Islet Secretory Granules Contain Cytochrome b_{561}

R. B. MACKIN, D. P. JONES, AND B. D. NOE

SUMMARY

A cytochrome has been detected in secretory granules prepared from anglerfish islets of Langerhans. The heme moiety was determined to be of the *b* type, and the dithionite-reduced cytochrome exhibited an α-band maximum at 561 nm with an extinction coefficient of 13.8 $mM^{-1} \cdot cm^{-1}$. The protein was present at a concentration of 40 ± 4 pmol/mg of secretory granule protein. The cytochrome was found to be an integral membrane protein and to be reduced by ascorbic acid but not by NADH, NADPH, reduced glutathione (GSH), or succinate.

Because of the similarity to previously characterized secretory granule cytochrome b_{561}'s from neuroendocrine tissues, this cytochrome is also referred to as cytochrome b_{561}. Although its function has not yet been elucidated, the apparent specificity for ascorbate suggests that it may be a component of the ascorbate-dependent peptidyl-glycine α-amidating monooxygenase system that functions in the amidation of islet hormones. DIABETES 1986; 35:881–85.

Many biologically active peptides are initially synthesized as larger propeptides that are either biologically inactive or have reduced biological activity. These precursors are subject to post-translational proteolytic cleavage that releases the biologically active peptide.[1-3] The sequence of the precursors to anglerfish islet insulin,[4] glucagon,[5,6] and somatostatin[7] and the human pancreatic polypeptide[8-10] are consistent with this model. More recently, it has become apparent that processing of two anglerfish islet prohormones may require enzyme activities other than those of the previously characterized proteases. Characterization of a neuropeptide Y/pancreatic polypeptide-like peptide[11] and glucagon-like peptide II[12] from anglerfish islets has indicated that both of these hormones can be modified by COOH-terminal amidation. These data suggest that a peptidyl-glycine α-amidating monooxygenase (PAM) may be required for amidation of these hormones.

The PAM system, as studied in the pituitary, requires intragranular ascorbate as a cofactor.[13] Granule transport of ascorbate has been studied most thoroughly in bovine adrenal chromaffin granules. These granules transport only dehydroascorbate across their membranes.[14] Recently, it has been demonstrated that ascorbate markedly stimulates the synthesis of norepinephrine by dopamine β-hydroxylase in preparations of isolated chromaffin granules, despite the observation that ascorbate was not transported into the chromaffin granules.[15] It has therefore been suggested that cytochrome b_{561} (also referred to as chromomembrin B[16]), an integral membrane protein previously characterized in neuroendocrine secretory granules isolated from bovine adrenal[17] and posterior pituitary[18] glands, may be involved in the transfer of electrons across secretory granule membranes to reduce intragranular dehydroascorbate (and/or semidehydroascorbate) to ascorbate.[18] Because of the similarities of these processing and secretory systems, anglerfish islet secretory granules were examined for the presence of cytochrome b_{561}. The results establish that such a cytochrome is an integral component of membranes of islet secretory granules and is reducible by ascorbate but not other common cellular reductants.

MATERIALS AND METHODS

Isolation and characterization of islet secretory granules.

Anglerfish islet tissue was obtained either from the Marine Biological Laboratory (Woods Hole, MA) or from the University of Connecticut Marine Station (Stonington, CT). Anglerfish islets were homogenized in 0.25 M sucrose containing 1 mM $MgCl_2$ and were subjected to differential discontinuous sucrose density-gradient centrifugation as previously described.[19,20] Subcellular fractions were routinely monitored for purity via marker enzyme assays and radioimmunoassays with results as previously reported from our laboratory.[20,21]

Department of Anatomy and Cell Biology (R.B.M., B.D.N.) and Department of Biochemistry (D.P.J.), Emory University School of Medicine, Atlanta, Georgia. Address reprint requests to Bryan D. Noe, Ph.D., Dept. of Anatomy and Cell Biology, Emory Univ. School of Medicine, Atlanta, GA 30322.
Received for publication 7 October 1985 and in revised form 5 February 1986.

Determination of absorption spectra. Secretory granules were suspended in 25 mM sodium phosphate buffer at pH 7.4 and subjected to six cycles of repeated freezing and thawing. Protein values were determined by the method of Bradford.[22] Aliquots were diluted with 25 mM Tris-HCl buffer pH 7.9, containing 20% glycerol and 0.5% deoxycholate, and spectra were recorded with an Aminco DW-2A spectrophotometer by methods previously described.[23] Samples were prepared with protein concentrations in the range of 1–2 mg/ml, except as indicated. Baseline spectra were obtained with identical preparations in both the sample and reference cuvettes. Reduced versus oxidized difference spectra were obtained by subsequent reduction of the sample with a few grains of sodium dithionite. Oxidation of the aliquot in the reference cuvette with potassium ferricyanide did not increase the magnitude of the difference spectrum and was therefore not performed routinely.

Determination of heme type and extinction coefficient. For determination of the type of heme moiety present in the cytochrome and for quantitation of the extinction coefficient, 100 μl of lysed secretory granule preparations (containing 0.5–1.6 mg of total secretory granule protein) were added to 400 μl of a solution of 0.1 N NaOH/20% pyridine. The absolute spectrum of the sodium dithionite–reduced sample was recorded, and heme *b* content was calculated with the extinction value of 32.4 $mM^{-1} \cdot cm^{-1}$ for the wavelength pair 557 vs. 575 nm.[23] Verification that the cytochrome contains a *b*-type heme, and not a covalently linked heme as in cytochrome *c*, was accomplished with the acetone/HCl extraction technique of Rieske.[24]

For routine quantitation of the cytochrome in secretory vesicles, a change in extinction coefficient ($\Delta\epsilon$) was calculated for the reduced cytochrome with the formula:

$$\frac{\Delta A_{561-570}}{\text{heme concn. (nmol/ml)}} = \Delta\epsilon(\mu M^{-1} \cdot cm^{-1})$$

with heme concentration determined as above. The reference wavelength (570 nm) was chosen because it appeared to be an isosbestic point in this preparation.

Membrane association of cytochrome b_{561}. Secretory granule preparations were lysed by repetitive freeze/thaw cycles in 500 μl of 25 mM sodium phosphate buffer, pH 7.4, and centrifuged for 5 min at 12,000 × *g*. The supernatant was removed, and the precipitate was suspended in 500 μl of 25 mM sodium phosphate, pH 7.4, containing 1.0 M KCl. The samples were then centrifuged for 5 min at 12,000 × *g*, and the supernatants were removed. Precipitates were then suspended in 500 μl of 25 mM sodium phosphate, pH 7.4. Aliquots containing similar concentrations of protein were then assayed for cytochrome content.

Binding of ligands to cytochrome. For CO binding, the sample cuvette was bubbled with CO for 1 min, and a CO (not reduced) versus air difference spectrum was recorded. Both sample and reference were then reduced with sodium dithionite, and a CO difference spectrum of the dithionite-reduced forms was thereby obtained.

For cyanide binding, aliquots were placed in the sample and reference cuvettes, and a baseline spectrum was taken. After this, 2 μl of 0.5 M KCN was added to the sample cuvette (final concn. 1 mM), and a CN-induced difference spectrum of the oxidized forms was recorded.

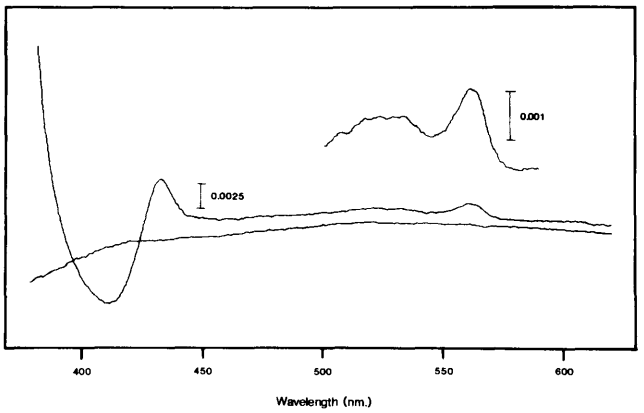

FIG. 1. Dithionite-reduced minus air-oxidized spectrum of secretory granule cytochrome b_{561}. Difference absorption spectrum of secretory granule hemoprotein obtained as described in MATERIALS AND METHODS. Concentration of total secretory granule protein is 1.62 mg/ml. Absorption units are indicated by calibration bars.

Determination of methemoglobin content. CO difference spectra of the cytosolic fractions showed that no hemoglobin was present. Aliquots were assessed for methemoglobin content by formation of cyanomethemoglobin. After recording a baseline spectrum, 1 μl of 0.5 M KCN was added to the sample cuvette (final concn. 1 mM) to provide CN (air)-versus air-oxidized difference spectrum. Quantitation of methemoglobin content was determined with an extinction coefficient of 12.5 $mM^{-1} \cdot cm^{-1}$ for the absorbance at 540 nm.[25]

Analysis of potential reductants. Identical aliquots of lysed secretory granule preparations were used to generate baseline spectra. Sample cuvettes were then treated to obtain the following final concentrations of possible reductants: 1 mM ascorbate (sodium salt), 2 mM NADH, 2 mM NADPH, 1 mM GSH, and 10 mM succinate (sodium salt). Samples were assayed for reduction by obtaining difference spectra at various time points between 0 and 45 min.

RESULTS

Detection of cytochrome b_{561}. To determine whether a cytochrome is present in islet secretory granules, an absorbance spectrum of dithionite-reduced versus air-oxidized granules was obtained. This spectrum had three absorbance maxima, at 433, 531, and 561 nm, corresponding to the Soret, β-, and α-bands, respectively (Fig. 1). Formation of the pyridine hemochrome by treatment with pyridine under alkaline conditions followed by reduction provided an absorption spectrum consistent with identification as a *b*-type heme.[24] However, covalently bound *c*-type heme groups absorb at 552 nm in this assay, and therefore an acetone/HCl extraction was performed to determine whether the heme was covalently bound to the protein. With this method, the pyridine hemochromogen detected the heme group in the soluble but not the precipitated fraction. This demonstrates that the heme group is not covalently linked to the protein. No spectral change was observed on addition of CN to the oxidized fraction, indicating that the hemoprotein was not adventitiously bound methemoglobin. Moreover, the hemoprotein

TABLE 1
Hemoprotein contents of subcellular fractions from anglerfish islets

Subcellular fraction	Major hemoproteins
Mitochondria/lysosomes	Cytochromes *a* and a_3, *b*, *c*, and c_1 (stoichiometry similar to that for mitochondria from liver and heart)
Secretory granules	Ascorbate-reducible cytochrome b_{561}, no CN-induced spectral change
Microsomes	CN-induced spectral change, predominantly cytochrome P_{420} (probably derived from P_{450})
Cytosol	Methemoglobin (or hemoprotein with similar absorption characteristics, e.g., indoleamine dioxygenase or tryptophan dioxygenase)

For explanation and references see text.

was not extractable with high salt, indicating that methemoglobin was not contributing significantly to the spectrum.

Islet secretory granule cytochrome b_{561} is an integral membrane protein. To determine whether the cytochrome b_{561} is localized to the lipid bilayer of the secretory granules, soluble proteins and membrane-associated proteins were sequentially extracted with buffers of low and high ionic strength, respectively. Dithionite-reduced versus air-oxidized difference spectra were then obtained for each fraction. Cytochrome b_{561} was detected only in the high-ionic-strength particulate fraction, indicating that this hemoprotein is an integral membrane protein (data not shown).

Quantitation of cytochrome b_{561}. The extinction coefficient of the cytochrome b_{561} was calculated for routine quantitation of the hemoprotein in the islet secretory granules. With the procedures outlined in METHODS, an extinction coefficient of $\epsilon = 13.8 \pm 2.6$ $mM^{-1} \cdot cm^{-1}$ was determined for the wavelength pair 561 vs. 570 nm. Because this value was determined on the granule fraction, which may be contaminated with other hemoproteins, it may be an underestimate. With this value, the concentration of the cytochrome in secretory granules was calculated to be 40 ± 4 pmol/mg of protein.

Cytochrome b_{561} is a secretory granule protein. The spectral characteristics of cytochrome b_{561} of the granule fraction were compared with properties of other subcellular fractions to determine whether the cytochrome b_{561} is unique to the granules. Previous studies in our laboratory have established techniques for preparation of subcellular fractions enriched in mitochondria (also containing lysosomes), secretory granules, microsomes, and cytosol.[19-21] Using these techniques, we fractionated tissue and examined the spectral characteristics of each fraction. Because of the interference by other hemoproteins, it was not possible to obtain quantitative measurements of the secretory granule cytochrome b_{561} in the other fractions. However, studies of spectral changes on reduction or addition of CO or KCN allowed qualitative analysis of cytochrome b_{561} distribution. The postmicrosomal supernatant (cytosol) contained a hemoprotein that reacted with CN to give absorption maxima characteristic of methemoglobin and, on reduction with dithionite, reacted with CO to give absorption maxima characteristic of carbonmonoxyhemoglobin. Because indolamine dioxygenase and tryptophan dioxygenase have similar absorbance characteristics,[26] we cannot yet identify this soluble hemoprotein with certainty. However, studies of the secretory granules showed that CN does not give a difference spectrum with the secretory granule cytochrome. Moreover, cytochrome b_{561} was not solubilized from the granules by high-salt treatment. Therefore the granule cytochrome is not an adventitiously bound soluble hemoprotein, and it appears unlikely that a significant amount of the secretory granule cytochrome b_{561} is present in the cytosolic fraction.

Spectral studies of the microsomal fraction revealed that the dithionite-reduced versus air-oxidized spectrum and the CO-induced difference spectrum of the reduced forms were very similar to those obtained with the granule fractions. However, the microsomal fraction also showed a CN-induced difference spectrum with an absorbance maximum of 420 nm. This 420-nm-absorbing chromophore was shown to account for much of the hemoprotein present in the microsomal fraction. Consequently, these results indicate that the microsomal fraction contains predominantly cytochrome P_{420} (probably derived from cytochrome P_{450}[27]) and at most only a small amount of cytochrome b_{561}.

The dithionite-reduced versus air-oxidized difference spectrum of the mitochondrial fraction contained absorbance maxima consistent with cytochromes *a* and a_3 (92 pmol/mg protein, calculated from extinction coefficients listed in ref. 23), cytochrome *b* (71 pmol/mg protein), and cytochromes *c* and c_1 (46 pmol/mg protein) in ratios comparable with those found in mitochondria from other cell types.[23] Therefore, little of the cytochrome b_{561} from the granules is present in this fraction.

These results are summarized in Table 1 and indicate that cytochrome b_{561} is predominantly localized in the secretory granules.

Determination of possible physiologic electron donors/acceptors. Several possible electron donors were tested to determine whether they could reduce cytochrome b_{561}. Of the reductants tested, only ascorbate was able to reduce cytochrome b_{561}. NADH, NADPH, GSH, and succinate at millimolar concentrations were without effect for up to 45 min. A summary of the characteristics of the islet secretory granule cytochrome b_{561} is given in Table 2.

DISCUSSION

A cytochrome b_{561} hemoprotein has been previously characterized in bovine adrenal medulla chromaffin granules[17,28] and posterior pituitary secretory granules.[18] This protein has been proposed to play a role in the shuttling of electrons across these secretory granule membranes.[29] In this capacity the cytochrome may play a role in the synthesis of neuro-

TABLE 2
Characteristics of cytochrome b_{561} in secretory granules

Characteristic	Value
Absorption maxima	
Dithionite reduced vs. oxidized	433, 531, and 561 nm
CO + dithionite vs. dithionite	420 nm
CN (oxidized) vs. oxidized	No change
Heme	type *b*
$\Delta\epsilon$ $b_{561-570}$	13.8 ± 2.6 mM^{-1}/cm
Reductants	Dithionite, ascorbate
Content in granules	40 ± 4 pmol/mg protein

transmitters and/or peptide hormones via either dopamine β-hydroxylase[30] or peptidyl-glycine α-amidating monooxygenase.[18] Pancreatic islets synthesize the COOH-terminally amidated form of pancreatic polypeptide,[31] and the proportion of amidated pancreatic polypeptide varies under different in vitro conditions.[32] Recently, anglerfish islets were shown to contain a peptide having some sequence homology with pancreatic polypeptide but more complete homology with porcine neuropeptide Y.[11] This peptide has therefore been termed *anglerfish peptide Y*. Anglerfish peptide Y is a candidate peptide for COOH-terminal amidation in this islet system. In addition, anglerfish islets also contain an amidated peptide derived from proglucagon II.[12] This peptide is located at the COOH-terminus of the precursor, shares significant sequence homology with glucagon II, and has been termed *glucagon-like peptide II*.

Because of the proposed function for cytochrome b_{561} in the other secretory granules, and the possible similarity of processing of pancreatic peptide hormones to peptide amidation in these other granules, anglerfish islet granules were examined for the presence of cytochrome b_{561}. A hemoprotein was detected in the secretory granules of the islets and was determined to have an α-band absorbance maximum of 561 nm and a heme moiety of the *b* type. This protein is therefore designated cytochrome b_{561}. The islet cytochrome b_{561} was determined to be an integral membrane protein. This observation is consistent with a possible role as an electron-transport protein involved in donating reducing equivalents to a peptide-processing enzyme.

The islet cytochrome was found to have an extinction coefficient of 13.8 ± 2.6 $mM^{-1} \cdot cm^{-1}$. Based on this value, the secretory granules contain ~40 ± 4 pmol cytochrome b_{561}/mg of total secretory granule protein. This value may be an underestimate if the cytochrome is unstable under the storage or assay conditions used. Instability during storage could also affect the calculated extinction coefficient and requires additional studies on purified cytochrome preparations to accurately assess granule cytochrome content. Duong et al.[18] calculated a concentration of 6.8 μg cytochrome b_{561}/mg membrane protein in secretory granules prepared from bovine posterior pituitary. Based on a molecular weight of 30,000 for their cytochrome b_{561}, this would be equivalent to 227 pmol cytochrome b_{561}/mg membrane protein. Anglerfish islet secretory granules contain roughly equal parts of soluble and integral membrane protein (unpublished observations). Therefore, anglerfish islets may contain less cytochrome b_{561}/mg secretory granule protein than do bovine posterior pituitary secretory granules. This may be consistent with the observation that anglerfish islets are composed of at least four different cell types.[33,34] It remains to be determined whether only the glucagon- and anglerfish peptide Y–producing cells contain islet cytochrome b_{561} or whether other islet cell types also contain the cytochrome.

Based on the concentration of cytochrome b_{561} in the secretory granule–enriched fractions from the islets and on the distinctively different properties of the cytochromes from the mitochondria/lysosome-, microsome-, and cytosol-enriched fractions, it is concluded that cytochrome b_{561} is present in islet secretory granules and is not found in the secretory granule–enriched fraction as a result of contamination from other subcellular organelles.

Although the results of our study do not directly demonstrate the physiologic relevance of the islet granule cytochrome b_{561}, reduction by ascorbate was rapid, and other physiologic reductants (NADH, NADPH, succinate, and GSH) did not reduce the cytochrome. Because reduced intragranular ascorbate is required as a cofactor for the PAM enzyme,[13] the cytochrome found in islet granule membranes may function in facilitating or modulating PAM activity. This hemoprotein could thereby be involved in regulating the amidation of anglerfish peptide Y and/or glucagon-like peptide II.

In conclusion, we have detected cytochrome b_{561} in the secretory granules of anglerfish islets. This protein has properties very similar to those determined for the cytochrome b_{561} found in bovine adrenal chromaffin granules and posterior pituitary secretory granules. Our study is the first to report the existence of a cytochrome b_{561} hemoprotein in the secretory granules of islet tissue. The protein found in islet granules is probably involved in the posttranslational processing of biologically active peptides. Studies designed to investigate the function of the islet granule cytochrome are in progress.

NOTE ADDED IN PROOF

Recently, evidence for ascorbate (or semidehydroascorbate)-dependent electron transfer across chromaffin granule membranes has been published in two separate reports[A,B]. In one of these studies the electron transfer was associated with changes in the spectrum of cytochrome b_{561}.[B]

[A]Beers, M. F., Johnson, R. G., and Scarpa, A.: Evidence for an ascorbate shuttle for the transfer of reducing equivalents across chromaffin granule membranes. J. Biol. Chem. 1986; 261:2529–35.

[B]Kelley, P. M., and Njus, D.: Cytochrome b_{561} spectral changes associated with electron transfer in chromaffin-vesicle ghosts. J. Biol. Chem. 1986; 261:6429–32.

ACKNOWLEDGMENTS

This research was supported by NIH Grants AM-16921 and AM-26378 to B.D.N. and by NIH Grant HL-30286 to D.P.J.

REFERENCES

[1] Gainer, H., Russell, J. T., and Loh, Y. P.: The enzymology and intracellular organization of peptide precursor processing: the secretory vesicle hypothesis. Neuroendocrinology 1985; 40:171–84.

[2] Loh, Y. P., Brownstein, M. J., and Gainer, H.: Proteolysis in neuropeptide processing and other neural functions. Annu. Rev. Neurosci. 1984; 7:189–222.

[3] Noe, B. D.: Converting enzymes. *In* Current Trends in Tumor Pathology. Vol. 1. Endocrine Tumors. Polak, J. M., and Bloom, S. R., Eds. Edinburgh, Churchill Livingstone, 1985:57–81.

[4] Hobart, P. M., Shen, L.-P., Crawford, R., Pictet, R. L., and Rutter, W. J.: Comparison of the nucleic acid sequence of anglerfish and mammalian insulin mRNA's from cloned cDNA's. Science 1980; 210:1360–63.

[5] Lund, P. K., Goodman, R. H., Dee, P. C., and Habener, J. F.: Pancreatic preproglucagon cDNA contains two glucagon-related coding sequences arranged in tandem. Proc. Natl. Acad. Sci. USA 1982; 79:345–49.

[6] Lund, P. K., Goodman, R. H., Montminy, M. R., Dee, P. C., and Habener, J. F.: Anglerfish islet pre-proglucagon II: nucleotide and corresponding amino acid sequence of the cDNA. J. Biol. Chem. 1983; 258:3280–84.

[7] Hobart, P., Crawford, R., Shen, L.-P., Pictet, R., and Rutter, W. J.: Cloning and sequence analysis of cDNAs encoding two distinct somatostatin precursors found in the endocrine pancreas of anglerfish. Nature 1980; 288:137–41.

[8] Boel, E., Schwartz, T. W., Norris, K. E., and Fiil, N. P.: A cDNA encoding a small common precursor for human pancreatic polypeptide and pancreatic icosapeptide. EMBO J. 1984; 3:909–12.

[9] Leiter, A. B., Keutmann, H. T., and Goodman, R. H.: Structure of a precursor to human pancreatic polypeptide. J. Biol. Chem. 1984; 259:14702–705.

[10] Takeuchi, T., and Yamada, T.: Isolation of a cDNA clone encoding pancreatic polypeptide. Proc. Natl. Acad. Sci. USA 1985; 82:1536–39.

[11] Andrews, P. C., Hawke, D., Shively, J. E., and Dixon, J. E.: A non-amidated peptide homologous to porcine peptide YY and neuropeptide YY. Endocrinology 1985; 116:2677–81.

[12] Andrews, P. C., Hawke, D. H., Lee, T. D., Legesse, K., Noe, B. D., and Shively, J. E.: Isolation and structure of the principal products of proglucagon processing, including an amidated glucagon-like peptide. J. Biol. Chem. In press.

[13] Eipper, B. A., Mains, R. E., and Glembotski, C. C.: Identification in pituitary tissue of a peptide α-amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. Proc. Natl. Acad. Sci. USA 1983; 80:5144–48.

[14] Tirrell, J. G., and Westhead, E. W.: The uptake of ascorbic acid and dehydroascorbic acid by chromaffin granules of the adrenal medulla. Neuroscience 1979; 4:181–86.

[15] Levine, M., Morita, K., Heldman, E., and Pollard, H. B.: Ascorbic acid regulation of norepinephrine biosynthesis in isolated chromaffin granules from bovine adrenal medulla. J. Biol. Chem. 1985; 260:15598–603.

[16] Apps, D. K., Pryde, J. G., and Phillips, J. H.: Cytochrome b_{561} is identical with chromomembrin B, a major polypeptide of chromaffin granule membranes. Neuroscience 1980; 5:2279–87.

[17] Silsand, T., and Flatmark, T.: Purification of cytochrome b_{561}: an integral heme protein of the adrenal chromaffin granule membrane. Biochim. Biophys. Acta 1974; 359:257–66.

[18] Duong, L. T., Fleming, P. J., and Russell, J. T.: An identical cytochrome b_{561} is present in bovine adrenal chromaffin vesicles and posterior pituitary neurosecretory vesicles. J. Biol. Chem. 1984; 259:4885–89.

[19] Noe, B. D., Baste, C. A., and Bauer, G. E.: Studies on proinsulin and proglucagon biosynthesis and conversion at the subcellular level. I. Fractionation procedure and characterization of the subcellular fractions. J. Cell Biol. 1977; 74:578–88.

[20] Fletcher, D. J., Quigley, J. P., Bauer, G. E., and Noe, B. D.: Characterization of proinsulin- and proglucagon-converting activities in isolated islet secretory granules. J. Cell Biol. 1981; 90:312–22.

[21] Fletcher, D. J., Noe, B. D., Bauer, G. E., and Quigley, J. P.: Characterization of the conversion of a somatostatin precursor to somatostatin by islet secretory granules. Diabetes 1980; 29:593–99.

[22] Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72:248–54.

[23] Jones, D. P., Orrenius, S., and Mason, H. S.: Hemoprotein quantitation in isolated hepatocytes. Biochim. Biophys. Acta 1979; 576:17–29.

[24] Rieske, J. S.: The quantitative determination of mitochondrial hemoproteins. Methods Enzymol. 1967; 10:488–93.

[25] Antonini, E., and Brunori, M.: Hemoglobin and Myoglobin in Their Reactions With Ligands. New York, North-Holland, 1971:45. (Frontiers in Biology, Vol. 21.)

[26] Hayaishi, O.: Properties and function of indoleamine 2,3-dioxygenase. J. Biochem. 1976; 79:13–21p.

[27] Omura, T., and Sato, R.: The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 1964; 239:2370–78.

[28] Duong, L. T., and Fleming, P. J.: Isolation and properties of cytochrome b_{561} from bovine adrenal chromaffin granules. J. Biol. Chem. 1982; 257:8561–64.

[29] Njus, D., Zallakian, M., and Knoth, J.: The chromaffin granule: proton-cyling in the slow lane. *In* Chemiosmotic Proton Circuits in Biological Membranes. Skulachev, V. P., and Hinkle, P. C., Eds. Reading, PA, Addison-Wesley, 1981:365–74.

[30] Njus, D., Knoth, J., Cook, C., and Kelley, P. M.: Electron transfer across the chromaffin granule membrane. J. Biol. Chem. 1983; 258:27–30.

[31] Kimmel, J. R., Hayden, L. J., and Pollock, H. G.: Isolation and characterization of a new pancreatic polypeptide hormone. J. Biol. Chem. 1975; 250:9369–76.

[32] Paquette, T. L., Gingerich, R., and Scharp, D.: Altered amidation of *pancreatic polypeptide in cultured dog islet tissue*. Biochemistry 1981; 20:7403–408.

[33] Johnson, D. E., Torrence, J. L., Elde, R. P., Bauer, G. E., Noe, B. D., and Fletcher, D. J.: Immunohistochemical localization of somatostatin, insulin and glucagon in the principal islets of the anglerfish (*Lophius americanus*) and the channel catfish (*Ictalurus punctata*). Am. J. Anat. 1976; 147:119–24.

[34] Johnson, D. E., Noe, B. D., and Bauer, G. E.: Pancreatic polypeptide (PP)-like immunoreactivity in the pancreatic islets of the anglerfish (*Lophius americanus*) and the channel catfish (*Ictalurus punctatus*). Anat. Rec. 1982; 204:61–67.