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Synthesis, intracellular distribution, and secretion of multiple forms of parathyroid secretory protein-I

(parathyroid hormone/membranes/precursor peptide/glycoprotein secretory mechanisms)

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ABSTRACT **Examination of whole cell extracts and subcellular fractions of dispersed porcine parathyroid cells incubated with [^{35}S]methionine indicates that two species of secretory protein-I, 72,000 and 64,000 daltons, respectively, are synthesized. Two secretory protein-I species of molecular weights equivalent to those in the cell but with slightly different isoelectric points were secreted; calcium suppressed the secretion of both of these. The secretory protein-I of cell and medium were shown to be related to each other and to previously identified secreted secretory protein-I by comparison of their ^{35}S-labeled tryptic peptides and location of methionine in positions 7, 15, and 32 of the peptide chains. Both of the cellular species appeared to be enclosed within membranes similar to those containing parathyroid hormone and its immediate biosynthetic precursor because they were associated with the membrane fraction of the cell, were not digested when the membranes were exposed to trypsin, and were extracted from these membranes, as were parathyroid hormone and proparathyroid hormone, with dilute sodium deoxycholate. We did not find an amino-terminal precursor form of secretory protein-I in an incubation as short as 2 min with [^{35}S]methionine, whereas [^{35}S]proparathyroid hormone was readily detected, indicating that processing of secretory protein-I involves a direct conversion of the pre-protein to the secretory protein-I. Posttranslational glycosylation or deletion of carboxy-terminal region of the secretory protein-I species might account for the differences in molecular weights and isoelectric points of the cellular and secreted forms.**

For many years studies on parathyroid gland function focused almost exclusively on parathyroid hormone (PTH), the principal calcium-regulating hormone of higher animal species, and on its immediate biosynthetic precursor, proPTH (1–3). To achieve better understanding of glandular function, recent attention has been directed at other glandular constituents that could be related to PTH metabolism and secretion. One such substance is secretory protein-I (SP-I) (4, 5), a glycosylated protein (6) with a monomer molecular weight of about 70,000 (4). It is rapidly synthesized in the parathyroid and its secretion, like that of PTH, is inhibited by calcium. It has been shown to be associated with and contained within membranous structures of the gland, as is PTH (4–6). Ravazzola *et al.* (7), demonstrated with fluorescent antibodies that SP-I can be located on or in secretory vesicles that contain PTH.

We showed that two different pools of PTH (8–10) and SP-I (9, 10) exist in the parathyroid, one containing "new" SP-I and PTH (those molecules synthesized no more than 20–90 min previously) and the other containing "mature" SP-I and PTH (those molecules synthesized more than 90 min previously). Hypocalcemia, the accepted physiological stimulus for PTH secretion, elicited secretion of SP-I and hormone from both pools whereas β-agonists elicited secretion from only the mature pool. These results support the concept that SP-I and PTH have similar processing and secretory routes. On the other hand, some data indicate that the secretion of SP-I and PTH may be dissociable. For example, when calcium concentration is increased, the secretion of newly formed and total SP-I is inhibited substantially more than that of PTH (5). Clearly, more information concerning the chemistry and processing of SP-I will be required if a functional role of this protein is to be discerned and before a case can be made for its involvement in the secretion or processing of PTH.

Here we report that SP-I exists within the cell in two membrane-associated forms and that the secreted protein likewise consists of two related species.

MATERIALS AND METHODS

Porcine parathyroid cells were prepared by the collagenase/DNase I procedure of Brown *et al.* (11) as modified (5, 6). Unless otherwise specified, the cells were incubated at a density of approximately 5×10^5 cells per ml in Krebs–Ringer supplemented buffer (5, 6) at 37°C containing 1 mM $MgSO_4$ and 0.5 mM $CaCl_2$. After 1 hr of acclimation, they were concentrated by gentle centrifugation and resuspended at a density of 1×10^6 cells per ml in incubation medium containing 0.25 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine per ml and incubated at 37°C for an additional 2 to 60 min. At the end of the incubation, cells and medium were separated by centrifugation.

In some experiments the radioactive proteins contained in cells and media were prepared directly by precipitation with trichloroacetic acid (5). In other experiments the cell pellet was homogenized with 10 strokes of a glass/Teflon homogenizer in 10 vol of 0.25 M sucrose/0.025 M Tris·HCl pH 7.4/0.025 M KCl/1 mM Mg acetate/1 mM methanesulfonyl fluoride. The homogenate was centrifuged at 111,000 × *g* for 45 min. The supernatant, termed "cytosol", was saved. The pellet was resuspended in 1 ml of fresh homogenizing buffer with the aid of a glass/Teflon homogenizer and recentrifuged at 111,000 × *g* for 45 min. This pellet contained the bulk of the cellular membranes and other postmitochondrial particulate and was termed "membranes". This fraction was dissolved in either 6 M urea/0.5 M acetic acid or the $NaDodSO_4$ sample buffer of Laemmli (12). Portions of the cytosol or the incubation medium were mixed with either the 6 M urea/0.5 M acetic acid or Na-

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Abbreviations: PTH, parathyroid hormone; SP-I, secretory protein-I.

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$DodSO_4$ sample buffer (20% sample/80% buffer). In some instances, the membranes were resuspended in the homogenizing buffer to a concentration of 2.5 mg of protein [Lowry *et al.* (13)] per ml. Portions of the suspension were treated with 0.01–0.2% sodium deoxycholate and centrifuged at 111,000 × *g* for 45 min. The supernatant fluid was retained for analysis. In other experiments, the membranes, before or after treatment with deoxycholate, were incubated with trypsin as described in *Results*.

Separation of radioactive proteins was accomplished in the system of Reisfeld *et al.* (14) as modified (5, 6) on 1.5-mm-thick slab gels with an acid/urea system or in the $NaDodSO_4$ system of Laemmli (12) on 1.5-mm-thick 7.5% acrylamide slab gels. The gels from either system were stained with Coomassie blue R-250 and destained. The stained acid/urea gels were sectioned into 1-mm segments and analyzed for the presence of radioactivity by liquid scintillation spectrometry; the stained $NaDodSO_4$ gels were dried and autoradiographed.

When indicated, the unstained gels were cut into 1-mm segments and the radioactive proteins were eluted with 0.05 M ammonium acetate, pH 7.0/0.1% $NaDodSO_4$/1 mM methanesulfonyl fluoride. The proteins were precipitated from the eluate by the addition of 5 vol of 0.1 M HCl/acetone at 4°C. The precipitates were washed with acidified acetone and lyophilized. Portions of the eluted radioactive proteins were subjected to sequence analysis (15) by Edman degradation and to isoelectric focusing (16). Another portion was subjected to trypsin digestion (TPCK-treated, Worthington), 10 μg/ml in 0.05 M ammonium carbonate (pH 8.5). The tryptic digests were lyophilized and the radioactive peptides were separated by ion exchange chromatography as described (17).

RESULTS

The synthesis and secretion of radioactive SP-I, PTH, and proPTH were studied initially by separating the proteins on acid/urea gels. Fig. 1 *Left* shows a gel pattern derived from a cell sample taken after 2 min of incubation with [^{35}S]methionine; Fig. 1 *Right* represents a portion of the medium after 60-min incubation of these same cells. The cell profile contained radioactive proPTH but not PTH because 2 min of incubation is insufficient for the latter to be formed (1). The profile of the medium differed from that of the cell in containing PTH and not proPTH because the latter is not secreted (9). In both patterns the major peak appeared to be SP-I. This assignment was based on the reported migration of SP-I on acid/urea gels (4–6).

In order to confirm the identification of the [^{35}S]methionine-labeled SP-I, it and the proPTH and PTH bands were eluted from gels described in Fig. 1 and subjected to 30 cycles of Edman degradation (Fig. 2). SP-I of the medium contained [^{35}S]methionine at cycles 7 and 15, in agreement with the reported position of these residues in the secreted protein (18). Cellular SP-I also contained methionine at positions 7 and 15, indicating that the cellular and secreted forms were identical at their amino termini. There was no evidence in the gels of a precursor form of SP-I containing an amino-terminal extension (that is, a proSP-I analogous to proPTH). As anticipated (15), [^{35}S]methionine was found at cycle 14 for proPTH and cycle 8 for secreted PTH.

When these samples or others from more extended incubations were examined by $NaDodSO_4$ gel electrophoresis, the patterns of radioactivity in the region expected to contain SP-I [about 70,000 daltons (4–6)] were more complex than they were on the acid/urea gels (Fig. 3). The medium exhibited two major bands in this region of the gel, one of 64,000 daltons and one of 72,000 daltons. The whole cell extract exhibited two bands of equivalent size. In addition, the cells contained a highly radioactive band of about 68,000 daltons. When the cell extracts were separated into membranes and cytosol, the 72,000- and 64,000-dalton bands were found predominantly in the membranes and the 68,000-dalton band, in the cytosol. The radioactivity in these bands of the cell and cell fractions relative to each other as shown in Fig. 3 is representative of several experiments. In all cases, the 72,000-dalton band was always more radioactive than the 64,000-dalton band. In the medium, however, the relative radioactivities of the 72,000- and 64,000-dalton bands varied substantially so that in some experiments the 72,000-dalton band was the predominant species, the reverse of that shown for medium in Fig. 3.

The 72,000- and 64,000-dalton proteins of cell and medium were chemically related as judged by the similarity of their [^{35}S]methionine-labeled tryptic peptides (Fig. 4). The 68,000-dalton protein appeared to be unrelated to the other two. That the 72,000- and 64,000-dalton proteins of the cell and medium were species of SP-I was established by radioactive micro-sequence-analysis. Methionine was located at positions 7 and 15 in each of the 72,000- and 64,000-dalton proteins of

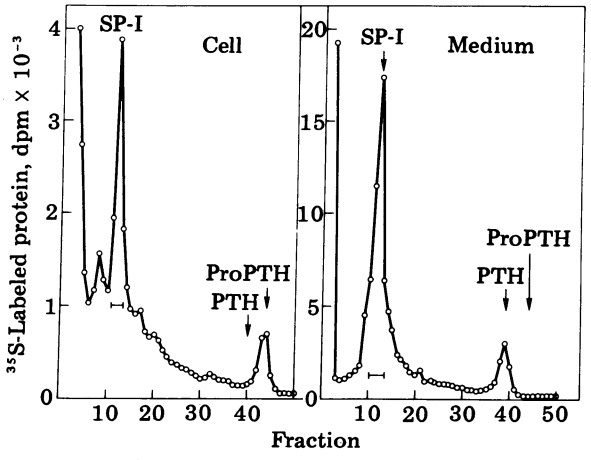

FIG. 1. Acid/urea polyacrylamide gel of whole parathyroid cell extract incubated with [^{35}S]methionine for 2 min (*Left*) or medium incubated for 60 min (*Right*). The horizontal marker under the SP-I peaks indicates the samples used for sequence analysis.

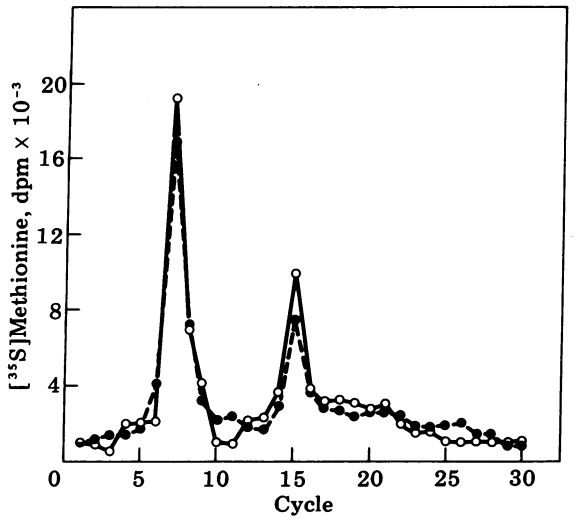

FIG. 2. Sequence analysis (micro method) of SP-I extracted from the gels of Fig. 1 (300,000 dpm of each applied). ○, From media; ●, from cells.

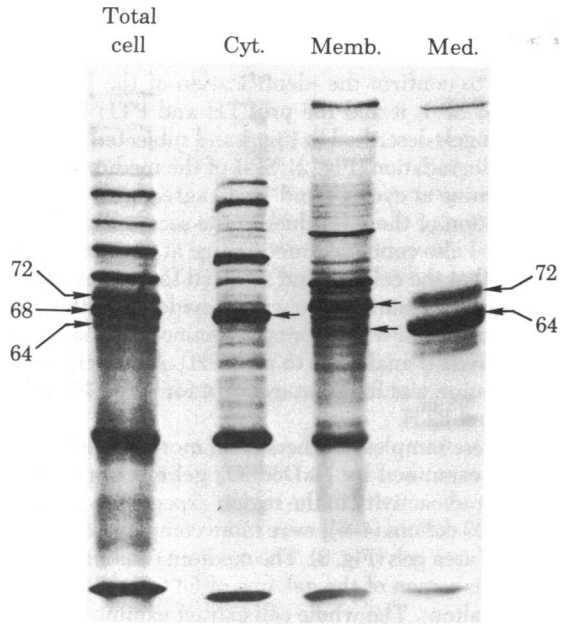

FIG. 3. Autoradiogram of 7.5% NaDodSO$_4$/polyacrylamide gel of total parathyroid cell extract, cytosol (Cyt.), membranes (Memb.), and incubation medium (Med.) from cells incubated with [^{35}S]methionine for 30 min. The figure is a composite. The track from total cells was exposed for 24 hr; the membrane, cytosol, and medium tracks were exposed for 72 hr. Because this gel does not resolve proteins smaller than 25,000 daltons, PTH and proPTH are not identified. Sizes are shown in kilodaltons.

the membranes and medium (data not shown). In addition, in an extended analysis (Fig. 5), methionine was also found in position 32 of the 72,000-dalton membrane protein. In contrast, no discrete peak of methionine was detected in the cytosolic 68,000-dalton protein through 34 cycles of Edman degradation.

Examination of the radioactive proteins present in the separated membranes and cytosol on acid/urea gels is shown in Fig. 6. In the membranes, there was a single asymmetric peak in the SP-I region of the gel that presumably contained the 72,000- and 64,000-dalton SP-Is. Because, in contrast to that in Fig. 1 *Left*, the cell sample for this figure had been from a sufficiently long incubation for radioactive PTH to be formed from proPTH (1–3), both of these proteins were also present. In the cytosol, the major radioactive protein (presumably the 68,000-dalton species) migrated slightly more slowly than did the SP-I of the membranes. Neither proPTH nor PTH was detected, establishing that the cytosolic fraction was not cross contaminated by membrane components. From this analysis it is apparent that the radioactive band previously separated on acid/urea gels from extracts of whole cells and referred to as SP-I (Fig. 1 *Left*) was a combination of two SP-I species and the cytosolic 68,000-dalton protein. The latter was not detected by radioactive sequence analysis because methionine, the radioactive amino acid used to label the protein, was not present in its amino-terminal region (or possibly because the amino terminus was blocked).

The isoelectric points of the cellular and secreted SP-Is and of the cytosolic protein were determined by isoelectric focusing to be: SP-I membrane, 72,000- and 64,000-dalton species, 4.7 and 4.4, respectively; both secreted SP-Is, 4.5; cytosol protein, 5.2. The closeness of isoelectric points of the cytosolic protein and the SP-Is accounts in part for their similar migration on acid/urea gels.

The 72,000- and 64,000-dalton species of the medium are

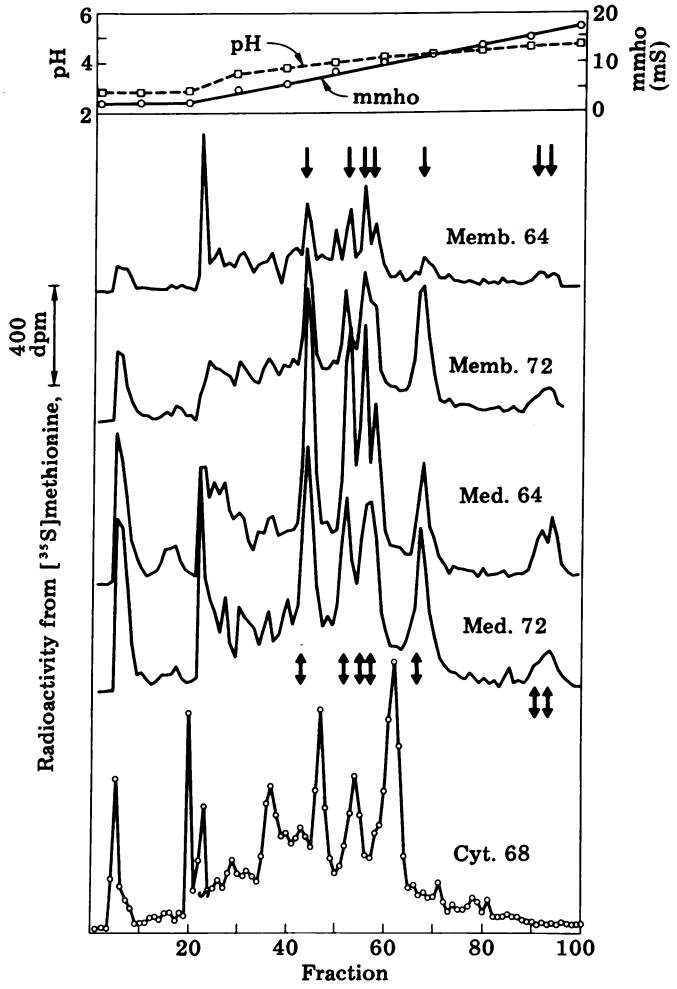

FIG. 4. Sulfopropyl-Sephadex chromatography of [^{35}S]methionine-labeled tryptic peptides. Arrows refer to the elution of peptides common to the membrane and medium proteins (see Fig. 3) and indicate the dissimilarity of these to the 68,000-dalton protein of the cytosol.

thought to be related to glandular function because both species were secreted in reduced amounts when the cells were incubated in medium containing a high calcium concentration (3.0 mM) (data not shown). In order to rule out the possibility that the 64,000-dalton SP-I was derived from the 72,000 dalton species by proteolytic conversion after secretion, the medium and cells were separated after a 40-min incubation and the medium was allowed to incubate for an additional 40 min. There was little change in amount or ratio of the two secreted proteins (data not shown).

An accepted procedure for deciding if a protein is contained within membranes or vesicles is its extractability with dilute detergent and resistance to digestion by proteolytic enzymes unless the membranes are first treated with detergent (18). According to these criteria, both of the cellular SP-Is appear to be contained within membranes. Fig. 7 compares the extraction from the membranes and identification on acid/urea gels of SP-I, proPTH, and PTH. Each protein was extracted completely by 0.2% sodium deoxycholate (with no detectable radioactive protein in the residue) and was about 50% extracted between 0.05% and 0.1%. At the lower concentrations of detergent, the SP-I was solubilized to a somewhat greater degree than was either PTH or proPTH. When the membranes were incubated with trypsin at 10 μg/ml for 30 min on ice and examined on acid/urea gels, the bulk of the SP-I, PTH, and

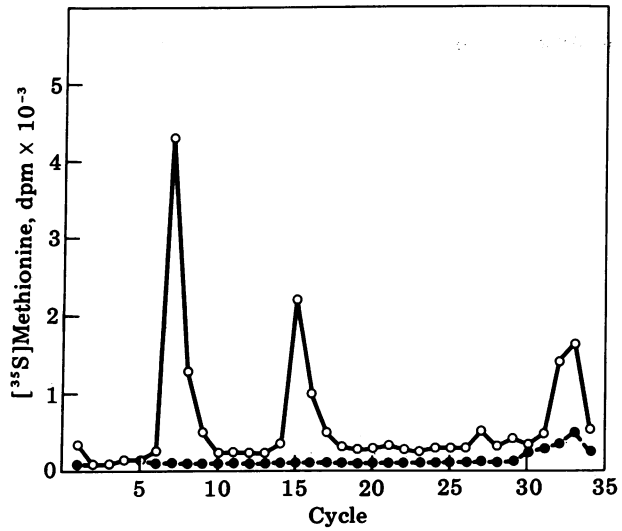

FIG. 5. Micro-sequence-analysis of 72,000-dalton membrane SP-I (○) and 68,000-dalton cytosol protein (●), both labeled with [35S]-methionine (60,000 dpm of membrane protein and 120,000 dpm of cytosol protein were analyzed).

proPTH resisted digestion unless the membranes were first treated with 0.2% deoxycholate: trypsin resistant without deoxycholate, 85%; with deoxycholate, <10%.

DISCUSSION

Our results establish that two species of SP-I, differing slightly in molecular weight and charge, are rapidly synthesized by the porcine parathyroid cells. Based on their association with the membrane fraction of the cell, their resistance to digestion by trypsin, and their solubilization by deoxycholate relative to PTH and proPTH, they appear to be enclosed within membrane structures equivalent if not identical to those containing hormonal proteins. In addition, two species of SP-I are secreted which have molecular weights equivalent to those in the cell. These four proteins are related chemically as judged by the similarity of methionine-containing tryptic peptides and by the identical locations of methionine residues within their amino-terminal regions.

The different molecular weights of the proteins could be due to varying lengths of the protein chain at the carboxyl end of the molecules or to different degrees of glycosylation as a result of posttranslational processing. Indeed, preliminary results from our laboratory indicate that residues including sulfate and ga-

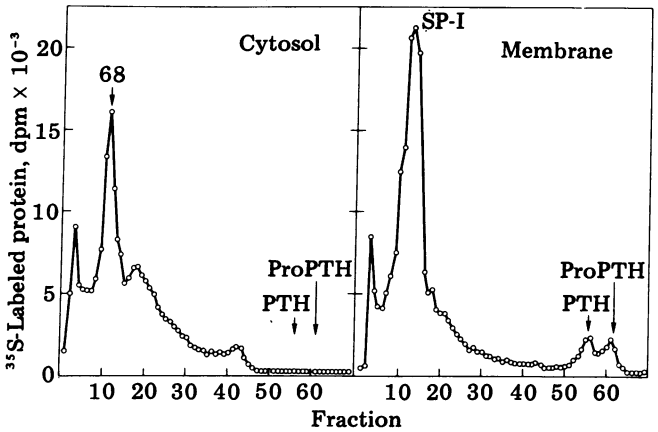

FIG. 6. Acid/urea polyacrylamide gel of [35S]methionine-labeled parathyroid cytosol and membrane proteins of Fig. 3.

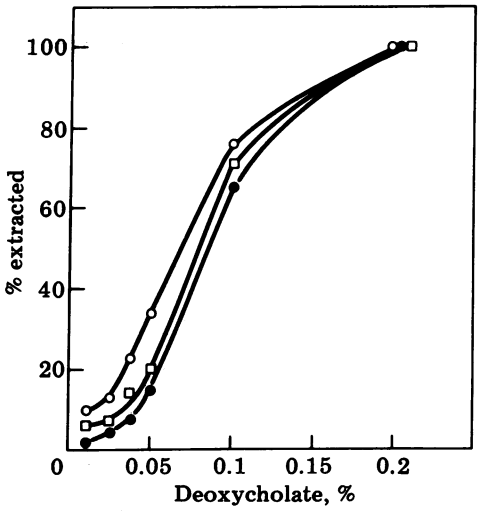

FIG. 7. Deoxycholate extraction of [35S]methionine-labeled SP-I (○), proPTH (●), and PTH (□) from parathyroid membranes.

lactose are added to SP-I during posttranslational processing and that glucosamine is added to SP-I during or just before its secretion from either the new or mature cellular pools.‡ With this in mind, and because of the slight differences in isoelectric points of the species, we think that the similarities in molecular size of the two pairs of cellular and secreted SP-I species are in part fortuitous and that these molecules will be found to differ somewhat in chemical composition and structure.

The existence of two cellular forms of SP-I is consistent with the report that, in cell-free reticulocyte and wheat germ lysate translational systems, multiple forms of "preSP-I" distinguishable by their different isoelectric points were generated (19). Sequences of the products of translation of the reticulocyte lysate system indicated that the amino-terminal region of preSP-I differed slightly from that of SP-I in having an 18-amino acid extension preceding the first residue of SP-I (19). The present work suggests that this entire amino-terminal extension is removed during processing, unlike the situation with preproPTH, because in those experiments in which the cells were incubated for brief periods (e.g., Figs. 1 and 2), we found no evidence of a "pro" form of SP-I although proPTH was readily detected.

The origin and significance of the multiple membrane-associated forms of SP-I remain to be established. It is possible that one form may be generated from the other by posttranslational glycosylation or protein deletion. Because PTH in both new and mature cellular pools is believed to be chemically identical (10), the distinguishing features of these pools could relate to the chemistry of the membranes enclosing the hormone or to the substances associated with the PTH within the membranes. In either case, the SP-I variants could provide the chemical signals that confer "newness" or "maturity" to the intracellular PTH pools. This interpretation requires that the PTH and SP-I coexist in the same structure or cellular environments. The finding by Ravazzola *et al.* (7) with fluorescent antibodies indicates that these proteins do in fact have similar subcellular structures—at least that portion of the PTH and SP-I that has been discharged from the Golgi. For other portions of the proteins (e.g., those still within the Golgi), evidence is lacking. Indeed, our data on extraction of SP-I, PTH, and proPTH with sodium deoxycholate (Fig. 7) show that the first species is more readily extracted at

‡ Morrissey, J. J., Hamilton, J. W. & Cohn, D. V. (1980) *Program Abst. Endocrine Society 62nd Annual Meeting*, June 18–20, 1980, Washington, DC, p. 187.

the lower concentrations of detergent. This could mean that these proteins are contained in different membrane structures but, admittedly, it might only be reflecting inherent differences in solubility of these proteins in the detergent.

A second possibility to account for multiple forms of SP-I is that the species are cosynthesized and comprise slightly different subunits of a native molecule, in accord with the report that SP-I consists of two or more 70,000-dalton subunits (4). This possibility seems less likely, however, because the ratio of radioactivity in the two secreted species varied relative to each other from experiment to experiment—and one would expect subunits to be synthesized and secreted in a fixed ratio.

A third possibility is that the two SP-I species represent slightly different gene products of two parathyroid cell types within a single gland or within the different parathyroid glands that are pooled to make a dispersed cell preparation. Indeed, isohormones of PTH that differ by one amino acid have been isolated from pools of parathyroid glands (20). Clarification of these points will aid in correlating the metabolism of SP-I with PTH synthesis and secretion.

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