated Edman degradation steps performed on a Beckman Sequenator. Thin layer (0.25 mm) cellulose chromatography plates (20 x 20 cm) were obtained precoated on glass from Brinkmann Instruments, Inc. (Westbury, New York).

Enzyme purification: TPP[1] was prepared from ovaries stored at -70°C from the time that they were removed from freshly-killed, pregnant sows. Partially-thawed ovaries were passed through a power meat grinder maintained at 4°C. A 1kg quantity of the ground tissue was suspended in 2 l of cold 1mM EDTA, pH 7.0. Temperatures were maintained at 0-4°C throughout the purification. The suspended tissue was homogenized in portions using a PT-20 Polytron homogenizer. The homogenate was immediately centrifuged at 11,000 g for 30 min. The supernatant (Super I) was removed and the precipitate resuspended in a volume of 1mM EDTA equal to that of Super I. The suspension was rehomogenized with the Polytron and centrifuged at 11,000 g for 60 min. The supernatant (Super II) was decanted and the pellet resuspended in 1% NaCl-1mM EDTA-0.1M formic acid-NaOH buffer, pH 3.5, using a volume equal to that of Super II. Following rehomogenization of the pellet, at which point the pH of the mixture was typically 4.0, a 20% aqueous solution of Triton X-100 was added to a final concentration of 1%, and the suspension was stirred overnight at 4°C. The acidic supernatant **(Super III)** was collected following centrifugation at 11,000 g for 60 min and used as an enriched source of TPP.

Solid ammonium sulfate was added to **Super III** to achieve 40% of saturation at 4°C; precipitated protein was immediately sedimented at 11,000 g for 30 min. The ammonium sulfate concentration of the supernatant was then raised to 70% of saturation and stirred overnight at 4°C. The protein precipitating between 40 and 70% saturated ammonium sulfate, the **40-70% SAS fraction,** was sedimented at 11,000 g for 30 min. The precipitate was redissolved in 1mM EDTA-0.1M acetic acid, pH 3.0, and dialyzed exhaustively against 1mM EDTA-1mM acetic acid, pH 4.0. An Amicon ultrafiltration unit equipped with a YM-10 membrane (Amicon Corp., Lexington, VA) was used to concentrate the **40-70% SAS fraction,** and to exchange residual salts with the column buffer used in the following step.

The **40-70% SAS fraction** was subjected to molecular exclusion chromatography on an Ultrogel AcA 34 column (2.5 x 93 cm) equilibrated with 0.1M NaCl-2mM 2-mercaptoethanol-0.1% Brij 35-0.1M acetic acid-NaOH buffer, pH 4.5. Effluent fractions

containing the peak of the TPP activity, which overlapped the leading half of the DPP I peak (M_r 200,000), were combined. The activity pool, **AcA 34 fraction**, was concentrated and equilibrated with the same column buffer, but with urea added to give 3M.

The **AcA 34 fraction** was subjected to chromatography on a column (2.5 x 45 cm) of Ultrogel AcA 34 equilibrated with the urea-containing buffer. The peak of TPP activity, which now emerged much later in the presence of urea (M_r <100,000), was recovered, dialyzed against 1mM EDTA-0.1% Brij 35-1mM acetic acid, pH 4.0, and concentrated by ultrafiltration. This **AcA 34/U fraction** was used for characterization studies.

Enzyme assays: Activity determinations on fluorogenic substrates were based on direct fluorometric rate measurements carried out on a Turner model 111 fluorometer calibrated with the appropriate leaving groups, e.g., 2-naphthylamine and 7-amino-4-methylcoumarin. A water-jacketted cuvette holder (for 12 x 75 mm tubes) was used to maintain reactions at 37°C. Reaction mixtures were continuously irradiated at 335 nm for naphthylamide substrates or 380 nm for coumarylamide substrates, and rates of increase in fluorescence intensity at 410 nm or 460 nm, respectively, were continuously monitored with a strip chart recorder and used to calculate absolute rates of hydrolysis. Hydrolysis rates on p-nitroanilide derivatives were followed colorimetrically by absorbance increase at 410 nm and converted to absolute rates using a value of 8.8 as the millimolar absorptivity of p-nitroaniline (12). Protein was determined by the Folin-Lowry procedure (13) using bovine serum albumin as a standard.

TPP was routinely assayed on Gly-Pro-Met-NNap at pH 4.5 by adding 20 µl of an appropriately-diluted sample to 4.0 ml of an incubation mixture that contained 2.0 mM naphthylamide substrate (or 0.15 mM methylcoumarylamide substrate) in .05% Brij 35-1mM EDTA-62mM acetic acid-NaOH buffer, pH 5.0. DPP I and II were assayed fluorometrically on Gly-Phe-NNap at pH 6.0 and Phe-Pro-NNap at pH 5.5, respectively, as previously described (1,2). Stock solutions of substrate (100-fold greater than concentration in reaction mixtures) were routinely prepared in dimethyl formamide and stored at -20°C.

Electrophoresis: Polyacrylamide (7.5%) gels were used to evaluate enzyme purity by means of electrophoresis under non-denaturing conditions at 8°C in a Hoefer vertical slab apparatus (Hoefer Instruments, Inc., San Francisco, California). Gels were polymerized in 0.37 M Tris-HCl buffers at pH 7.0 or 8.8 (14). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

Time course analysis of poly(Gly-Pro-Ala-) digests by thin layer chromatography: A chromatographic analysis of the split products was conducted on thin layers (0.25 mm) of Avicel microcrystalline cellulose.

Reactions were initiated by combining 500 µg of freeze-dried protein, **AcA 34/U fraction**, with 200 µl of a 1.5% (w/v) solution of poly(Gly-Pro-Ala-) (average M_r about 14,000) in 1M acetic acid-pyridine buffer, pH 5.0. Immediately thereafter, a 1-µl aliquot was spotted at "zero time" and at intervals of 2, 4, 8, 12 and 24 hr. A 100-µl aliquot of the 24-hr reaction mixture was then freeze dried, reconstituted in 85 µl of the same volatile pyridine-acetate buffer, and the time course continued by adding 15 µl (20 munits) of ovarian DPP II contained in the same buffer. A 1-µl aliquot was spotted at 0, 1, 2 and 4 hr, which corresponded to intervals of 24, 25, 26 and 28 hr on the continuous time scale. The enzyme control was handled in an identical manner, except that poly(Gly-Pro-Ala-) was omitted from the buffered substrate solution used for the digest. The enzyme control was spotted at 0, 24, and 28 hr. Reactions were maintained at 37°C, and standards (Gly-Pro-Ala, Gly-Pro and alanine) were applied in 3-nmole quantities.

Plates were developed with 90% aqueous phenol and allowed to air dry overnight. Peptides were detected with a ninhydrin reagent that contained 50 ml of 0.2% ninhydrin in absolute ethanol, 10ml glacial acetic acid, and 2ml of 2,4,6-collidine. The plates were heated at 110°C for 2 min, and stored in the dark at room temperature for 24 hr to allow for full color development.

Preparation of dipeptidyl peptidase II: Pregnant hog ovaries were also used for the purification of DPP II. The method used was a modification of a procedure originally developed for the purification of the bovine pituitary enzyme (1). The procedure used to purify porcine DPP II, a species form of DPP II with an extraordinary preference for prolyl bonds (15), will be reported elsewhere.

Subcellular fractionation: Ovaries from freshly-killed sows were chopped and homogenized in cold 0.25M sucrose. The procedure of Wattiaux et al. (16) was used to isolate lysosomes from a light mitochondrial fraction by means of isopycnic centrifugation

in a discontinuous Metrizamide gradient. The gradient was fractionated into 1-ml serial aliquots using a density gradient fractionator, model 185 (Instrumentation Specialties Co., Lincoln, Nebraska).

RESULTS

Purification and molecular weight: The purification procedure outlined under Methods yielded a highly-enriched preparation of TPP (Table I). The elimination of two successive aqueous extracts (supernatants I and II) removed a high proportion of ovarian protein and lysosomal proteases, thus justifying a loss of 40-50% of the TPP activity in the two discard supernatants. The activity present in **Super III** was subsequently purified an additional 58-fold by fractionation with ammonium sulfate and two cycles of molecular exclusion chromatography on Ultrogel AcA 34. In the first chromatographic step, TPP was recovered as an aggregate ($M_r \sim 250,000$) that was considerably larger than the endogenous DPP I peak that contaminated **Super III** and served as useful internal, M_r 200,000 standard. The TPP fractions were pooled so as to purposely include about half of the DPP I peak, and exclude the lower-M_r cysteine and aspartic proteinases. The concentrated pool was then rechromatographed on a similar AcA 34 column but under dissociating (3M urea) conditions. Under these conditions, the monomeric form of TPP emerged later than DPP I, which continued to serve as a useful M_r 200,000 standard despite considerable losses of activity caused by the denaturing effect of the 3M urea.

TABLE 1. Partial purification of tripeptidyl peptidase from 1 kg of pregnant hog ovaries

Fraction	Yield		Specific activity	Purification
	munits[a]	mg	mU/mg	fold
Supernatant III ("**Super III**") (pH 4, 1% Triton X-100)	133,000	14,900	8.9	1.0
$(NH_4)_2SO_4$ fraction, 40-70% sat'ed ("**40-70% SAS fraction**")	49,000	1060	46.2	5.2
Gel filtration ("**AcA 34 fraction**") (Peak $M_r \sim 250,000$)	22,000	108	204	23
Gel filtration, 3 M urea ("**AcA 34/U**") (Peak M_r 55,000)	16,000	31	516	58

[a]One milliunit hydrolyzes 1 nmole Gly-Pro-Met-NNap per min at pH 4.5, 37°C.

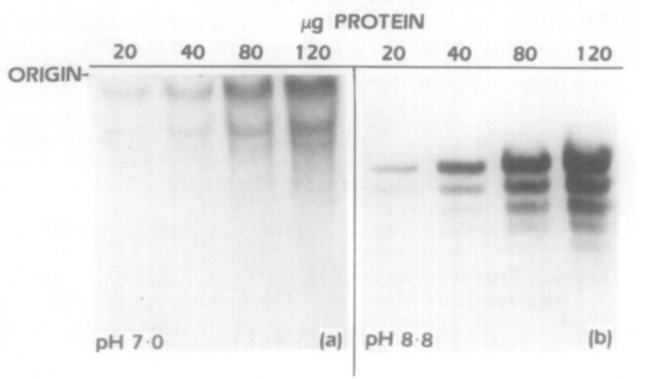

Figure 1. Comparison of patterns obtained by electrophoresis of tripeptidyl peptidase (fraction AcA 34/U) in 7.5% polyacrylamide gels at pH 7.0 (Fig. 1a) and pH 8.8 (Fig. 1b) at 100 volts (25 ma) for 3 hr.

Calibration of the Ultrogel/urea column with molecular weight standards indicated that TPP behaved as a M_r 55,000 protein under these dissociating conditions.

Rate response to pH and substrate concentration: A different pH optimum was observed on each of the three arylamide derivitives of Gly-Pro-Met-. At 37°C, the 2-naphthylamide derivative was hydrolyzed most rapidly at pH 4.5 (K_m 0.45 mM; V_{max} 722 nmoles/min/mg protein), the 7-(4-methyl)coumarylamide at pH 5.0 (K_m 0.17 mM; V_{max} 294 nmoles/min/mg protein), and the p-nitroanilide at pH 4.3.

Assessment of purity in polyacrylamide gels: Electrophoresis under non-denaturing conditions at pH 7.0 and 8.8 gave very different results (Figure 1). At pH 7.0 about three different protein species were detected in the partially-purified TPP, whereas at pH 8.8, at least five components were seen. However, the concentration gradient and uniform spacing of the bands (at pH 8.8) were attributed to polymerization or aggregation —a phenomenon that also occurred during purification when molecular exclusion chromatography was carried out in the absence of urea.

Specificity on fluorogenic and chromogenic peptide derivatives: The action of TPP on a range of naphthylamide, coumarylamide, and nitroanilide substrates (Table II) revealed an N-terminal exopeptidase specificity that was restricted to the removal of tripeptide units provided the P_3 residue was unsubstituted. Although rates were highest on tripeptide derivatives containing a hydrophobic (methionyl or leucyl) residue in the P_1 position, substantial rates seen on alanyl and arginyl linkages suggested a relativley broad specificity.

TABLE II. Rates of hydrolysis of peptide arylamide substrates by porcine tripeptidyl peptidase at pH 5.0 and 37°C

Substrate	Activity	
	Specific (mU/mg)	Relative (%)
2-Naphthylamides (2 mM)		
Gly-Pro-Met-NNap	604	100
Met-NNap	0	0
Suc-Gly-Pro-Met-NNap	2	0.3
Gly-Pro-Leu-NNap	175	29
7-(4-Methyl)coumarylamides (0.15 mM)		
Gly-Pro-Met-NMec	124	100
Pro-Met-NMec	0	0
Met-NMec	0	0
Suc(MeO)-Gly-Pro-Met-NMec	0	0
Gly-Pro-Arg-NMec	10	8
Z-Arg-Arg-NMec	0	0
Arg-NMec	0	0
p-Nitroanilides (3 mM)		
Gly-Pro-Met-$NPhNO_2$	1105	100
Gly-Pro-Ala-$NPhNO_2$	221	20
Pro-Ala-$NPhNO_2$	0	0
Ala-$NPhNO_2$	0	0
Boc-Gly-Pro-Ala-$NPhNO_2$	0	0
Val-Pro-Arg-$NPhNO_2$	124	11
Bz-Val-Pro-Arg-$NPhNO_2$	0	0

Depolymerization of poly(Gly-Pro-Ala-): A time course chromatographic analysis of the split products produced by the action of TPP on $(Gly\text{-}Pro\text{-}Ala)_n$ at pH 5.0 is reproduced in Figure 2. The chromatogram revealed the accumulation of a fragment that was identified as Gly-Pro-Ala on the basis of its mobility compared to a standard. The polymeric substrate reacted poorly with ninhydrin, and was seen only as a yellowish zone. The addition of porcine DPP II to the reaction mixture resulted in the breakdown of the Gly-Pro-Ala product and the simultaneous appearance of products identified as Gly-Pro and free alanine.

Effect of inhibitors: The results summarized in Table III show that TPP was most sensitive to organophosphorus (Dip-F), sulfonyl fluoride (PMSF), and active-site-directed chloromethyl ketone (Gly-Pro-MetCH_2Cl) inhibitors. The sulfhydryl-blocking reagents showed only partial inhibition at relatively high concentrations. Consistent with a serine catalytic mechanism, TPP was unaffected by EDTA and pepstatin.

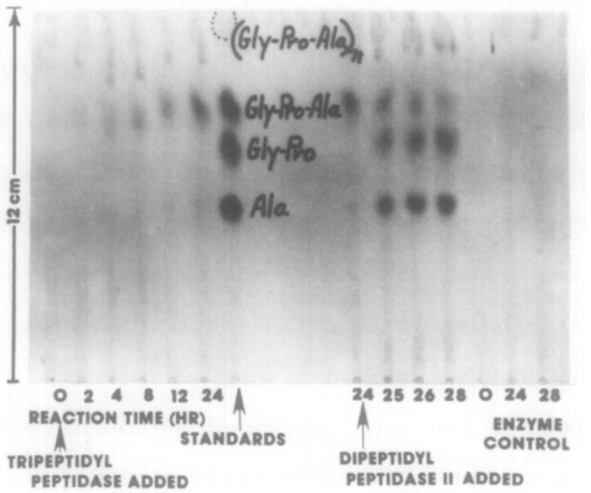

Figure 2. Time course analysis by chromatography on thin layer cellulose of the products of poly(Gly-Pro-Ala-) degradation at pH 5.0 by the sequential action of tripeptidyl peptidase and dipeptidyl peptidase II.

Subcellular localization: TPP activity was located in subcellular fractions prepared from fresh porcine ovaries. The sedimentation and latency characteristics of the enzyme were typically lysosomal when compared to those for established marker enzymes such as acid phosphatase and DPP I.

TABLE III. Comparison of potential inhibitors on porcine tripeptidyl peptidase activity

Compound	Final Conc. (mM)	Inhibition (%)
Diisopropyl fluorophosphate (Dip-F)	10.0 1.0	100 82
Alkali-inactivated Dip-F	1.0	0
Phenylmethylsulfonyl fluoride (PMSF)	1.0	7
p-Chloromercuriphenyl sulfonate (PCMS)	1.0	53
Iodoacetate	10.0	46
EDTA	1.0	0
Gly-Pro-MetCH_2Cl	0.01 0.001	100 64
Pepstatin	0.01	0
Leupeptin	0.01	0

Reaction rates were recorded fluorometrically at pH 5.0 following the addition of Gly-Pro-Met-NNap to enzyme/buffer mixtures preincubated with inhibitor for 30 min at 37°C.

DISCUSSION

The relatively broad specificity of TPP seen on arylamide derivatives of Gly-Pro-X-, and the extensive degradation of poly(Gly-Pro-Ala-) catalyzed by the coupled activities of TPP and DPP II strongly suggest a role for these exopeptidases in the lysosomal digestion of phagocytosed (17) and newly synthesized (18) collagen. In view of the fact that proline and hydroxyproline constitute 20-25% of the residues in collagen (7), and because none of the known lysosomal proteinases is capable of cleaving these linkages, it would appear that the coupled exopeptidase mechanism described here could very well make a significant contribution to the overall process of intracellular collagen degradation. Further investigations of such a mechanism are now in progress.

Apart from its propensity for aggregation and its stability to freeze-drying, the porcine ovarian TPP reported here shows several properties that are notably similar to the bovine pituitary "tripeptidyl aminopeptidase" described by Doebber et al. (6). Both show a unique tripeptide releasing activity on tripeptide arylamides and large polypeptides, similar pH optima and molecular weights, a lysosomal localization, and limited solubility in the absence of detergent. Whereas the porcine enzyme exhibits a serine catalytic mechanism, the catalytic class of the bovine enzyme has apparently not yet been established. Both are, however, unaffected by EDTA and pepstatin, and show anomalous (irreversible) sensitivity to high (1mM) levels of a particular sulfhydryl-blocking reagent, p-chloromercuriphenyl sulfonate.

By analogy with the trivial term "dipeptidyl peptidase" assigned by the I.U.B. Nomenclature Committee to the dipeptide-releasing aminopeptidases (DPP I-IV), it is suggested that the lysosomal tripeptide-releasing aminopeptidases thus far identified in the bovine pituitary and porcine ovary be tentatively included under the name tripeptidyl peptidase I (TPP I), and that the extralysosomal "tripeptidyl aminopeptidase" recently identified in rat liver by Bålöw et al. (19) be termed tripeptidyl peptidase II (TPP II). These two enzymes would thus constitute the first two members of a new class of exopeptidases that would systematically be termed, by analogy, the **tripeptidylpeptide hydrolases.**

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REFERENCES

1. McDonald, J.K., Leibach, F.H., Grindeland, R.E., and Ellis, S. (1968) J. Biol. Chem. 243, 4143-4150.
2. McDonald, J.K., Zeitman, B.B., Reilly, T.J., and Ellis, S. (1969) J. Biol. Chem. 244, 2693-2709.
3. McDonald, J.K., Callahan, P.X., Ellis, S., and Smith, R.E. (1971) In: Tissue Proteinases (A.J. Barrett and J.T. Dingle, eds.), pp. 69-107, North-Holland Publishers, Amsterdam.
4. Fukasawa, K., Fukasawa, K.M., Hiraoka, B.Y., and Harada, M. (1983) Biochim. Biophys. Acta 745, 6-11.
5. McDonald, J.K., and Schwabe, C. (1980) Biochim. Biophys. Acta 616, 68-81.
6. Doebber, T.W., Divor, A.R., and Ellis, S. (1978) Endocrinology 103, 1794-1804.
7. Fietzek, P.P. and Kuhn, K. (1976) Int. Rev. Conn. Tiss. Res. 7, 1-60.
8. McDonald, J.K., Eisenhauer, D.A., and Hoisington, A.R. (1985) In: Intracellular Protein Catabolism (E.A. Khairallah, J.S. Bond, and J.W.C. Bird, eds.), Alan R. Liss, Inc., New York, in press.
9. McDonald, J.K. and Hoisington, A.R. (1983) Fed. Proc. 42, 1781 only.
10. Bloom, S.M., Dasgupta, S.K., Patel, R.P., and Blout, E.R. (1966) J. Am. Chem. Soc. 88, 2035-2041.
11. Oriel, P.J., and Blout, E.R. (1966) J. Am. Chem. Soc. 88, 2041-2045.
12. Erlanger, B.F., Kokowsky, N., and Cohn, W. (1961) Arch. Biochem. Biophys. 95, 271-278.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Chrambach, A., and Jovin, T.M. (1983) Electrophoresis 4, 190-204.
15. Eisenhauer, D.A., Chilton, B.S., and McDonald, J.K. (1983) Endocrinology 112S, 295 only.
16. Wattiaux, R., Wattiaux-de Coninck, S., Ronveaux-Dupal, M.F., and Dubois, F. (1978) J. Cell. Biol. 78, 349-368.
17. Svoboda, E.L.A., Brunette, D.M., and Melcher, A.H. (1979) J. Anat. 128, 301-314.
18. Bienkowski, R.S. (1983) Biochem. J. 214, 1-10.
19. Bålöw, R.-M., Ragnarsson, U., and Zetterqvist, O. (1983) J. Biol. Chem. 258, 11622-11628.