Isolation of Parathyroid Hormone after Extraction with Phenol

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(Received for publication, July 17, 1959)

In 1925, Collip (1) reported that an endocrine substance, extracted from parathyroid tissue with hot hydrochloric acid, corrected the hypocalcemia which followed parathyroidectomy. Extracts made by this method had been important in early biological studies, but have been difficult to purify, and have seemed to contain several active, but unstable components (2, 3).

Later other solvents have been used in an effort to obtain the active principle in more homogeneous form. A potent fraction has been prepared with weak acid in the cold (4), but simple aqueous solutions generally have been poor extractants. Rasmussen (5, 6) has obtained highly purified, although incompletely stable material from extracts made with hot 80% acetic acid, a solvent advanced for this purpose by Davies and Gordon (7).

Strong phenol solutions have been used to extract a more stable, uniform substance in high yield (8). The product obtained by this means was easily purified to a form suitable for further chemical and biological studies, and its isolation is described herein.

EXPERIMENTAL

The modification of Lowry (9) was used for measuring protein concentration with Folin reagent. As previously described (10), fractions prepared in gelatin medium were assayed against parathyroid injection, U.S.P. (Lilly) as a standard by measuring the calcium in the serum of parathyroidectomized rats 4 hours after injection.

RESULTS

Fat was removed from acetone-dried glands1 (708 g equivalent to 5084 g of fresh glands) by homogenizing successively with acetone (2.5 ml per g), and twice with chloroform (each with 2.5 ml per g). After a final extraction with acetone (1.7 ml per g), the material was washed with acetone and dried in air; 708 g yielded 499 g of crude gland powder. The method of extraction employed earlier (8) was simplified by the use of a concentration of phenol greater than 73%. Crude gland powder was stirred with 90% weight per volume phenol (liquefied phenol, U.S.P., 10 ml per g of tissue) for 2 hours at room temperature; 5 volumes of a solution of acetic acid-acetone, 1:4, and 0.004 M sodium chloride were added, and the mixture was cooled at 5° for 1 hour. The inert solids were removed by filtration, and an equal volume of ether was added to the filtrate. After standing overnight in the cold, the active precipitate was collected on a filter, washed with acetone, and dried in a vacuum. The crude extract assayed 20 units per mg and averaged 5.6% of the weight of defatted powder in six experiments. The yield was 20% higher than that obtained from smaller amounts of crude powder (8).

The dried crude extract (4 to 6 g) was dissolved in 80% acetic acid (20 ml per g), 3 volumes of water were added, and the mixture was filtered with the aid of Celite. Sodium chloride, added to a concentration of 6% in the filtrate, caused formation of a precipitate which was allowed to settle and was then removed by filtration. The precipitate was re-extracted with 80% acetic acid (20 ml per g of crude extract), 3 volumes of water were added, and inert matter was precipitated with 6% sodium chloride. To the combined filtrates, trichloroacetic acid was added to a concentration of 3%, and the mixture was cooled at 5° for 1 hour. The precipitate, collected by centrifugation, was washed once with 3% trichloroacetic acid, suspended in 0.02 N hydrochloric acid (10 ml per g crude extract), and extracted five times with 1.5 volumes of peroxide-free ether. The clear solution was treated with freshly washed IRA-400 acetate resin and lyophilized. In seven experiments the weight of this product averaged 6.3 (2 to 10)% that of the crude extract and assayed 340 (200 to 600) units per mg. Approximately 10% of the total activity of the crude extract was discarded in the second salt precipitate.

The partially purified material was dissolved in 20% acetic acid at a concentration of 4 to 5 mg per ml and sodium chloride was added to 3%. After saturating the solution with n-butanol it was loaded into the first three tubes of a countercurrent train (10 ml per phase per tube). The pattern shown in Fig. 1 was obtained after 100 transfers had been carried out in a system of n-butanol against 20% acetic acid-3% sodium chloride; 80 to 90% of the activity was found in the central region, which corresponded to a distribution coefficient of 0.85. The remainder of the activity was divided equally among the other two peaks. Redistribution for 56 transfers of the material recovered from a 100-tube fractionation gave a pattern close to theoretical (Fig. 2). The active fraction was recovered by evaporating the solvents to dryness, suspending the solids in weak acetic acid, and precipitating the hormone with 3% trichloroacetic acid. In two preliminary experiments recovery was tested with a method reported by Dixon (11). The hormone was adsorbed onto a 2 × 6 cm column of Amberlite XE-64 resin in the hydrogen form from a 6% sodium chloride solution adjusted to pH 3.0 to 3.5 with acetic acid. Salts were washed from the column with

1 Generously supplied by Dr. C. Irwin, Eli Lilly Company, Indianapolis, Indiana.
5% acetic acid and the hormone was then successfully eluted with 50% acetic acid but still contained some salt. The countercurrent products, lyophilized as the acetate, were soluble in water, acid, alkali, or salt solutions and contained 13.8% nitrogen (not corrected for water content). Altogether nine distributions of from 56 to 250 transfers each were carried out and the distribution coefficient averaged 0.85 (0.75 to 0.90).

The active substance from 100-tube distributions appeared as a single component with $R_p$ 0.5 to 0.6 when chromatographed on paper in butanol-water-acetic acid, 50:50:18.

The activity of products at each stage of purification was not altered significantly when tested 2 months after storage as dry powders at room temperature. Data from several statistically valid assays of purified material are shown in Table I. Though less than optimal, the precision of these assays was surprising in view of the small number of animals used. The potency of the final product was similar in comparison with the bioassay standard when tested by measuring phosphorus excretion in parathyroidectomized rats (13).

**DISCUSSION**

Parathyroid hormone seemed tightly bound in glandular tissue and could not be removed with simple aqueous solvents which readily dissolve the purified substance (3). But it was observed, in extension of the findings of Rasmussen, that treatment of the tissue with hot 0.2 N hydrochloric acid, conditions likely to hydrolyze nucleic acids, freed adenine and guanine as well as the active principle. This suggested that an important factor in extraction of the hormone may be its release from closely associated nuclear matter. The use of phenol to separate proteins from nucleic acids (14, 15), and its successful application for extraction of parathyroid hormone, supported this thesis. The active principle in crude extracts had been shown to be stable to and soluble in phenol by Tweedy (16). Aqueous solutions of 60% guanidine or 100% trichloroacetic acid, useful solvents for nucleoprotein, were also effective extractants, but the product seemed unstable after treatment with these agents.

Active fractions from either of the two commonly used hot acid extraction methods could be adsorbed on carboxymethyl cellulose (0.7 meq per g) from solution in 1 N acetic acid. However, the phenol-extracted material seemed to be a less basic compound and was not retained under these conditions. Dissimilar behavior between extracts made with acetic acid and of those made with hydrochloric acid have been reported (17), and it is likely that the active fractions derived from the several extraction procedures are different but probably closely related. The stable nature of the fractions described in the present report was difficult to explain in view of the findings of Rasmussen (18). He observed losses of activity from 50 to 70% when purified acetic acid material was stored as a dry powder for 1 to 2 weeks. Glycine added before drying partially prevented the losses and after inactivation potency could be restored by treatment with cysteine. Thus inactivation seemed related to oxidation, but this does not fully explain the greater stability of products prepared with phenol. Structural studies may resolve the differences between materials derived by different methods.

Bioassays indicated that crude phenol extracts were slightly

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**Table I**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Assay No.</th>
<th>Potency†</th>
<th>Index of precision λ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Limits of potency $p = 0.05$</td>
</tr>
<tr>
<td>4-22</td>
<td>a</td>
<td>2430</td>
<td>1,250–7,100</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1380</td>
<td>480–5,850</td>
</tr>
<tr>
<td>5-14</td>
<td>a</td>
<td>3080</td>
<td>1,250–16,000</td>
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<tr>
<td></td>
<td>b</td>
<td>4080</td>
<td>2,320–8,880</td>
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<tr>
<td></td>
<td>c</td>
<td>3800</td>
<td>1,140–24,600</td>
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<tr>
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<td>a</td>
<td>1450</td>
<td>740–3,120</td>
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<tr>
<td></td>
<td>b</td>
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<tr>
<td>Average</td>
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<td>1,460–11,600</td>
</tr>
</tbody>
</table>

* Calculated according to Gaddum (12) from 4-point assays with four animals per group.

† U.S.P. units per mg dry weight (not corrected for ash content which ranged from 2 to 20%).

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more active than the commercial material used as a standard, and that potency was easily increased 15-fold by salt fractionation. The further 10-fold concentration achieved by countercurrent distribution gave a product 2800 times as active as crude gland powder. Within the limits of the assays used the activity of the final product agreed well with this degree of concentration and indicated that large losses were not sustained.

Although 100-transfer distributions were used for preparative purposes, the major impurities were widely separated from the fraction of interest, and 6 to 8 transfers should allow recovery of the hormone in sufficient purity for most purposes.

Early crude extracts which had marked effects on calcium metabolism also enhanced the excretion of phosphate in the urine (19). Other investigators had proposed that a distinct hormone may account for the latter effect. The isolation of a single substance, a potent mediator of both biological effects, seems to end this controversy.

**SUMMARY**

Parathyroid hormone was prepared from extracts made with phenol at room temperature; the active principle, isolated by countercurrent distribution, was stable and appeared homogeneous on paper chromatograms. Bioassays indicated that little loss of activity occurred during fractionation and the potency of the final product was 2800 units per mg. Both calcium-mobilizing and phosphaturic properties were attributed to a single hormonal substance.

**Acknowledgment** The author is indebted to Dr. E. B. Astwood for much helpful advice during the course of this work. The expert assistance of Miss Lola Bock is gratefully acknowledged.

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