Further Studies on the Isolation and Characterization of Parathyroid Polypeptides*

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Notable advances have been made recently in the isolation and characterization of the bovine parathyroid hormone (1-6). However, its complete characterization has not been achieved. Criteria have been published (1-6) indicating that several related polypeptides possessing hormonal activity have been isolated in a relatively pure state. However, in the one instance in which one of these was subjected to starch gel electrophoresis (7), evidence of inhomogeneity was detected. Because of the increasing interest in these hormonal polypeptides, it becomes of importance to have reliable methods for their isolation as homogeneous preparations. This is particularly necessary because, by all of the published methods, a varying yield of an unstable product is obtained, at least partially because of the rapidity of its oxidative inactivation (5, 8).

For these reasons, we have continued to study this problem in an effort to improve the ease and reproducibility of isolation. One necessary adjunct to the success of this endeavor has been the development of a reliable method for the routine detection of polypeptide inactivation (8). For these reasons, we have continued to study this problem in an effort to improve the ease and reproducibility of isolation. One necessary adjunct to the success of this endeavor has been the development of a reliable method for the routine detection of polypeptide inactivation (8).

Experimental Procedure and Results

Starch Gel Electrophoresis—Starch gel electrophoresis was carried out by a modification of the technique of Edelman and Poulik (9). The most important change from their conditions was in the use of disodium ethylenediaminetetraacetate in the buffer mixtures. The importance of this addition is illustrated in Fig. 1, which represents the patterns obtained when samples of hormonal extracts of varying states of purity were subjected to electrophoresis in the presence and absence of disodium EDTA. It is readily apparent that the problem of trailing was completely overcome by this means with considerable improvement in the resolution of the various bands. Our procedure was identical with that of Edelman and Poulik except that the original buffer (0.2 M formic acid-0.05 M NaOH-8 M urea, pH 2.9) contained in addition 0.01 M disodium EDTA. For electrophoresis, 1 mg of each peptide sample was taken up in 0.1 ml of 0.05 M formic acid-0.01 M NaOH-8 M urea and applied to the gel. A voltage gradient of 8 volts per cm was applied, and electrophoresis was conducted at 25° for 20 hours. Slicing, staining, and fixing of the gels were done as described by Edelman and Poulik (9).

Initial Extraction—Dilute hydrochloric acid was the first agent employed to dissociate parathyroid activity from crude gland extracts (10), but successful dissociation required heating to temperatures of 70-100° (10). This procedure has been shown to lead to a partial hydrolysis of the hormonal polypeptide and yields a complex mixture which is difficult to fractionate (2). Unfortunately, extraction with cold acid does not bring about the desired dissociation even though it is known that the hormonal peptide is stable in cold dilute mineral acid. In view of our previous observation that hormonal activity is stable in concentrated urea solutions (8), it was considered possible that the addition of urea to solutions of dilute mineral acid might overcome this difficulty. This proved to be the case. A number of other extracting agents studied were either no better or less good than the standard phenol extraction procedures (1, 4). The urea-HCl solutions proved to be superior as illustrated in Table I. There was a significant improvement in both the specific and total hormonal activity extracted. A further improvement in yield was achieved by the addition of cysteine-HCl at two steps in the isolation procedure (Table I). This final method was adopted.

Two liters of 8 M urea and 0.1 M cysteine (cysteine addition, Step 1) in 0.2 N hydrochloric acid were added to 200 g of dried, defatted bovine parathyroid powder (12). The mixture was stirred and allowed to stand for 1 hour. Glacial acetic acid and acetone, 2 liters each, were added with constant stirring, and the mixture was allowed to stand for another hour. Acetone, 6 liters, and 1 M sodium chloride, 50 ml, were added with vigorous stirring. The resulting mixture was allowed to stand until the precipitate had completely settled. The solution was then filtered through four layers of cheesecloth. The precipitate was dis-
carded. The filtrate was divided into portions and placed in two 12-liter flasks, to each of which were added 5 liters of peroxide-free diethyl ether. A precipitate formed and was allowed to settle. The supernatant was decanted and carded. The precipitate was collected by centrifugation and washed twice with ether-acetone, 1:1. The washed precipitate was dissolved in 1 liter of 20% (v/v) acetic acid containing 0.01 M cysteine (cysteine addition, Step 2) and stirred with a magnetic stirrer for 30 to 40 minutes to ensure maximal solution of the precipitate. Complete solution of the ether precipitate did not occur. Dry sodium chloride, to give a final solution of 5% NaCl, was added to this slightly turbid solution, and the mixture was stirred until complete solution of the salt was achieved. During the course of the dissolution of the NaCl, a fine precipitate gradually formed. This was separated by centrifugation at 6000 × g and discarded. Sufficient 45% trichloroacetic acid was added to the supernatant to produce a final concentration of 7.5%. The precipitate which formed was collected by centrifugation and washed twice with 5% trichloroacetic acid and once with peroxide-free ether. It was then dissolved in approximately 1.5 liters of 0.02 M hydrochloric acid. This solution was extracted three times with an equal volume of peroxide-free ether. The dissolved ether in the remaining aqueous solution was removed on a rotary evaporator. The solution was then passed through a column (2 × 30 cm) of ion exchange resin, Amberlite IRA 400 (10 to 50 mesh), in the acetate form. The effluent was lyophilized. The resulting powder (trichloroacetic acid powder) was a very light brown. The solution was then passed through a column (2 × 30 cm) of ion exchange resin, Amberlite IRA 400 (10 to 50 mesh), in the acetate form. The effluent was lyophilized. The resulting powder (trichloroacetic acid powder) was a very light brown. The usual yield was 1.2 to 1.6 g. The entire operation was carried out at 4°. All the solvents and chemicals were reagent grade and were made up in deionized water. The solutions and solvents were cooled to 4° before use.

**Gel Filtration**—All gel filtration experiments were carried out at 4°. Sephadex G-100 in the bead form (Pharmacia) was employed for the initial separation of the trichloroacetic acid powders. The resin was washed repeatedly with distilled water, the fine particles were removed by decantation, and the beads were taken up in the buffer and poured in sections into a column 4.0 × 150 cm. The developing buffer (0.2 M ammonium acetate, pH 4.7) was allowed to flow by gravity through the column with an average flow rate of 35 to 40 ml per hour. Fractions were collected at 20-minute intervals and analyzed at 277 mp in a Zeiss spectrophotometer. The trichloroacetic acid powder (750 mg) was taken up in 40 ml of buffer. The small amount of insoluble material was removed by centrifugation and re-extracted with 10 ml of buffer. The combined, clear supernatants were added to the column and washed onto the resin bed before development was initiated. The patterns obtained with various isolation procedures are shown in Fig. 2. Phenol extracts (Fig. 2D), prepared as previously described (3), and urea-HCl extracts prepared with (Fig. 2C) and without cysteine (Fig. 2A and D) at the different steps in the isolation procedure were separated under nearly identical circumstances. As is evident in Fig. 2, the best separations were achieved with trichloroacetic acid powders prepared, as described in the previous section, with the cysteine-urea-HCl method. Five distinct and well separated peaks were

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**Table I**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Yield</th>
<th>Biological activity</th>
<th>Total yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>1.4-1.6</td>
<td>150-170</td>
<td>225,000</td>
</tr>
<tr>
<td>Urea-HCl</td>
<td>1.2-1.8</td>
<td>180-200</td>
<td>250,000</td>
</tr>
<tr>
<td>Urea-HCl-cysteine</td>
<td>1.2-1.6</td>
<td>222-240</td>
<td>280,000</td>
</tr>
</tbody>
</table>

*Average of three separate extractions.*
FIG. 3. Gel filtration of trichloroacetic acid powder (urea-cysteine-HCl) on a column of Sephadex G-75, 4 X 150 cm. Each fraction was 12 ml, and the flow rate was approximately 45 ml per hour. Material in tubes 112 to 128 corresponded to Peak 4, Fig. 2C.

FIG. 4. The gel filtration patterns observed when the various peaks obtained from filtration on Sephadex G-100 (Fig. 2C) were lyophilized and then rerun on a G-25 column, 2 X 150 cm, at a flow rate of 40 ml per hour. Each fraction represented approximately 5 ml. The small numbers under the peak (cf. 2a) represent the material recovered for biological and chemical characterization, and the small vertical lines (B to D) represent the width of the band recovered. A, pattern obtained when Peak 2 of Fig. 2C was rerun; B, Peak 3; C, Peak 4; and D, Peak 5. Peaks 2a, 3a, 4a, and 5a appear as a single peak when rerun on the G-25 columns.

FIG. 5. Pattern obtained after starch gel electrophoresis of (left to right) trichloroacetic acid powder, Peak 5a after gel filtration on both Sephadex G-100 and G-25 (Fig. 4D), Peak 4a after similar processing (Fig. 4C), Peak 4 after gel filtration on G-100 (Fig. 2C), and Peak 5 after similar processing (Fig. 2C). Note that the second and third bands (after the second column) are sharper than their counterparts, bands 3 and 4.
cates that this hormone also produces striking effects upon calcium release, phosphate uptake, and phosphate-dependent respiration when added in vitro to isolated mitochondria (13-15). These various responses are now being studied in an effort to develop a simple and reliable assay technique for this hormone in vitro. However, one of the difficulties encountered was the discovery that peptides 2 and 5 possessed some but not all of these biological properties (Table II).

The results of the various assay techniques have been reported as relative values, with the response to peptide 4 used as the standard, because at present only one of the methods (11) is in widespread use and generally accepted as a method of assay for the parathyroid hormone. Phosphaturic activity was determined by means of a technique similar to that described by Cotlove (16) and employed by MacIntyre, Boss, and Troughton (17). The mitochondrial assays were carried out under the conditions previously described (13-15). The results of a typical mitochondrial assay with peptides 2, 4, and 5 are shown in

**Fig. 6. Schematic representation of patterns of peptide bands obtained on starch gel electrophoresis.**

Crude trichloroacetic acid powder (TCA) (urea-cysteine-HCl): 2, Peak 2, Fig. 2C; 2a, Fig. 4A; 3, Peak 3, Fig. 2C; 3a, Fig. 4B; 4, Peak 4, Fig. 2C; 4a, Fig. 4C; 5, Peak 5, Fig. 2C; 5a, Fig. 4D.

**Table II**

Relative potency of peptides 2, 4, and 5 when assayed by various techniques in vivo and in vitro

See the text for a description of these techniques. All data are presented as percentage of effectiveness compared to the response obtained with peptide 4.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>In vivo (parathyroidectomized rat)</th>
<th>In vitro (mitochondria)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcium-mobilizing</td>
<td>Phosphate excretion</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>60</td>
</tr>
</tbody>
</table>

* The peptide was not tested in this system.

**Fig. 7. The effects of peptides 2a (●), 4a (○), and 5a (□) upon the accumulation of 32P in isolated mitochondria.** Incubation was carried out at 30°C as previously described (14) in a medium containing liver mitochondria equivalent to 0.7 mg of nitrogen, 4 μmoles of sodium phosphate, pH 7.1, 50 μmoles of imidazole buffer, pH 7.4, 610 μmoles of sucrose, 0.015 μc of 32P, 20 μmoles of MgCl2, 30 μmoles of sodium glutamate, 2 μmoles of ATP, and 5 μg of oligomycin B in a total volume of 3.0 ml. After 5 minutes of incubation, varying amounts of the respective peptides were added in 100 μl of 0.01 M acetic acid, and samples were removed 15 minutes later to be plated, dried, and counted as previously described (14).

**Fig. 7.** It is particularly noteworthy that even though peptide 2 gives only 75% of maximal response obtained with peptide 4, it is effective at lower concentrations than peptide 4.

**DISCUSSION**

The development of improved techniques for the isolation and identification of bovine parathyroid hormone has led to the quite unexpected identification of several polypeptides possessing biological properties similar in one or more respects to those of the polypeptide considered to be the parathyroid hormone. It is important to point out that these newly isolated peptides are distinct from the several isolated previously (2-6). Those previously isolated were obtained by the extraction of crude gland powder with hot acetic acid, hot hydrochloric acid, or phenol solutions. By all indications the peptide isolated from phenolic extracts corresponds to peptide 4 of the present study, and the peptides isolated from the two acid extracts represent partially hydrolyzed fragments of this peptide. They all possessed both calcium-mobilizing and phosphaturic activity when given to normal or parathyroidectomized rats. In contrast, the present peptides 2 and 5 coexist with peptide 4 either in urea-HCl extracts or in phenol extracts. In the latter case, peptides with properties similar to those described for peptides 2 and 5 have been isolated from phenol extracts in these laboratories during the course of the development of the present isolation technique.

It is not yet possible to decide whether these two new peptides, 2 and 5, are fragments of peptide 4 or normal constituents of the parathyroid glands. Our preliminary studies of their chemical composition lead us to believe that they are not fragments, par-
particularly in view of the fact that initial extraction with either phenol or urea-HCl leads to a nearly identical mixture of peptides. If these two peptides do exist normally, then the question of their physiological significance becomes of considerable interest, and the issue of more than one parathyroid hormone needs reconsideration.

The present results re-emphasize the paramount importance of having well characterized and homogeneous peptides for any study of the mode of their action. In the present case, this becomes even more important because of our recent findings concerning Peak 3. As noted in Fig. 6, this peak has not been completely resolved into its separate components, but none of its constituents possesses any of the activities attributed to peptide 4. Nevertheless, the material obtained from Peak 3 has the ability to promote aerobic glycolysis when added in vitro to isolated Ehrlich ascites tumor cells. This is of considerable interest in view of the reports that the administration of crude parathyroid extract to rabbits induces, in bone slices obtained from the animals 18 to 24 hours later, an increased aerobic glycolysis (18, 19) and an increased conversion of 14C-glucose to 14CO2. This stimulation of glycolysis has been proposed as one of the primary actions of this hormone upon bone metabolism. In view of the above findings, it is obviously necessary to re-examine critically this and other responses with the use of purified hormonal peptides.

A dichotomy between effects with steroid hormones in vivo and in vitro has been well established, but has been noted less frequently in the case of peptide hormones. However, recent studies with bovine growth hormone have uncovered a situation somewhat analogous to the present one. Manchester and Wallis (20) have separated three fractions of an ox growth hormone preparation, all of which possess nearly identical activities in vitro but vary greatly in their ability to promote growth in hypophysectomized rats.

The development of a method of starch gel electrophoresis applicable to our particular problem was the single most important step in the results achieved. The method of starch gel electrophoresis in strong urea solutions was chosen in order to minimize any possible interactions between the peptides in the mixture. However, as illustrated in Fig. 1, the resolution was far from ideal even under these circumstances until EDTA was employed as well. The most likely explanation for the striking improvement in resolution effected by this agent is that the EDTA complexed heavy metal ions present both in the urea and in the starch and thereby reduced interactions between the peptide and the supporting medium.

The present isolation procedure represents a considerable improvement in both reliability and reproducibility over those previously described. The method should be applicable to the isolation of peptides from other complex mixtures. The use of the urea-HCl offers several distinct practical advantages over that of phenol, particularly with regard to ease and safety of handling. In our hands the product obtained is more stable and more homogeneous on starch gel electrophoresis. It is to be noted that during the course of this work, mention was made by Bereon et al. (21) of a method of isolation in which urea solutions were used for extraction of this hormone. Unfortunately no details were given, nor has any publication of the method appeared subsequently, so that it is not possible to compare its effectiveness with that of the present technique.

1 A. Tenenhouse and H. Rasmussen, unpublished observations.
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