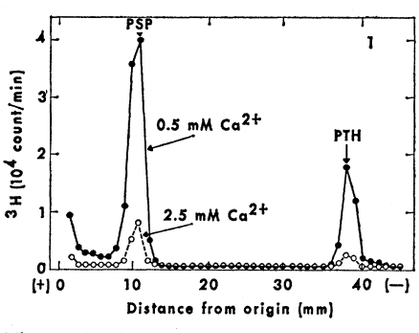
Parathyroid Secretion: Discovery of a Major Calcium-Dependent Protein

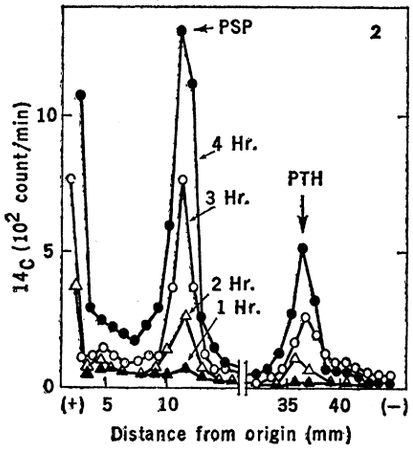
Abstract. *Bovine parathyroid tissue incubated in vitro secretes a protein that is distinct from both parathyroid hormone and proparathyroid hormone and comprises about 50 percent of the total secreted protein. This protein appears to be an aggregate consisting of two or more subunits of molecular weight 70,000 as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Although the function of this protein is unknown, the secretion rates of both the protein and parathyroid hormone respond in parallel to changes in the concentration of calcium in the medium.*

The intracellular compartmentalization within secretory or zymogen granules of proteins destined for secretion appears to be a common cellular mechanism (*1*). In addition to the recognized secretory product, other proteins involved in the secretory mechanism are also present in the granules and are secreted. During norepinephrine secretion, dopamine-β-hydroxylase and chromogranins as well as adenosine triphosphate are also secreted (*2*); the inactive connecting peptide of the insulin precursor is secreted in equimolar amounts with insulin (*3*); and the neurophysins, proposed carrier proteins, are secreted with oxytocin and vasopressin (*4*). In studies of bovine parathyroid slices in vitro, we noted that parathyroid hormone (PTH) accounted for only 20 percent of the protein secreted into the medium. We now report that parathyroid tissue secretes another major protein, which comprises about 50 percent of the total radioactive protein released. Secretion of this protein is inhibited in parallel with that of PTH by increasing concentrations of calcium in the incubation medium.

Calf parathyroid glands from freshly killed animals were dissected free of adjacent fat and connective tissue. Slices of the glands were incubated at 37°C with either ^{14}C-labeled amino acids or [^{3}H]leucine in Hanks balanced salt solution or minimum essential medium (*5*). After incubation, proteins in the medium were analyzed by polyacrylamide gel electrophoresis (*5*), or by gel filtration through Sephadex G200.

Two major peaks of radioactivity (Fig. 1) were observed after gel electrophoresis of proteins secreted by bovine parathyroid slices during 4 hours of incubation with [^{3}H]leucine. One peak comigrates with PTH and contains about 20 percent of the radioactivity on the gel. The other peak, parathyroid secretory protein (PSP), migrates more slowly and contains about 50 percent of the radioactivity. The labeled medium was analyzed electrophoretically on several polyacrylamide gel systems: (i) at *p*H 4 (*5*); (ii) at *p*H 4 with 8*M* urea (Fig. 1); (iii) at *p*H 9 (*6*); (iv) at *p*H 9 with 8*M* urea; (v) at *p*H 7 with sodium dodecyl sulfate and 8*M* urea (*5*); and (vi) at *p*H 9 with sodium dodecyl sulfate (*7*). In each case, a single major peak contained about 50 percent of the radioactivity. By these criteria, PSP is a homogeneous protein.





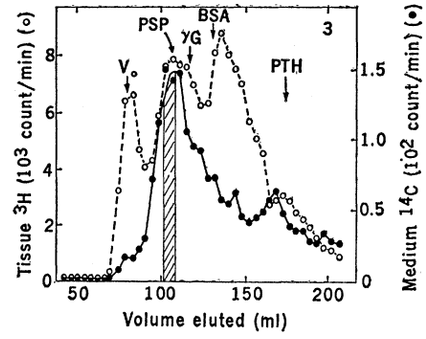


Fig. 1. Analysis by polyacrylamide gel electrophoresis of medium from an incubation of parathyroid slices with [^{3}H]leucine. Calf parathyroid glands, obtained from freshly killed animals at a local abattoir, were sliced and incubated (*5*) at 37°C for 4 hours in leucine-free minimum essential medium (Grand Island Biological) containing 0.5 or 2.5 m*M* calcium chloride, [^{3}H]leucine (12.5 μc/ml) (New England Nuclear, 33 c/mmole), and 5 percent fetal bovine serum. After incubation the medium was removed, two volumes of 10 percent trichloroacetic acid were added, and the precipitate was collected by centrifugation at 10,000*g* for 10 minutes. To remove free [^{3}H]leucine the pellet was twice dissolved in 0.1*N* sodium hydroxide, reprecipitated with 10 percent trichloroacetic acid, and collected by centrifugation at 10,000*g*. The final pellet was resuspended in 2 ml of distilled water and lyophilized to remove acid; the powder was analyzed by polyacrylamide gel electrophoresis at *p*H 4.4 as described (*5*). Fig. 2. Secretion of radioactive protein by parathyroid slices as a function of time. Parathyroid slices were incubated in Hanks balanced salt solution (Grand Island Biological) containing 0.5 m*M* calcium chloride, ^{14}C-labeled amino acids (2 μc/ml) (New England Nuclear), and 5 percent fetal bovine serum. The medium was analyzed as in Fig. 1. Portions of the polyacrylamide gel containing PSP and PTH are shown superimposed for incubations of 1 to 4 hours. Fig. 3. Gel filtration through Sephadex G200 of incubation medium and a parathyroid tissue extract. An extract of parathyroid slices incubated with [^{3}H]leucine for 4 hours and medium from a 4-hour incubation of parathyroid slices with ^{14}C-labeled amino acids were analyzed by gel filtration through a 2.5 by 40 cm column of Sephadex G200. The extract was prepared by homogenizing about 200 mg (wet weight) of parathyroid slices in a Waring blender in 4 ml of the column eluant buffer [0.1*M* tris(hydroxymethyl)aminomethane (*p*H 7.5), 0.1*M* potassium chloride, 0.01*M* magnesium chloride, and 1 percent fetal calf serum]. The homogenate was centrifuged at 10,000*g* for 10 minutes. The supernatant was combined with 0.5 ml of medium and applied to the column, and 4-ml fractions were collected. Portions (0.2 ml) of the column fractions and 200 μg of bovine serum albumin were added to 1 ml of 10 percent trichloroacetic acid, and precipitates were collected on glass fiber filter pads and assayed for radioactivity by liquid scintillation counting. The elution volume of the marker proteins—PTH, bovine serum albumin (BSA), and rabbit gamma globulin (γG)—were determined in separate analysis on the same column. The ratio of PTH to PSP eluted from the gel filtration column appears to be lower than that seen upon polyacrylamide gel electrophoresis because of adsorptive losses of PTH during filtration (*20*).

When calcium chloride in the medium was decreased from 2.5 m*M* (or higher) to 0.5 m*M* [a procedure that stimulates PTH secretion (*8*)], secretion of both PTH and PSP increased about fivefold (Fig. 1). This calcium effect on secretion of both proteins was invariably observed in several experiments. Secretion of both proteins also responded in parallel to graded changes in calcium concentration from 0.5 to 5.0 m*M* (which includes the physiological range) despite four- to fivefold changes in secretion rates (Table 1).

The regulation of secretion of both PTH and PSP by calcium suggests that both are present in parathyroid secretory granules and are cosecreted. Analysis of the kinetics of the appearance of PTH and PSP in the medium supported this hypothesis. No detectable radioactive PTH or PSP was secreted during the first hour; secretion of both proteins increased in parallel in hours 2 to 4 (Fig. 2). Furthermore, secretion of the two proteins was inhibited to the same extent by increasing magnesium chloride from 0.8 to 5.0 m*M* or by adding 10^{-4}*M* vinblastine sulfate. [^{3}H]Threonine was readily incorporated into PSP (not shown) but not into PTH (which does not contain threonine); this indicates that PSP is not simply an aggregate of PTH. Although PSP might be secreted independently of PTH, perhaps by a second cell type in the parathyroid gland, these data strongly suggest that PSP is cosecreted with PTH.

A molecular weight of 70,000 was determined for PSP by electrophoresis on discontinuous polyacrylamide gels containing sodium dodecyl sulfate (*7*). However, PSP eluted before rabbit gamma globulin from a column of Sephadex G200, which indicates a molecular weight greater than 150,000 (Fig. 3). In polyacrylamide gel electrophoresis, the mobility of PSP relative to bovine serum albumin was 0.45 at *p*H 9 (*6*) and increased to 1.2 in 8*M* urea. These observations suggest that PSP is composed of two or more subunits which are dissociated in denaturing solutions. The ratio of the incorporation of either [^{3}H]leucine or ^{14}C-labeled amino acids into PTH compared to PSP is about 0.4. Since the molecular weight of PTH is 9600, about three molecules of PTH are secreted for each subunit of PSP.

We next demonstrated the presence of PSP in parathyroid tissue. An extract prepared from tissue incubated with [^{3}H]leucine for 4 hours and medium from a similar incubation of tissue with ^{14}C-labeled amino acids were combined and examined by gel filtration (Fig. 3). A ^{3}H-labeled protein coeluted with ^{14}C-labeled PSP from the medium. When fractions comprising the PSP peak from the Sephadex G200 column (cross-hatching in Fig. 3) were pooled and analyzed by polyacrylamide gel electrophoresis, most of the ^{3}H had the same mobility as PSP from the medium. Similar analyses of other column fractions revealed several other ^{3}H peaks, but none corresponded to PSP. Thus, PSP is synthesized intracellularly and apparently is not modified substantially during secretion.

Table 1. Effect of calcium on secretion of PTH and PSP by parathyroid tissue. Slices of parathyroid tissue were incubated with [^{3}H]leucine as described for Fig. 1, with various concentrations of calcium chloride in the medium. The medium was analyzed as in Fig. 1 and the radioactivity in the peaks of PSP and PTH was determined. A second experiment gave similar results.

Calcium (m*M*)	Radioactivity (10^3 count/min per milligram of protein)		Ratio, PTH/PSP
	PTH	PSP	
0.5	1050	2670	0.41
1.0	995	2580	.39
1.5	822	2120	.39
2.0	245	599	.42
2.5	175	421	.42
5.0	210	525	.41

In previous gel filtration analyses of radioactive proteins secreted by rat or bovine parathyroid tissue in vitro, some radioactivity was associated with proteins that were larger than PTH and eluted in the void volume (*9*); increasing the calcium concentration in the medium inhibited secretion of both PTH and the void volume proteins. Since PSP accounts for most of the protein larger than PTH secreted by parathyroid tissue, most of this void volume protein was probably PSP. Other proteins are probably also secreted in lesser amounts—for example, we observed two peaks of [^{3}H]threonine-labeled proteins migrating near PTH and also some proteins migrating slower than PSP. Also, Licata *et al.* (*10*) have shown that after a 48-hour incubation of rat parathyroids in vitro with [^{3}H]glucosamine, radioactivity is found in at least three proteins in the medium, the largest amount in a protein slightly smaller than PTH, but some in several larger proteins. Although our initial studies indicate that glucosamine may be incorporated into PSP, glucosamine-containing proteins are only a small percentage of proteins secreted by rat parathyroids while 50 percent of the protein secreted by the bovine gland is PSP. Thus, the relation of the glucosamine-containing proteins secreted by the rat parathyroids to PSP is uncertain.

There are several possible functions of PSP. (i) It might bind PTH and facilitate its transport in the cell as proposed for neurophysin in vasopressin and oxytocin transport (*4*). (ii) PSP might be an enzyme responsible for posttranscriptional modification of PTH, for example, conversion of proparathyroid hormone to PTH. (iii) PSP might be related to a high molecular weight form of PTH like those of other polypeptide hormones such as gastrin (*11*), adrenocorticotrophic hormone (*12*), insulin (*13*), and growth hormone (*14*). (iv) It might bind calcium (*15*). (v) PSP might be a structural protein related to the secretory granule and as such might not be solely confined to the parathyroid gland.

Our studies thus far do not support any of these possibilities. We found no PTH when the PSP peak in Fig. 3 was analyzed by polyacrylamide gel electrophoresis in 8*M* urea. No binding of ^{125}I-labeled PTH or proparathyroid hormone to partially purified PSP at 4° or 25°C was detected when PSP was separated by gel filtration or dialysis with dextran-coated charcoal (*16*), but low-affinity binding would not be detected under the conditions used. No conversion of ^{14}C-labeled proparathyroid hormone to PTH was observed after incubation at 37°C for 2 hours with partially purified PSP. Two sensitive antiserums against PTH did not react with PSP, and PSP cannot be proparathyroid hormone, which has a molecular weight of 12,000 (*5, 17*) or less (*18*). No calcium binding was observed by gel filtration on Sephadex G200 after incubation of [^{45}Ca]calcium chloride with a tissue extract containing PSP. Therefore, we have no evidence about PSP function, or even about whether PSP is secreted in vivo. However, the control of PSP secretion by calcium and the relatively large amounts synthesized by the bovine parathyroid cell (10 to 15 percent of total protein synthesis) suggest that it has some function in secretion.

When tissue from other bovine or-

gans that secrete proteins (pituitary, pancreas, thyroid, thymus, liver, and salivary gland) was incubated under conditions identical to the parathyroid incubation, less than 0.5 percent of the radioactive protein in the medium had the electrophoretic mobility of PSP. If PSP is unique to the parathyroid gland, it may be the basis of a new assay for parathyroid gland activity. Since PSP is a large protein, it is likely to be highly immunogenic. A radioimmunoassay for PSP might be developed to complement that for PTH, a situation analogous to the sensitive radioimmunoassay for neurophysin which measures the secretory activity of the posterior pituitary gland (*19*).

BYRON KEMPER
Department of Biology, Massachusetts Institute of Technology, Cambridge 02139
JOEL F. HABENER
Endocrine Unit and *Department of Medicine, Massachusetts General Hospital,* and *Harvard Medical School, Boston 02114*
ALEXANDER RICH
Department of Biology, Massachusetts Institute of Technology
JOHN T. POTTS, JR.
Endocrine Unit and *Department of Medicine, Massachusetts General Hospital,* and *Harvard Medical School*

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Benzo[a]pyrene Metabolites: Efficient and Rapid Separation by High-Pressure Liquid Chromatography

Abstract. *High-pressure liquid chromatography can separate eight metabolites of benzo*[a]*pyrene formed by rat liver microsomes. This method offers major advantages over previous techniques used for the separation of oxygenated polycyclic aromatic hydrocarbons.*

Polycyclic aromatic hydrocarbons (PAH's), which cause cancer in experimental animals, are found in the atmosphere, waterways and oceans, soil, marine life, and in the food chain. Sources of these pollutants include emissions from transportation, heat and power generation, refuse burning, industrial processes, and oil spills (*1*). Mammals and many lower organisms metabolize polycyclic hydrocarbons, primarily by enzymatic oxygenation, a process which converts the hydrocarbons into organic solvent–soluble polycyclic phenols, dihydrodiols, quinones, and water-soluble conjugates (*2, 3*). Some of these metabolites are formed through epoxide intermediates (*2*). These PAH's are also covalently bound to cellular macromolecules (*4*). Oxygenation is primarily catalyzed by a microsomal enzyme complex aryl hydrocarbon hydroxylase which contains cytochrome P-450. This enzyme complex has a major function in both the detoxification of PAH's (*5*) and the activation of some to toxic derivatives or to active carcinogens (*6*). In order to understand the relation between the formation of specific metabolites and the induction of cancer, it is necessary to describe for each PAH its profile of metabolites and to characterize the biological activity of each product. Thin-layer chromatography or column chromatography have been the usual methods of separation (*3, 7*). Although useful in specific cases, these methods are often inadequate. The separations are often incomplete; because the metabolites are often labile when exposed to light and air, they are easily destroyed. Gas chromatography has been used successfully for the separation of the parent PAH, but is inadequate for the hydroxylated metabolites, which are destroyed by pyrolysis during the vaporization stage. Recently, trimethylsilylation of methylcholanthrene metabolites has permitted their

Table 1. High-pressure liquid chromatographic separation of benzo[*a*]pyrene metabolites. The following are abbreviations used: BP, Benzo[*a*]pyrene; 9,10-diol, 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene; 7,8-diol, 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene; 4,5-diol, 4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene; 3-OH, 3-hydroxybenzo[*a*]pyrene; 9-OH, 9-hydroxybenzo[*a*]pyrene; 1,6-quinone, Benzo[*a*]pyrene-1,6-dione; 3,6-quinone, benzo[*a*]pyrene-3,6-dione; 6,12-quinone, benzo[*a*]pyrene-6,12-dione.

Metabolite	Retention time (min)	MW*
9,10-diol-BP	8.5	286
4,5-diol-BP	15.5	286
7,8-diol-BP	18.0	286
1,6-quinone-BP	25.5	282
3,6-quinone-BP	26.0	282
6,12-quinone-BP†	28.0	
9-OH	35.0	268
3-OH	37.0	268
BP	48.0	252

* Molecular weight (MW) determinations were performed on a Jeol JMS-01SG-2 at 70 electron volts, with a solid probe; the temperature ranged from 90° to 150°C. † Insufficient material for complete analysis, and therefore tentative.