Further Studies on the Isolation and Characterization of Parathyroid Polypeptides*

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ABSTRACT: Three separate bovine parathyroid polypeptides have been isolated by gel filtration and are homogeneous by several strict criteria. These peptides include parathyroid hormone (PTH), a peptide which stimulates aerobic glucose and pyruvate oxidation in isolated Ehrlich ascites tumor cells, and a peptide which stimulates *in vitro* the uptake of phosphate into mitochondria and mitochondrial respiration but lacks the other activities possessed by PTH. The amino acid compositions, molecular weights, and results of the NH_2-terminal residue determinations are reported.

Following the introduction by Aurbach (1959a) of phenol as an extracting agent, the isolation of an apparently homogeneous parathyroid polypeptide was reported by Rasmussen and Craig (1959) and by Aurbach (1959b). The further characterization of this peptide was carried out by Rasmussen and Craig (1961) who presented a tentative empirical formula. Their isolation was achieved by the use of countercurrent distribution (CCD).[1] In later work, Rasmussen and Craig (1962) introduced the use of Sephadex G-50 to achieve peptide isolation. Material obtained by this technique appeared homogeneous by several criteria, and a revised tentative formula was presented. However, when apparently homogeneous parathyroid hormone (PTH), prepared by Sephadex G-50, was subjected to starch-gel electrophoresis by Barrett *et al.* (1962) it appeared inhomogeneous. The Sephadex method was improved by substituting Sephadex G-100 for G-50 (Rasmussen *et al.*, 1964; Aurbach and Potts, 1964). In addition, improvements in resolution and recovery were obtained by adding the reducing agent, cysteine, at several key steps in the isolation procedure, by substituting urea–HCl mixtures for phenol as the original extracting agent, and by carrying out the gel filtration at 4° rather than 18° (Rasmussen *et al.*, 1964). The elution patterns obtained upon subjecting crude gland extracts to gel filtration upon columns of G-100 revealed several distinct peaks other than that identified as the parathyroid hormone. Some of these peaks were found to have interesting biological properties (Rasmussen *et al.*, 1964). The hormonal peak and some of the others were further purified and were reported to be homogeneous (Rasmussen *et al.*, 1964). Homogeneity was based primarily upon evidence from starch-gel electrophoresis. However, refinements in this technique as well as the use of disk-gel electrophoresis revealed varying degrees of heterogeneity. Further purification with various types of Sephadex and carboxymethylcellulose (CMC) columns has led to the preparation of homogeneous preparations of the parathyroid hormone, peak 4; a protein which mimics some of the effects of the hormone upon mitochondria *in vitro*, peak 2; and a protein which stimulates the conversion of glucose or pyruvate to CO_2, peak 3S. These three peptides have been characterized by amino acid, end group, and ultracentrifugal analyses. This paper reports the purification and characterization of these three peptides.

Materials and Methods

Extractions. The procedure for the extraction of PTH from bovine parathyroid glands has been described (Rasmussen *et al.*, 1964). This procedure employs an extraction solution of 8 M urea–0.2 N HCl–0.1 M cysteine. Concentrated phenol solutions have been used in the past for extraction of PTH (Aurbach, 1959a,b; Rasmussen and Craig, 1961). This paper also reports the use of 80% phenol with 0.1 M cysteine as the initial extracting solution, all other steps being the same as described by Rasmussen *et al.* (1964).

Gel Filtrations. Sephadex G-100 was used in the initial fractionation of the trichloroacetic acid (TCA) precipitate prepared by both the urea–HCl–cysteine and phenol–cysteine methods. The procedure was previously described in detail by Rasmussen *et al.* (1964). All gel filtrations were performed at 4°.

Further purifications of the various peaks obtained in the G-100 fractionation were made on G-100 and

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[1] Abbreviations used in this work: PTH, parathyroid hormone; TCA, trichloroacetic acid; CMC, carboxymethylcellulose; CCD, countercurrent distribution; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

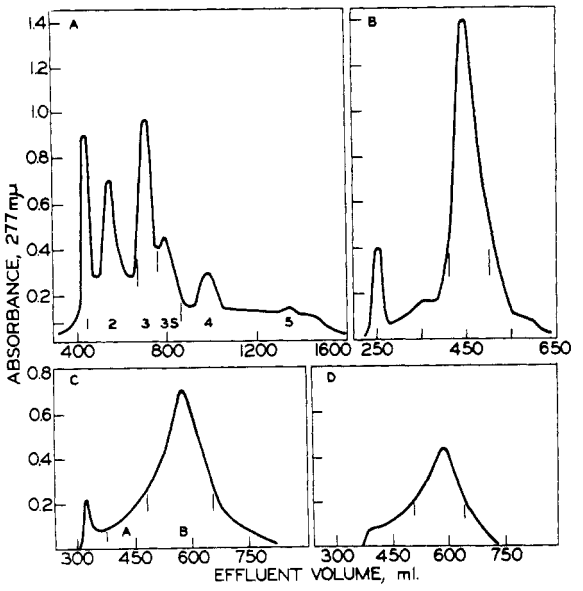

FIGURE 1: The elution patterns of crude urea–HCl–cysteine-prepared TCA powder. A, pattern obtained when subjected to gel filtration on a 4.0 × 150 cm column of Sephadex G-100 employing a 0.2 M ammonium acetate buffer, pH 4.6, as eluent; B, pattern obtained when peak 3S was rerun on a 2.5 × 200 cm G-100 column; C, pattern obtained after the main peak (Figure 1B) was subjected to gel filtration upon a 2.5 × 200 cm column of Sephadex G-25 employing 0.01 M acetic acid; D, pattern after peak B (Figure 1C) was rerun on a column of G-25.

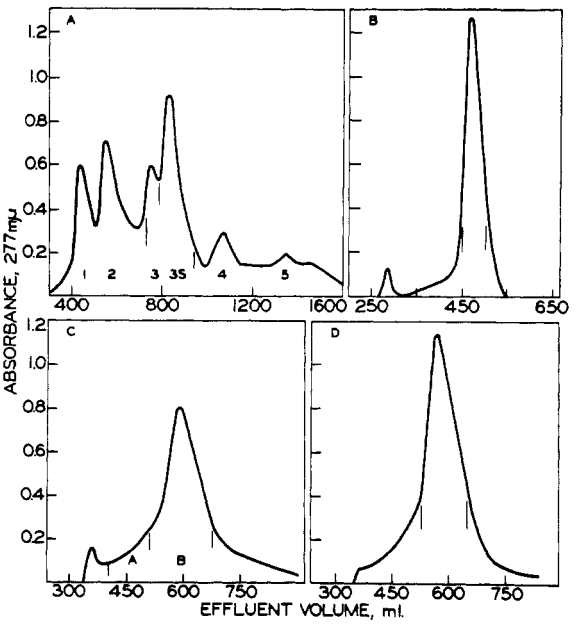

FIGURE 2: The elution patterns of crude phenol–cysteine-prepared TCA powder. A, pattern obtained when subjected to gel filtration on a 4.0 × 150 cm column of G-100; B, C, and D, the further purification of peak 3S on G-100 and G-25 columns under identical conditions with those employed in Figure 1.

G-25. The columns employed were either 2.5 × 200 cm G-100, 2.5 × 200 cm G-25, or 2.0 × 150 cm G-25. Ammonium acetate buffer (0.2 M, pH 4.6) was used for all G-100 columns. With all G-25 columns, 0.01 M acetic acid was used. Samples of 350 mg of protein were used on the 2.5 × 200 cm G-100 and G-25 columns, and 250 mg on the 2.0 × 150 cm G-25 column. Fractions of 6 ml were collected with the G-100 columns and smaller G-25 columns, while 7.5-ml fractions were collected from the 200-cm columns of G-25. The flow rates were 20 ml/hr for G-100 columns and 40 ml/hr for G-25 columns. All fractions were analyzed at 277 mμ in a Zeiss spectrophotometer. The protein was recovered by lyophilization after dilution of the buffer solution 1:1 with distilled H_2O. Two successive lyophilizations were necessary to remove the ammonium acetate.

CMC Chromatography. Chromatography on columns of CMC was performed using a concentration gradient of ammonium acetate buffer (pH 5.0) in the following manner. CMC (Chromatography Corp. of America, Morton Grove, Ill.) was washed several times with water and then equilibrated with the starting buffer for the gradient. The method of Hirs *et al.* (1953) was used to find suitable distribution coefficients for chromatography. The initial and final buffers for the gradient were determined by equilibrating 2 ml of packed CMC (by centrifugation in 15-ml glass centrifuge tubes) with 3.5 ml of buffers of a range of concentrations from 0.01 M to 2.0 M. Peptide (2 mg) was placed in each tube and equilibrated with the resin. The CMC was then packed by centrifugation and the supernatant was analyzed at 277 mμ to determine if the protein was bound to the CMC or free in the supernatant. In this way an initial buffer concentration just below the eluting concentration and a final concentration just above it were determined for each peptide. For the three peptides studied, these concentrations were the same. The initial buffer concentration was 0.1 M ammonium acetate (pH 5.0). The gradient elution device of Bock and Ling (1954) was employed. A linear gradient developed such that the final concentration of buffer passing through the column was 1.05 M, pH 5.0.

A 2.5 × 25 cm column of CMC was packed using air pressure above the buffer head, and was then equilibrated at 4° with starting buffer. A 200–400-mg quantity of protein was used per sample and was dissolved in 5–7 ml of the starting buffer and washed onto the column. Fractions of 15 ml were collected at a flow rate of approximately 4 ml/min. A total gradient volume of 1800 ml was used. Analysis of the effluent was carried out by spectrophotometric means employing a wavelength of 277 mμ.

Starch-Gel Electrophoresis. Starch-gel electrophoresis was carried out by the method of Edelman and Poulik (1961) with the modifications described by Rasmussen *et al.* (1964), except that the conditions were changed

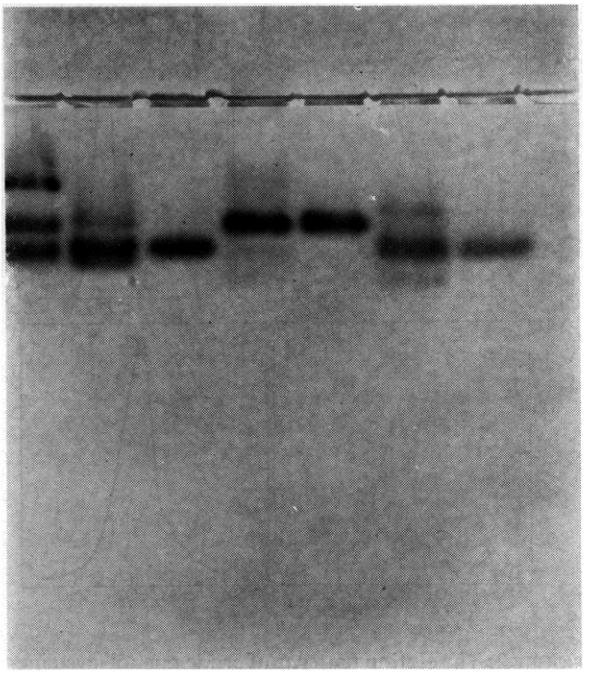

FIGURE 3: Starch-gel electrophoresis of bovine parathyroid polypeptides. Left to right the samples are: TCA precipitate, peak 2 from the G-100 fractionation (peak 2, Figure 1A), pure peptide 2 (Figure 5C), peak 3S from the G-100 fractionation (peak 3S, Figure 1A), pure peptide 3S (Figure 1D), peak 4 from the G-100 fraction (peak 4, Figure 1A), and pure peptide 4 (Figure 6B). The procedures for electrophoresis are given in the text.

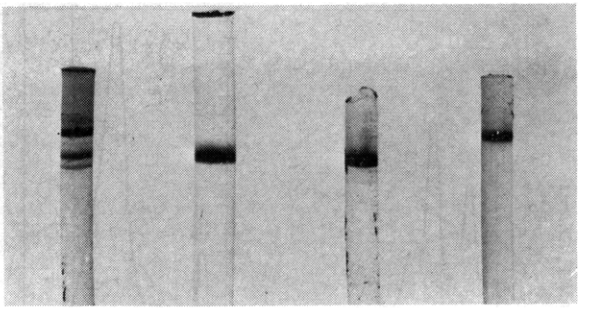

FIGURE 4: Disk electrophoresis of bovine parathyroid polypeptides. Left to right the samples are TCA precipitate, pure peptide 2, pure peptide 3S, and pure peptide 4. The procedures for electrophoresis are given in the text.

from an 8 M urea starch gel run at room temperature for 20 hr to a 4 M urea gel run at 4° for 24 hr. The pH of the gel buffer was 2.9–3.0. Such gels gave improved resolution of the parathyroid polypeptides.

Disk Electrophoresis. The procedure for disk electrophoresis was modified from the technique of Ornstein and Davis (1958) as follows. The gels were prepared from monomer and catalyst solutions here described: solution A, 144 g of urea and 1.12 g of EDTA made up to 300 ml with deionized water; solution B, 1.34 g of potassium hydroxide, 8.65 ml of glacial acetic acid, and 2.0 ml of *N,N,N′,N′*-tetramethylethylenediamine made up to 50 ml with solution A; solution C, 60.0 g of acrylamide and 0.80 g of *N,N′*-methylenebisacrylamide made up to 150 ml with solution A; monomer solution, made by mixing solutions B and C; catalyst solution, 0.28 g of ammonium persulfate made up to 100 ml with solution A. These solutions were filtered into dark-glass bottles, stored in the cold, and used no longer than 2 weeks. All organic chemicals in these solutions were obtained from Distillation Products Industries, Rochester, N. Y.

The samples were applied in 0.5 ml of a 20% solution of sucrose in electrophoresis buffer rather than in a large-pore gel. The electrophoresis buffer (pH 4.6) consisted of 31.2 g of β-alanine and 0.8 ml of glacial acetic acid made up to 5 l. with deionized water. The spacer gel consisted of 0.25 ml of a suspension of Sephadex G-200 in the 20% sucrose-in-buffer solution. Electrophoresis was carried out at 4° for 3 hr at 3 ma/gel. The gels were stained for 1 hr in a 1% amido black 10B solution in the electrophoresis buffer described above. They were then destained as described by Ornstein and Davis (1958), but if additional destaining was found to be necessary, they were placed in tubes containing 7% acetic acid and a small amount of cellulose *N,N*-diethylaminoethyl ether (Distillation Products Industries) for 24 hr.

Amino Acid Analyses. Amino acid analyses were performed on an automatic amino acid analyzer (Technicon Chromatography Corp., Chauncey, N. Y.) using the Technicon modifications of the method of Spackman *et al.* (1958). Peptides were hydrolyzed with constant-boiling HCl at 110° in sealed, evacuated tubes for various lengths of time. The tube was flushed twice with nitrogen gas (high purity, dry) before the final evacuation in order to minimize the presence of oxygen during hydrolysis. After hydrolysis, the tubes were opened and the sample was reduced to dryness by evacuation in a desiccator containing phosphorus pentoxide. The sample was redissolved in the starting buffer for the analysis and aliquots of hydrolysate corresponding to 0.5–0.75 mg of peptide were analyzed. The analyses took 22 hr and employed a pH gradient of 2.875–5.00. A single 0.6 × 125 cm column of Chromobeads Type A ion-exchange resin (Technicon) was used, and all procedures were as prescribed by Technicon. An internal standard of 0.25 μmole of norleucine was used.

Performic acid oxidations were performed according to the method of Moore (1963) for the improved detection of cystine in the peptides. One modification was employed. After the reaction, the sample was concentrated to dryness by freezing and lyophilization rather than in the rotary evaporator at 40°. This was to prevent loss of sample through foaming. The samples were hydrolyzed and amino acid analyses performed.

Tryptophan was determined spectrophotometrically

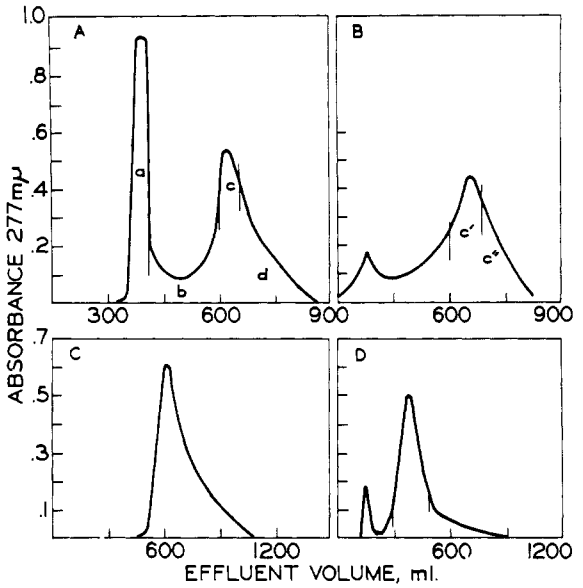

FIGURE 5: Elution patterns of various peaks. A, pattern obtained after peak 2 (Figure 1A) was passed through a 2.5 × 200 cm column of G-25 employing 0.01 M acetic acid as eluent; B, rechromatography of peak c (Figure 5A) under the same conditions; C, the further purification of peak c′ (Figure 5B) by gradient elution chromatography on a 2.5 × 25 cm column of CMC; D, elution pattern obtained when peak 3S (Figure 1A) was subjected to gradient elution chromatography on a 2.5 × 25 cm column of CMC.

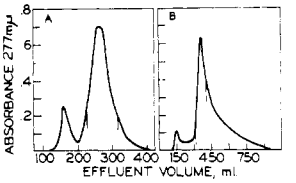

FIGURE 6: The elution patterns of peak 4 (Figure 1A). A, pattern obtained when subjected to gel filtration on a 2.0 × 150 cm column of Sephadex G-25 employing 0.01 M acetic acid as eluent; B, the further purification of the main peak from Figure 6A by gradient elution chromatography on a 2.5 × 25 cm column of CMC using a linear gradient from 0.1 to 1.05 M ammonium acetate, pH 5.0, and a total gradient volume of 1800 ml. The material between 310 and 400 ml was recovered and characterized further.

by the method of Goodwin and Morton (1946). The absorbancy was measured at 280 and 294.4 mμ, and also at 340 and 370 mμ in order to make the correction for nonspecific absorption which these authors discuss.

Molecular Weight Determinations. The molecular weights of the peptides were determined by equilibrium centrifugation according to the method of Ginsburg *et al.* (1956). The partial specific volumes used were 0.746 ml/g for peptide 2, 0.746 ml/g for peptide 3S, and 0.737 ml/g for peptide 4.

End Group Determinations. The dinitrophenyl (DNP) method of Sanger (1949), modified for use with a pH-Stat, has been outlined in detail for the complete identification of the NH_2-terminal residue (Fraenkel-Conrat *et al.*, 1955). Ether-extractable derivatives were identified by two-dimensional paper chromatography. The first dimension (ascending) was toluene–pyridine–chloroethanol–ammonia (Biserte and Osteux, 1951) and the second dimension (descending) was 1.5 M potassium phosphate buffer, pH 6.0 (Levy, 1954). The nonether-extractable fraction of DNP derivatives was examined by descending paper chromatography with a phthalate–*t*-amyl alcohol system (Blackburn and Lowther, 1951). Authentic DNP amino acids (Mann Research Laboratories, Inc., New York 6, N. Y.) were used as reference compounds in the chromatography. The Sakaguchi reaction (Acher and Crocker, 1952) was employed for the identification of DNP-arginine. Edman degradations were carried out by the micro method of Fraenkel-Conrat *et al.* (1955) or with 2 N HCl at 70° (Rovery *et al.*, 1953).

Biological Assays. The five assays of parathyroid activity as mentioned above have been previously described (Rasmussen *et al.*, 1964). The assay for peak 3S was carried out in isolated Ehrlich ascites carcinoma cells (LePage strain) by measuring the increase in production of $^{14}CO_2$ from 6-^{14}C-glucose or 3-^{14}C-pyruvate (Volk Radiochemicals, Chicago, Ill.). Female Swiss white mice weighing approximately 20 g were injected intraperitoneally with the strain of cells. The cells were used within 5–7 days after injection. The mice were sacrificed by cervical dislocation and a small nick was made in the peritoneal cavity. The cells were removed by aspirating with a Pasteur pipet fitted with a 2-ml rubber bulb. Usually 2–4 ml of ascitic fluids were obtained per animal and this volume was made up to 12 ml with the incubation medium (see below). The mixture was centrifuged at 300*g* for 2 min. The cells were washed and centrifuged three more times, the final centrifugation being 900*g* for 2 min. One volume of cells was then diluted to 12 volumes with incubation medium.

The incubations were carried out in double side-arm Warburg vessels in a standard Warburg apparatus at 37° with air as the gas phase; 0.5 ml of diluted cells was used per vessel. The amount of peptide tested was dissolved in 0.1 ml of 0.0001 M acetic acid and placed in the vessel. The vessel was then made up to a volume of 3.0 ml with the incubation medium which had the following composition: 126 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1.2 mM $MgCl_2 \cdot 6H_2O$, 5 mM pH 7.4 phosphate buffer, and 0.1 M pH 7.4 Tris buffer. One side arm contained 0.1 ml of either 1 mM glucose (containing 3×10^5 cpm of 6-^{14}C-glucose) or 1 mM pyruvate (containing 10^5 cpm of 3-^{14}C-pyruvate). The other side arm contained 0.1 ml of 60% perchloric acid. The center well contained a folded piece of filter paper and 0.2 ml of 20% KOH to trap the liberated CO_2.

After 10 min of preincubation, the substrate was

TABLE I: The Amino Acid Composition of Three Purified Bovine Parathyroid Polypeptides.[a]

Amino Acid	Peptide 2[b]		Peptide 3S[c]		Peptide 4[d]		Peptide 4[e]
	Obsd	Taken	Obsd	Taken	Obsd	Taken	(CCD)
Aspartic acid	12.20	12	10.92	11	7.61	8	8
Threonine	8.09	8	7.76	8	0.65	1	1
Serine	9.19	9	12.87	13	6.07	6	7
Glutamic acid	20.13	20	6.25	6	9.58	10	10
Proline	8.71	9	6.14	6	2.10	2	2
Glycine	21.12	21	9.04	9	3.71	4	4
Alanine	27.00	27	20.15	20	6.30	6	6
Valine	13.34	13	12.01	12	6.67	7	6
Cystine	0	0	0	0	0	0	0
Methionine	0	0	0.77	1	1.67	2	2
Isoleucine	9.16	9	0	0	2.68	3	3
Leucine	24.44	24	20.25	20	7.28	7	7
Tyrosine	3.86	4	2.95	3	0.99	1	1
Phenylalanine	2.19	2	6.88	7	1.83	2	2
Amide N (NH_3)	...		6.08	6	7.14	7	7
Lysine	23.64	24	11.99	12	7.11	7	7
Histidine	6.19	6	10.00	10	3.22	3	3
Arginine	19.77	20	3.19	3	4.33	4	4
Tryptophan	(0)	0	(1.06)	1	(1)	1	1
		208		142		74	74

[a] Values are expressed as residues per mole. For those amino acids that decrease during hydrolysis, notably threonine and serine, and for ammonia which increases, the values given were extrapolated to zero time on basis of results at indicated times of hydrolysis. [b] Nine separate analyses at 15, 30, and 60 hr of hydrolysis. [c] Fourteen separate analyses at 16, 24, 50, and 72 hr of hydrolysis. [d] Ten separate analyses at 16 and 72 hr of hydrolysis. [e] The composition of parathyroid hormone reported by Craig and Rasmussen (1962).

tipped in from the side arm. After 30 min of incubation, the perchloric acid was tipped in to stop the reaction. The flasks were further incubated for 10 min to allow the quantitative displacement of CO_2 from the main chamber to the center well of the vessel. The center well was then washed with water several times and the washings were pooled. To this was added Na_2CO_3 as carrier and $BaCl_2$ to precipitate the carbonate as $BaCO_3$. An amount of Na_2CO_3 was used such that the $BaCO_3$ precipitate was between 30 and 35 mg. This precipitate was transferred to tared stainless steel cup planchets which were lightly coated with lanolin to hold the $BaCO_3$ precipitate to the planchet. The planchets were dried, weighed, and counted at infinite thickness in a gas-flow Nuclear Chicago counter, Model D48, equipped with scaler and automatic sample changer. The results were expressed as counts/min per mg of $BaCO_3$.

Alternatively, 0.2 ml of Hyamine (hydroxide of Hyamine, 10-X, Packard Instrument Co., Inc., La-Grange, Ill.) was placed in the center well to trap the liberated CO_2. After the incubations, the center well was washed with a total of 2 ml of Hyamine and the washings were pooled. The solutions were made up to a constant volume and equal aliquots were placed in scintillation vials. The liquid scintillator (10 ml), which contained 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP)/l. in toluene (Herberg, 1960), was added to each vial. The vials were counted for 10 min in a Packard Tri-Carb liquid scintillation spectrometer, Model 314-EX, equipped with read-out and sample-changing system. Results were expressed as total counts per minute for each incubation flask.

Results

The elution pattern for fractionation of the urea–HCl–cysteine-prepared TCA precipitate on Sephadex G-100 is shown in Figure 1A. This pattern is similar to that reported by Rasmussen *et al.* (1964), except that more prominent shoulders of the third peak (labeled 3S) are now obtained. The Sephadex G-100 pattern for the phenol–cysteine-prepared TCA precipitate is shown in Figure 2A. This pattern is essentially the same as that of Figure 1A, except that the relative amounts of material in peaks 3 and 3S are reversed, the shoulder 3S having considerably more material and appearing as a

TABLE II: The Relative Potency of Parathyroid Polypeptides When Assayed by a Variety of *in Vivo* and *in Vitro* Techniques.[a]

	In Vivo (PTX rat)[b]		*In Vitro* (Mitochondria)			*In Vitro*
Peptide or peak	Calcium Mobilizing[c]	Phosphate Excretion	Calcium Release	Phosphate Uptake	Respiration	(ascites cells) Glycolysis[d]
2	0	0	—[e]	75	75	−50[f]
3S	0	0	0	0	0	255
4	100	100	100	100	100	0

[a] See text for description of these techniques. [b] PTX means parathyroidectomized. [c] All data for parathyroid assays are presented as percentage of effectiveness compared to the response obtained with peptide 4 (PTH). [d] Data for glycolysis are expressed as per cent stimulation of the $^{14}CO_2$ formation where the control (no peptide added) gives 0% stimulation. [e] Indicates peptide has not been tested in particular system. [f] Inhibition of glycolysis observed with this peptide.

peak, and peak 3 having relatively less and appearing as a shoulder. The relative amounts of the other peaks obtained were the same for the two methods. All of these peaks were recovered for further purification and analysis.

Peak 3S was further purified and studied because it was found to be active in stimulating CO_2 production from pyruvate or glucose in the isolated Ehrlich ascites tumor cells (see below). The method of purification of the peak obtained from the initial G-100 fractionation consisted of one passage through a 2.5 × 200 cm G-100 and two passages through a 2.5 × 200 G-25 column. These patterns are indicated in Figures 1B, C, and D for peak 3S of urea–HCl–cysteine origin and in Figures 2B, C, and D for that of phenol–cysteine extraction. These patterns are similar except that there was relatively more of the 3S peptide and less of "contaminating" peptides when phenol–cysteine rather than urea–HCl–cysteine was used as the initial extracting agent. The resulting G-100 and G-25 purifications of the phenol–cysteine 3S material show smaller initial peaks and much sharper main peaks. When the purified 3S peptide was subjected to starch-gel electrophoresis and disk electrophoresis (Figures 3 and 4), a single band indicating homogeneity was obtained in each case. However, when the 3S material from the initial G-100 fractionation of the TCA precipitate was put directly on the 2.5 × 25 cm CMC column as described above, the pattern shown in Figure 5D was obtained. When the main peak was recovered as shown and rerun on the CMC column, a homogeneous peptide was obtained also, according to the starch-gel and disk electrophoresis. This peptide was identical with that prepared on Sephadex and was equally active. The peak was eluted from the CMC in the gradient concentration range of 0.39–0.56 M ammonium acetate with the tip of the peak eluting at 0.46 M. Therefore, use of the CMC column instead of repeated Sephadex gel filtrations appears to be the easiest and fastest way to purify this peptide.

Peak 4 had all of the parathyroid activities previously described (Rasmussen *et al.*, 1964). It was further purified by passage through a single 2.0 × 150 cm Sephadex G-25 column. This pattern is given in Figure 6A. The main peak was then recovered as indicated by the small vertical lines and passed through a single 2.5 × 25 cm CMC column using the gradient and methods described. The main peak was recovered as shown in Figure 6B. It was eluted in the gradient concentration range of 0.33–0.46 M ammonium acetate, with the tip of the peak eluting at 0.39 M. This eluting concentration of buffer in the gradient was highly reproducible. The purified peptide was subjected to starch-gel and disk electrophoresis. The results are shown in Figures 3 and 4, respectively. They show that a single peptide band was obtained for peptide 4 after the CMC step.

Peak 2 was active in two *in vitro* assay systems as previously described by Rasmussen *et al.* (1964). It was further purified by two passages through a 2.5 × 200 cm G-25 column. The results are shown in Figures 5A and 5B. Fraction c was recovered from the peak as shown in Figure 5A and rerun as shown in 5B. The peak c′ which was recovered showed a single band on both types of electrophoresis (Figures 3 and 4), indicating homogeneity of the peptide. The areas d and c′′ in Figures 5A and 5B could be passed through G-25 again to recover more purified peptide 2. When a sample of the purified peptide 2 was passed through the 2.5 × 25 cm CMC column, a single peak was obtained as shown in Figure 5C. The peak was eluted from the CMC at a buffer gradient concentration range of 0.45 to 0.75 M, with the tip of the peak eluting at 0.51 M.

The amino acid compositions of the three purified proteins are summarized in Table I. The three peptides differ considerably both in size and composition. The composition of PTH (peptide 4) as determined from the product of the CMC chromatography was identical with the composition previously reported for that obtained by countercurrent distribution (Rasmussen and Craig, 1962) and by Sephadex G-50 (Rasmussen and Craig, 1962), except for an added valine residue and

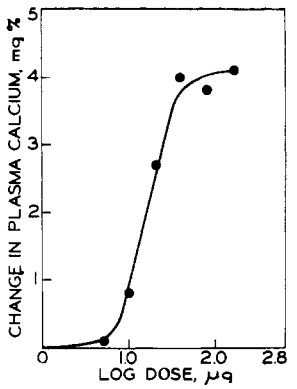

FIGURE 7: Log dose–response curve obtained by measuring the concentration of plasma calcium in groups of seven 100-g Holtzman rats 6 hr after parathyroidectomy by cautery and the intraperitoneal injection of parathyroid hormone (G-100 peak 4, G-25, CMC product) dissolved in 0.001 M acetic acid at dose levels of 5, 10, 20, 40, 80, and 160 μg. Plasma calcium values are plotted as the mean increment of increase above that obtained in a simultaneously studied, untreated, parathyroidectomized group of rats. The estimated potency of this preparation when compared with USP Parathyroid Extract (Lilly) is approximately 3000 units/mg. The standard deviations of the values obtained were $\leq \pm 1.0$ mg %.

one less serine residue. The two prolines obtained agreed with that for countercurrent distribution, but not the Sephadex G-50 product. Difficulties in hydrolysis of valine, the extrapolation of serine values to account for losses, and fluctuations in proline values due to ninhydrin variations may explain why the previous results deviated from those now reported.

The other two peptides have compositions that are excellent indications of homogeneity. Peptide 2 contains no cystine, methionine, or tryptophan. Peptide 3S contains no cystine or isoleucine.

The spectrophotometric determination of tryptophan showed that PTH contained one residue, peptide 3S one residue, and peptide 2 none, the tyrosine to tryptophan ratio being greater than 20:1. In addition, the performic acid oxidations verified that all three peptides lacked cystine or cysteine.

The molecular weight determinations by ultracentrifugation yielded 23,100 for peptide 2 compared to 22,526 based on amino acid analysis, 16,200 for peptide 3S compared to 15,172 based on amino acid analysis, and 10,000 for PTH compared to 8366 based on amino acid analysis. For the first two peptides these values agreed very well. For PTH the discrepancy noted cannot be presently explained. However, it may be possible that the molecular size based on amino acid analysis is too small. These molecular weights also correspond very well to those expected on the basis of the Sephadex G-100 elution pattern.

NH_2-Terminal residue determinations were attempted on the three peptides as described. The DNP method showed the end group of parathyroid hormone to be alanine, agreeing with that previously reported (Rasmussen and Craig, 1962b). The DNP method also showed the NH_2-terminal residue of peptide 3S to be valine. However, end group determinations by both the DNP method and Edman degradation (using both 3 N HCl at 40° and 2 N HCl at 70° for the cyclizing agent) were uniformly unsuccessful in revealing any NH_2-terminal residue for peptide 2. Preliminary tryptic digestions (Ingram, 1958) of this peptide have consistently yielded a ninhydrin-negative, but Sakaguchi-positive (Acher and Crocker, 1952) spot on the peptide maps. This is a further indication that there is no free amino group present at the NH_2 terminus of the peptide.

Various biological assays indicative of parathyroid activity were performed on the peaks obtained in the Sephadex G-100 fractionation of the TCA precipitate and on the three purified peptides. Also, the assay for glycolytic activity in the ascites cells was performed with each peptide. The results are summarized in Table II. They show that PTH was active in the five parathyroid assays, but not upon CO_2 production. Peptide 2 was partially active in mitochondrial respiration and phosphate uptake but not active in the two *in vivo* parathyroid assays, nor in stimulating CO_2 production. Peptide 3S was not active in any of the five parathyroid assays, but was very active in promoting CO_2 production from glucose or pyruvate.

A dose–response assay of the purified PTH from CMC chromatography in the calcium mobilization system of Munson (1955) is shown in Figure 7. The control group of rats (parathyroidectomized, not treated with PTH) had a mean plasma calcium value of 5.3 mg %. The treated groups received 5, 10, 20, 40, 80, and 160 μg/rat, respectively, the PTH being dissolved in 0.01 M acetic acid. An increase in plasma calcium of 2.7 mg % was obtained with 20 μg and the full increase of 4 mg % was obtained with 40 μg of PTH. The activity of this purified PTH was estimated to be 3000 USP units/mg when compared with the USP parathyroid extract (Lilly), and is just as active if not more so than previous preparations (Rasmussen and Craig, 1962).

Discussion

The use of phenol–cysteine as the initial extraction solution in the preparation of the TCA precipitate has been described. The Sephadex G-100 elution patterns are very similar for both phenol–cysteine and urea–HCl–cysteine material, except for peaks 3 and 3S. In this case, greater yields of peak 3S are obtained. The purified peptides obtained by both methods are equivalent chemically and biologically. Except for the increased yields of peptide 3S with phenol–cysteine, there is no other advantage to be seen in using this solution. Indeed, because of the dangers that can be encountered with severe burns from concentrated phenol solutions, the

urea–HCl–cysteine method is routinely used in this laboratory.

By several strict criteria, the three peptides under discussion have been shown to be homogeneous. They are eluted from Sephadex G-25 and carboxymethylcellulose as single peaks. These two methods of purification supplement each other in that the first is made on the basis of molecular size and conformation, while in the second the purification depends on the ionic character of the molecule.

The three peptides (2, 3S, and 4) gave single sharp bands when subjected to starch-gel electrophoresis or polyacrylamide disk electrophoresis. The latter method was considerably more sensitive and had a greater resolving power than other electrophoretic techniques. In our hands, this method has on several occasions resolved peptide mixtures that had appeared as a single band on starch-gel electrophoresis. Electrophoretic homogeneity at the widely separated pH values of the disk and starch-gel methods are additional indications of molecular homogeneity.

Further excellent criteria for homogeneity are: (1) the absence of particular amino acids such as cystine in peptide 4, cystine and isoleucine in peptide 3S, and cystine, methionine, and tryptophan in peptide 2; (2) a single amino acid which was found NH_2 terminal in peptides 4 and 3S, as well as the absence of a free NH_2-terminal residue for peptide 2; (3) the close correspondence between the molecular weight values obtained by ultracentrifugal and amino acid analysis.

The finding that one of the peptides (3S) is active in the stimulation of CO_2 production in ascites tumor cells is of considerable interest. Parathyroid hormone has been linked to glycolysis and the production of CO_2 and lactate by various workers. Vaes and Nichols (1962) injected large doses of parathyroid extract into mice and showed *in vitro* stimulation in aerobic glycolysis of bone chips incubated in a glucose-containing medium, as measured by lactic acid production. Cohn and Forscher (1962) have shown that parathyroid extract when injected into rabbits stimulates the production of $^{14}CO_2$ from 6-^{14}C-glucose in bone slices and kidney slices incubated *in vitro*. Cohn (1964) also showed that the rabbit bone slices from animals treated with parathyroid extract converted less succinate, fumarate, citrate, and glutamate to $^{14}CO_2$. A stimulation of glycolysis has been proposed as one of the primary actions of parathyroid hormone upon bone metabolism, in support of the hypothesis advanced by Neuman and Neuman (1958). In view of the isolation of a peptide that stimulates CO_2 production from glucose, it is necessary to critically reexamine these biological responses employing the purified hormonal peptides. Our results also point out a major disadvantage of using crude parathyroid extracts in *in vitro* studies in that they are a mixture of several biologically active peptides and might give confusing results.

The amino acid compositions of the three peptides indicate that parathyroid hormone cannot be a fragment of peptide 3S or 2 since it contains amino acids that are lacking in both of these peptides. In addition, peptide 3S cannot be completely included as a piece of peptide 2 since it also has amino acids that are absent in the latter peptide. However, these facts do not exclude the existence of a larger molecule of which these peptides are fragments.

The isolation of several parathyroid polypeptides with different activities is of considerable interest. The structural, immunological, and functional interrelationships between the peptides, their physiological significance, and the issue of more than one parathyroid hormone are important aspects which make necessary further study on their structure and function.

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Biosynthesis of Phosphatidyl Myoinositol Phosphates in Rabbit Brain*

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ABSTRACT: Rabbit brain slices were incubated separately with orthophosphate-^{32}P and myoinositol-2-^{3}H, and the incorporation of label into the phosphoinositides was determined by analysis of the intact lipids separated by DEAE-cellulose chromatography. The results are consistent with the hypothesis that the phosphatidylmyoinositol moiety remains intact during the metabolic interconversions of the phosphoinositides. Two different methods of isolation of the lipids gave similar results. Analysis of the incorporation of myoinositol-2-^{3}H by either method failed to provide any evidence for an alternative biosynthetic relationship among the phosphoinositides. Each of the different extracts obtained in the Folch procedure for isolating the phosphoinositides [Folch, J. (1949), *J. Biol. Chem. 177*, 505] contains phosphatidylmyoinositol, but only the petroleum ether extract contains the polyphosphoinositides. Mitochondria incubated with orthophosphate-^{32}P are shown to incorporate the label into all three phosphoinositides.

The incorporation of labeled precursors (orthophosphate-^{32}P, myoinositol-^{3}H, and glycerol-2-^{14}C) into the three components of the polyphosphoinositide complex by rabbit brain slices has been described (Brockerhoff and Ballou, 1962a,b). The results indicate that the glyceryl–phosphoryl–myoinositol structure is retained intact in the metabolism of the inositides, *i.e.*, phosphatidyl-L-myoinositol (I) → phosphatidyl-L-myoinositol 4-phosphate (II) → phosphatidyl-L-myoinositol 4,5-diphosphate (III). The results were obtained from analysis of the deacylated lipids. Since these observations were made, other workers (Hölzl and Wagner, 1964; Andrade and Huggins, 1964; Ellis and Hawthorne, 1962) have confirmed the data for the incorporation of radioactive orthophosphate, but conflicting results have been reported (Palmer and Rossiter, 1964) concerning the inositol incorporation. In an attempt to resolve this conflict, we have repeated and extended our studies by analyzing directly the intact lipids. The results provide further support for the biosynthetic pathway we have proposed.

Since the method of isolation of the lipids may affect the results, we have used the experimental procedure of Palmer and Rossiter (1964) and have reexamined our own technique. Similar results were obtained by both methods. It had been assumed (Brockerhoff and Ballou, 1962a) that, in the original Folch (1949) procedure, the phosphoinositides are obtained only in the petroleum ether (bp 60–70°) extract. To check this assumption, all of the fractions obtained by this multiple solvent extraction method have been analyzed for inositides. Each is shown to contain I, although the polyphosphoinositides are found only in the petroleum ether extract. Our results establish that rabbit brain mitochondria, like brain slices, are able to incorporate orthophosphate-^{32}P into all of the phosphoinositides.

Experimental Procedure

Materials and Methods. Myoinositol-^{3}H was obtained from New England Nuclear Corp. and was shown to be isotopically homogeneous by paper chromatography. Phosphorus was determined by Bartlett's method (1959) and myoinositol was assayed micro-

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