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CONCENTRATION OF PARATHYROID HORMONE ACTIVITY BY CHROMATOGRAPHY ON CARBOXYMETHYLCELLULOSE*

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SUMMARY

A method for the further purification of a fraction salted-out from a dilute hot hydrochloric acid extract of untreated ground bovine parathyroid glands is described. Chromatography on carboxymethylcellulose using a NaCl gradient rising to 1 *M* at pH 4.68 results in an active Ca-mobilizing fraction with a 6-fold increase in specific activity in moderate yield. This can be further increased 2- to 6-fold by recycling and eluting with a gradient rising to 0.5 *M* salt. The method is applicable to starting material containing either high or low amounts of activity.

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INTRODUCTION

The concn. of a fraction with Ca-mobilizing activity directly from a dilute hydrochloric-acid extract of untreated ground frozen bovine parathyroid glands has been reported[1]. The present work describes the further purification of this salted-out fraction by chromatography on carboxymethylcellulose. Active fractions with potencies between 600 and 1000 U.S.P. units/mg nitrogen have been obtained on a single run through the column and the procedure is capable of providing approx. a 12-fold increase in purity of the starting material when a recycling operation is included.

MATERIALS AND METHOD

Starting material

The dialyzed fraction used for chromatography was prepared by the method described previously[1]. Prior to chromatography 30 to 120 ml of this frozen fraction was thawed, then lyophilized, and the thoroughly dry material in the flask was dissolved in the original vol. of fresh ice cold acetate buffer pH 4.68 (0.0049 *M* sodium acetate + 0.0051 *M* acetic acid). Where indicated, this buffer was used throughout this work.

Biological assays and chemical analyses

Fractions were tested for biological activity by measuring the serum calcium 6 h after subcutaneous injection into acutely parathyroidectomized rats[2]. In general, the potencies reported here were obtained by testing the active fractions at 2 dose levels with a 4-fold difference in protein concn. in groups of 6 rats. U.S.P. Parathyroid Extract (Eli Lilly) containing 100 units/ml was used as the reference standard. A dose of not more than 0.1 mg N was used for testing fractions from the initial chromatographic separation and 0.05 mg for fractions from a recycling operation. Nitrogen was determined by the procedure of LOWRY *et al.*[3] using an Armour standard and the protein content of a sample estimated by using a conversion factor (N × 6.25).

Column chromatography

Carboxymethylcellulose (CMC) was prepared either by the method of PETERSON AND SOBER[4] or ELLIS AND SIMPSON[5]. The thoroughly dried powder was sieved through a 300-mesh screen prior to use. Prior to chromatography the total protein of the redissolved starting material was determined and 4 times this weight of CMC was suspended in 30 ml of ice-cold buffer. This suspension was pipetted into a chromatography tube (10 mm inside diameter) which had one end loosely plugged with pyrex glass wool. After settling, the CMC was packed under positive air pressure, using about 10 column vols. of buffer. Preparation of the column as well as operations such as chromatography and dialysis were carried out in a cold room at 4°.

Aliquots of the starting material were allowed to pass through the column by gravity until all had been applied. This was followed by a wash of 8 to 10 column vols. of buffer. The adsorbed protein was then eluted by gradient elution in a closed system[5] under conditions where the rate of flow into and out of the mixing chamber were equal. The mixing vessel contained 250 ml of buffer and the reservoir about

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300 ml of 1 *M* NaCl in the same diluent. The system was adjusted so that the column delivered 1 drop every 9 sec and eluates of approx. 6.8 ml/tube were collected on an automatic fraction collector. In practice, only the first 25 tubes were collected. Tubes 6 to 10 which contained the major portion of the adsorbed Ca-mobilizing activity were pooled and dialyzed against 1-l aliquots of cold distilled water with mechanical agitation inside and outside the sac. The water was changed at 30 to 45 min intervals for a total of 3 h. The dialyzed solution was then lyophilized and redissolved in about one-third the original vol. of buffer. The total protein of the redissolved material was determined and 10 times this weight of CMC was prepared into a column, using the same diameter chromatography tube. During the recycling process, the conditions of adsorption, washing and gradient-elution were similar to those described previously except that the reservoir now contained 0.5 *M* NaCl. About 75 to 110 tubes containing eluates between 2.5 and 3.5 ml were collected. An aliquot of each tube (0.1 ml) was tested for its nitrogen content and the major protein peak located. This material was divided into 3 consecutive pooled fractions. Each pool was dialyzed, lyophilized, and redissolved in a smaller vol. of water adjusted to pH 3 by the dropwise addition of 1.5 *N* HCl. On assay, the most active Ca-mobilizing fraction eluted between 0.225 and 0.275 *M* salt, which corresponded to the central portion of this peak.

RESULTS

In test-tube expts. with varying amounts of CMC, it was found that a quantity of adsorbant equiv. to 4 times the weight of the total protein in the starting material effectively retained a portion of the Ca-mobilizing activity at pH 4.68 which could then be removed by stepwise elution with NaCl. Larger quantities of adsorbent bound the activity more tightly to give poorer yields. Best results as well as the sharpest resolution were obtained when this quantity of adsorbent was packed into

TABLE I

EFFECTIVENESS OF CHROMATOGRAPHY ON CARBOXYMETHYLCELLULOSE (CMC)

Assays 189, 193, 196 and 205 are evaluations of pooled material from tubes 6 to 10 obtained from first runs through the CMC column. The left hand column in (d) represents the increase in specific activity of these fractions as compared with the original starting material. Assays 200, 204 and 202 show results obtained upon recycling several such fractions from that used in assay 205. In this instance, the left hand column in (d) represents the total increase in specific activity over the original starting material whereas the right hand column shows the increase afforded by the recycling operation. Each assay represents data from a separate distinct chromatographic run through the CMC column.

Assay No.	*(a)* *Specific activity* *μ PTH/Mg N*	*(b)* *Factor for standard error of assay*[13] $\left(\frac{\times}{\div}\right)$	*(c)* *Yield* *(% recovery)*	*(d)* *Purity* $\frac{CMC}{Starting\ material}$	*(d)* *Purity* $\frac{Recycle\ CMC}{CMC}$
189	1370	1.72	6	6	—
193	616	1.24	72	5.5	—
196	684	1.24	59	5.7	—
205	175	1.22	22	6	—
200	416	1.28	3	13	2.5
204	320	1.57	4	12	1.8
202	1200	1.87	8	34	6.0

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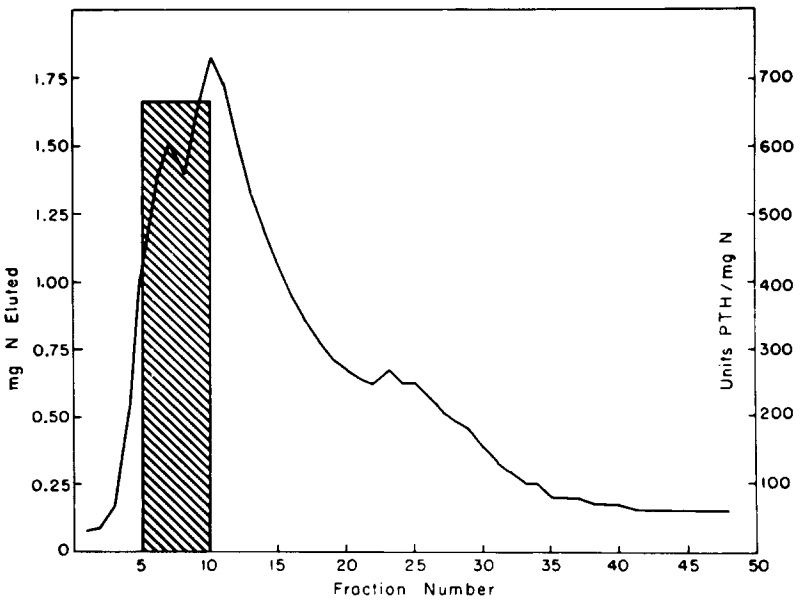

Fig. 1. Chromatography of 0.3–0.4 saturated ammonium sulfate fraction on carboxymethylcellulose. 128 mg protein (120 units PTH/mg N) adsorbed on column. Ratio protein to CMC-1:4. Effluent collected in 6.8-ml fractions. Gradient rising to 1 *M* NaCl in acetate buffer pH 4.6, 0.01 μ. Mixing chamber vol., 250 ml. Temp. 4°.

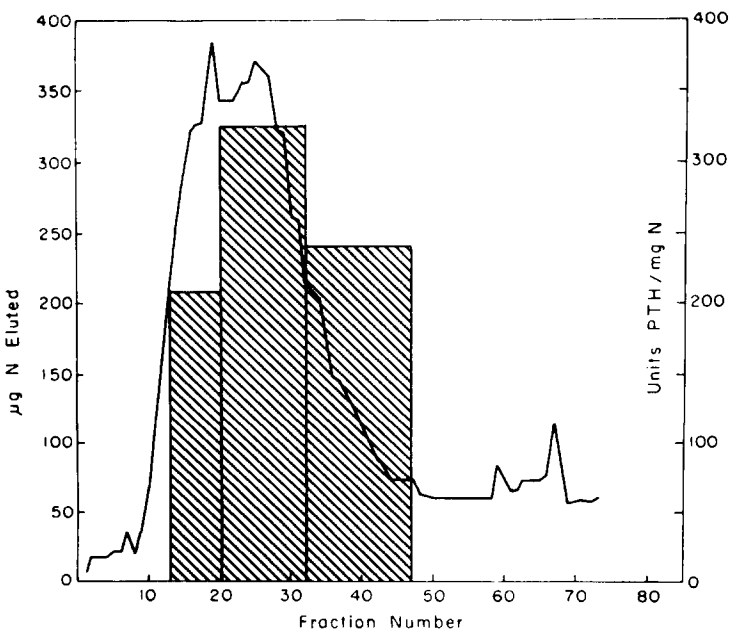

Fig. 2. Recycling of active fraction on carboxymethylcellulose. 61 mg protein (175 units PTH/mg N) adsorbed on column. Ratio protein to CMC, 1:10. Effluent collected in 3.5-ml fractions. Gradient rising to 0.5 *M* NaCl in acetate buffer pH 4.6, 0.01 μ. Mixing chamber volume 250 ml. Temp. 4°.

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a column and a sharp gradient rising to 1 *M* salt applied. Two types of starting materials were used in this work. Either of them, when chromatographed on CMC provided active material with approx. a 6-fold increase in specific activity on a single run through the column (Table I, col. (d)). The first, containing 120 to 150 units parathyroid hormone (PTH)/mg N, was prepared from the usual hot acid extract. Active fractions obtained from this starting material following chromatography had an average potency of 890 U.S.P. units PTH/mg N at a yield of 45 % (Table I, assays 189, 193, 196). Two additional such fractions prepared similarly during exploration of this method had estimated potencies within the range reported in Table I (about 1000 units) when their assay values were extrapolated to a standard curve.

A typical elution diagram is shown in Fig. 1. A major portion of the Ca-mobilizing activity was found to elute with an early part of the main protein peak between tubes 6 to 10. In some runs, pools made from tubes 1 to 5 and 11 to 15 had smaller amounts of activity indicating trailing. On continued elution, about 85 to 92 % of the adsorbed nitrogen but no further activity could be recovered. The active fraction had the u.v. spectrum for a protein, with a high absorption at wave lengths below

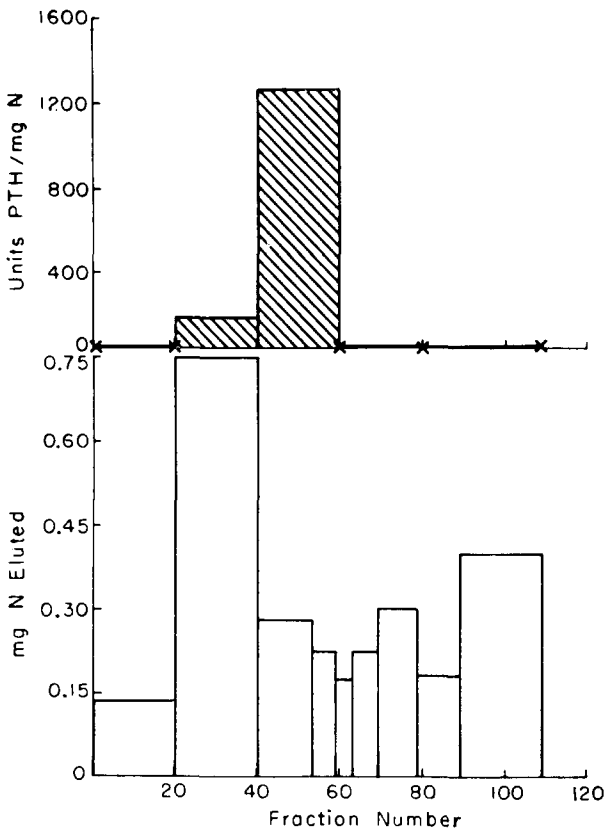

Fig. 3. Recycling of active fraction on carboxymethylcellulose. 28.3 mg protein (100 units PTH per mg N) adsorbed on column. Ratio protein to CMC, 1:10. Effluent collected in 2.5 ml vols. Gradient rising to 0.5 *M* NaCl in acetate buffer, pH 4.6, 0.01 μ. Mixing chamber vol. 250 ml. Temp. 4°. Fractions were pooled as indicated and tested.

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230 mμ followed by another peak at 275 mμ with a 280/260 mμ ratio of 1.3. Representative samples also possessed an equiv. level of phosphaturic activity[6] indicating that no separation from the Ca-mobilizing principle occurs at this stage of purification. Short term dialysis expts. under the conditions described here showed that at pH's between 4.6 to 5.5, over 90 % of the N was retained in the dialysis sac, although this does not exclude the possibility that some of the activity was lost during this process.

The second type of starting material contained 28 units /mg N. It was prepared from an extract that initially had a negligible amount of activity (less than 10 units). Active fractions prepared from this batch averaged 175 units /mg N at a yield of 22 % (assay 205). In an attempt to increase the potency of this fraction, it was recycled on a column using a gradient rising to 0.5 *M* salt. The results of these runs are shown in Table I, assays 200, 204 and 202 and elution diagrams are shown in Figs. 2 and 3. Division of the main protein peak into 3 fractions gave central portions (between 0.225 and 0.275 *M* salt) with a further 2- to 6-fold increase in specific activity at about a 7 % yield.

DISCUSSION

The purification procedure outlined here offers the advantage of requiring few manipulative steps to produce a potent parathyroid extract in moderate yield from starting material with either a low or high amount of activity. A single pass through the column provides a 6-fold increase in specific activity. The low activity fraction may be further purified by recycling to give additionally an approximate 2- to 6-fold rise in specific activity and it is reasonable to assume that high activity starting material would behave similarly. The overall process therefore provides a 25 to 30-fold increase in purity of the hot acid extract and several of the preparations have exceeded 1000 U.S.P. units PTH /mg N. On this score, this simple method can provide material whose activity equals or surpasses those previously described for preparing a potent parathyroid extract from a dilute hydrochloric acid solution of bovine glands[7,8,9]. It compares favorably with the high potency extract obtained by zonal-electrophoresis of an ultrafiltrate of an acetic acid solution of these glands[10]. The possibility of stabilizing these extracts and extending their potency by the incorporation of cysteine has recently been reported[11]. The method further attests to the usefulness of cellulose ion-exchange adsorbents for hormone purification[5,12].

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THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

III. MECHANISM OF THE REVERSIBLE POLYMERIZATION OF THETIN-HOMOCYSTEINE METHYLPHERASE AND ITS RELATION TO THE MECHANISM OF METHIONINE SYNTHESIS

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SUMMARY

1. Purified horse liver thetin-homocysteine methylpherase undergoes a reversible polymerization reaction in addition to catalyzing the synthesis of methionine by transmethylation from dimethylthetin and homocysteine.

2. The mechanism of the 2 reactions has been investigated in a variety of ways.

3. The reversible polymerization appears to be due to the formation and cleavage of intermolecular disulfide bonds. Separate sites of the protein molecule seem to be involved in the polymerization and the transmethylation reactions.

4. There is no evidence to suggest that a methylated enzyme functions as an intermediate in the transmethylation reaction.

INTRODUCTION

Thetin-homocysteine methylpherase has been purified from horse liver[1] and it invariably has been found to consist of a mixture of polymers of varying molecular weight. Treatment with any one of several thiols is required to transform all of the polymerized

* 15th paper in a series on enzymic mechanisms in transmethylation.
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