PURIFICATION OF BOVINE PARATHYROID HORMONE BY GEL FILTRATION

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SUMMARY

A method is described for the isolation of bovine parathyroid hormone by the technique of gel filtration.

This material has an amino acid composition similar to that of material isolated by countercurrent distribution. A revised estimate of the amino acid residues per molecule (75) and minimum molecular weight (8447) are given. The empirical formula is: Lys_7, His_3, Arg_4, Asp_8, Thr, Ser_7, Glu_{10}, Pro_3, Gly_4, Ala_6, Val_6, $Meth_2$, $Ileu_3$, Leu_7, Tyr, Phe_2, Try, and $(CONH_2)_7$.

INTRODUCTION

Using the technique of countercurrent distribution, we have isolated, in relatively pure form, a family of parathyroid polypeptides[1-3], ranging in molecular weight from 3800 to 8500. Our data indicate that the smaller ones are active fragments of the larger "native" hormone having a molecular weight of at least 8500.

Although the technique of countercurrent distribution has proven to be most useful in these purification studies, repeated attempts have been made by ourselves and others[4-6], to develop more convenient and less time consuming methods for the isolation of the parathyroid hormone. To date, none have been completely successful.

The results of PORATH[7], using the technique of gel filtration on beds of dextran gels, suggested that purification of parathyroid hormone might be achieved by this means. Our initial studies with the technique were only partially successful[2]. However, by changing the nature of the eluting agent, we have now been able to isolate the parathyroid hormone from crude extracts of bovine glands using this technique. It is the purpose of this paper to describe the results of these studies.

MATERIALS AND METHODS

The starting material for the filtration experiments was a trichloroacetic acid precipitate prepared from phenol extracts of defatted bovine parathyroid glands, according to AURBACH[8,9]. It had a biological activity of 275–375 U.S.P. units/mg

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when assayed by the technique of MUNSON[10], the assay method used throughout this study.

The dextran resin (Sephadex G-50) manufactured by Ly Pharmacia, Uppsala, Sweden, was screened dry through a 120-mesh sieve and the material which passed through was screened on a 200-mesh sieve. The material remaining on the 200-mesh screen (10–15% of the original resin) was taken up in buffer, allowed to settle for 10 min and the supernatant decanted. This was repeated three or four times until all the fine resin particles had been removed. The wet resin was mixed with an equal volume of buffer and poured into a column in 30-cm sections. The columns employed were 0.9 × 150 cm, 2.0 × 210 cm, and 3.0 × 210 cm. Various eluting solutions were studied, ranging from 0.2 *M* acetic acid to a variety of ammonium acetate buffers. The buffer finally chosen was prepared by mixing equal volumes of 0.6 *M* acetic acid and 0.4 *M* ammonium hydroxide, pH 4.72 ± 0.05. To recover the various fractions, the column eluent was diluted with an equal volume of distilled water and then lyophilized. The resulting powder was difficult to recover because of a marked tendency to adhere to the side of the lyophilization flask. For this reason, it was dissolved in 0.1 *M* acetic acid and lyophilized a second time.

To prepare the sample for the column, 300 mg of crude hormone powder was taken up in 10 ml of buffer. A moderate amount of the powder was insoluble. This was separated from the supernatant by centrifugation and reextracted with 10 ml of buffer, centrifuged again, and the second supernatant combined with the first. The combined supernatants (17–18 ml) were placed onto a column 3.0 × 210 cm and allowed to flow into the resin bed. It was washed onto the resin with small volumes of buffer, 2–3 ml, and then elution began with the level of buffer in the reservoir at the level of the top of the column. The flow rate was approx. 20 ml/h, and the effluent was collected in 5-ml fractions. All experiments were carried out at a constant temperature of 18°.

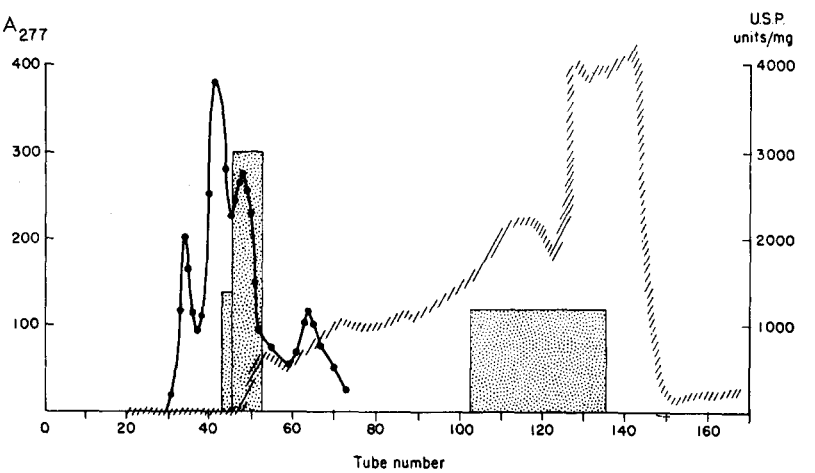


Fig. 1. Gel filtration of crude parathyroid extract. The solid line, ———, represents the effluent pattern observed when 0.2 *M* ammonium acetate, pH 4.72 was used as the eluent; the shaded line, ////////, is that obtained when 0.2 *M* acetic acid was employed. The stippled areas represent the location of hormonal activity under the two different conditions. The column was 3.0 × 210 cm of Sephadex, G-50.

Material obtained from a single countercurrent distribution either in *n*-butanol, 0.1% acetic acid, pyridine (5:12:3.5) or *n*-butanol, 0.15 *M* ammonium acetate in 0.1% acetic acid, pyridine (5:12:5.4) was also subjected to gel filtration.

Protein concentration was determined by ultraviolet absorption at 277 mμ in a Beckman DU spectrophotometer. Amino acid analysis was performed by the method of SPACKMAN, STEIN AND MOORE[11]. Tryptophan was determined by the method of GOODWIN AND MORTON[12]. The film dialysis studies were carried out as previously described[13].

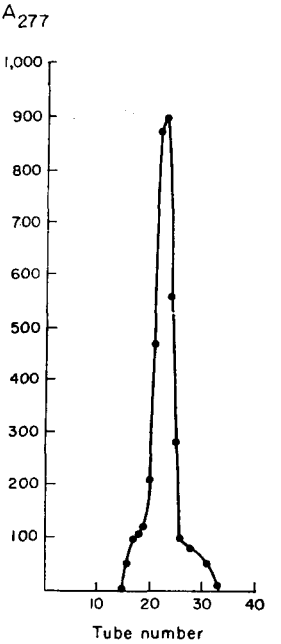


Fig. 2. Gel filtration of the material recovered from tubes 44–51, Fig. 1. The column was 0.9 × 150 cm.

RESULTS

The results, obtained when 300 mg of crude hormone powder was run on a 3.0 × 210 cm column are shown in Fig. 1. For comparison, the effluent pattern obtained when a similar experiment was done using 0.2 *M* acetic acid as the eluent is recorded (shaded line). The hormonal activity was located predominantly in tubes 47–53, when 0.2 *M* ammonium acetate was the eluent. When the material from tubes 47–53 was rerun on a column 0.9 × 150 cm the pattern shown in Fig. 2 was observed. The protein in tubes 20–24 was recovered, assayed for its biological activity, and analysed for its content of amino acids. A comparison of these values with those obtained on a sample of hormone obtained by countercurrent distribution is given in Table I.

To test the efficacy of this technique as compared to that of countercurrent distribution[2], equal aliquots of the same crude preparation were purified by the two means. The yield of pure material from distribution was only 65% of that obtained by gel filtration.

TABLE I

THE AMINO ACID COMPOSITION OF BOVINE PARATHYROID HORMONE

The column headed CCD is the composition calculated from 14 separate analyses of eight different samples obtained over a period of three years by countercurrent distribution[3]. The column headed Sephadex is the composition calculated from 7 separate analyses of four different samples obtained over a period of six months. The residues per molecule were calculated from the values of arginine, lysine, aspartic acid, glutamic acid, leucine, and alanine, rather than on the basis of tyrosine alone, as previously done[2].

Amino acid	*CCD*	*Sephadex*
Lysine	7.06 (7)	7.11 (7)
Histidine	2.75 (3)	2.77 (3)
Ammonia	7.58 (7)	—
Arginine	3.98 (4)	3.98 (4)
Aspartic	7.71 (8)	7.29 (8)
Threonine	0.78 (1)	1.12 (1)
Serine	6.79 (7)	5.55 (7)
Glutamic	9.99 (10)	9.96 (10)
Proline	2.22 (2)	2.71 (3)
Glycine	3.82 (4)	3.89 (4)
Alanine	6.11 (6)	5.99 (6)
Valine	6.02 (6)	5.69 (6)
Methionine	1.66 (2)	1.67 (2)
Isoleucine	2.57 (3)	2.76 (3)
Leucine	6.93 (7)	7.20 (7)
Tyrosine	0.88 (1)	1.03 (1)
Phenylalanine	1.85 (2)	1.92 (2)
Tryptophan	(1)	(1)
	74	75
Biological activity U.S.P. units/mg	2400–3200	2200–3000

Minimum molecular weight: 8447

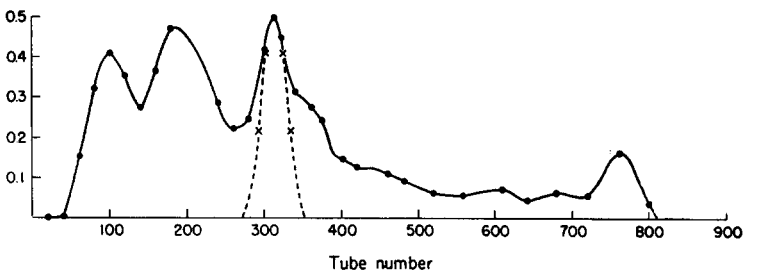


Fig. 3. Countercurrent distribution of crude parathyroid extract in pyridine–*n*-butanol–0.15 *M* ammonium acetate in 0.1 % acetic acid (4:5:12.5). The hormonal activity was located in the peak with a partition coefficient of 0.31 (tubes 285–350).

Some comparison of the resolving power of the two methods is apparent from Figs. 3 and 4. Illustrated in Fig. 3 is a distribution pattern obtained after a crude extract had been subjected to 1300 transfers in the solvent system *n*-butanol–pyridine–0.15 *M* ammonium acetate (5:4:12.5). When the material from tubes 290–350 was chromatographed on a 0.9 × 150 cm column of Sephadex, the pattern shown in Fig. 4 was obtained. The material from the major peak (tubes 19–22) had

an amino acid composition and biological activity comparable to those recorded in Table I.

As reported previously[2], and illustrated in Fig. 5, the major hormonal activity of a crude extract distributed with a partition coefficient (K) of 0.255 in the system

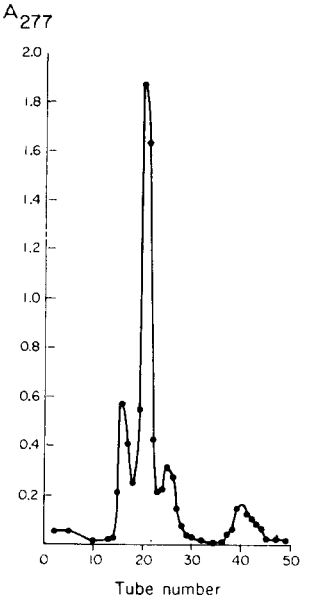


Fig. 4. Gel filtration of material recovered from tubes 290–350, Fig. 3, on a column of Sephadex, G-50, 0.9 × 150 cm. The major peak contained 90% of the estimated original hormonal activity.

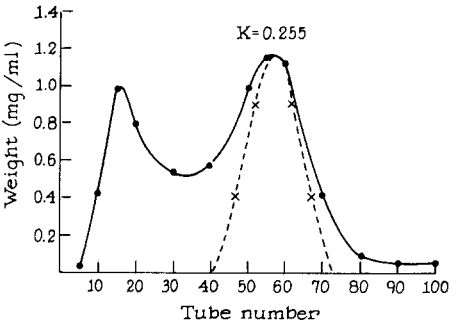
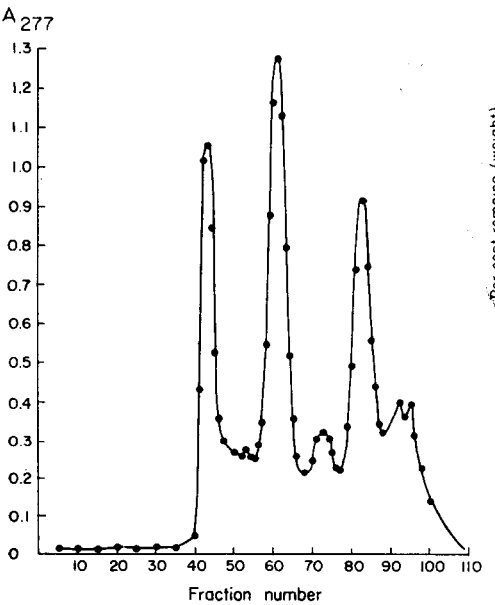


Fig. 5. The countercurrent distribution pattern of a crude extract of parathyroid glands in a system of n-butanol–0.1% acetic acid–pyridine[2].



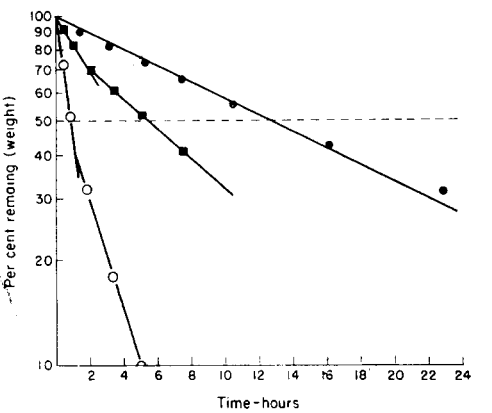


Fig. 7. The rate of dialysis of parathyroid hormone through a cellophane membrane (Visking No. 20/32) in 0.2 M ammonium acetate, pH 4.73, ●—●; 0.2 M acetic acid, ■—■; and 0.01 M acetic acid, ○—○.

Fig. 6. The filtration diagram of the partially purified parathyroid extract obtained on a 2.5 × 210 cm column of Sephadex, G-50, using a 0.2 M ammonium acetate buffer, pH 4.8. The starting material was from tubes 26–42, Fig. 5.

n-butanol–pyridine–0.1% acetic acid (5:3.5:12). However, the material in tubes 26–42 contained considerable biological activity (400–800 units/mg). This material was also subjected to gel filtration with the results shown in Fig. 6. The material from the largest peak (tubes 58–65) had the highest biological activity (2000–2600 units/mg) and had an amino acid composition similar to that recorded in Table I. The fourth peak had moderate biological activity (600–1000 units/mg) and was not further characterized.

The purified material obtained by gel filtration was studied by the techniques of membrane diffusion using different solvents. The results are shown in Fig. 7, and indicate that the rate of diffusion of the pure hormone is approximately half as fast in 0.2 *M* ammonium acetate, and approx. four times as rapid in 0.01 *M* acetic acid as in 0.2 *M* acetic acid.

DISCUSSION

It is apparent that the purification of bovine parathyroid hormone can be achieved using gel filtration rather than countercurrent distribution. In fact, it would appear that the resolving power of this technique compares favorably with that of countercurrent distribution, as illustrated in Figs. 3 and 4. The higher recoveries obtained using filtration probably indicate that even under the best conditions yet obtained, there is some transformation of hormone during prolonged countercurrent distribution.

The material obtained after a single filtration should be adequate for most physiological and biochemical studies, but a second filtration step seems necessary to obtain material suitable for detailed chemical analyses.

No attempt has been made to study all the parameters which influence the filtration process. However, from the data presented in Fig. 1, it is apparent that the process is far more complex than simple molecular sieving. The marked influence that a change in the eluent, from 0.2 *M* acetic acid to 0.2 *M* ammonium acetate, had upon both the separation of the individual components, and their rate of elution from the column suggests that several factors may be operative. The most obvious possibilities being changes in the gel structure of the resin, changes in its ion-exchange capacity, and changes in the conformation of the polypeptide molecules due to the altered environment. The latter factor seems to be the predominant one as indicated by the marked change in the rate of diffusion of the hormone through a porous cellophane membrane (Fig. 7) with changes in the ionic environment. A change from dilute to more concentrated acetic acid, to ammonium acetate resulted in a progressive decrease in the rate of passage through the membrane and therefore presumably a decreased rate of penetration into the resin particles. Whether this factor is responsible for the sharpening of the peaks in the filtration process cannot be answered at present.

Regardless of which of these factors is operative, the data indicate that changes in pH and ionic strength markedly influence the filtration behavior of larger polypeptides, and suggest that by the proper selection of eluting agents, it should be possible to separate mixtures of large polypeptides by this technique. These results plus those of PORATH *et al.*[10,14,15] indicate the possibilities for the widespread use of these gels for polypeptide purification.

The previously reported values for the number of amino acid residues per molecule were obtained by using the tyrosine values as the basis of calculation. However, further experience has indicated that these values are often 20% too low and therefore

that the estimated number of residues of several amino acids is too high. The first column in Table I was calculated on the assumption that there are ten glutamic acid, six alanine, seven leucine, four arginine, seven lysine and two phenylalanine residues per molecule. Using this basis, the calculated residues per molecule are quite consistent, and represent a more accurate estimate of the amino acid composition of the bovine parathyroid hormone. There are minor discrepancies between the composition of the material obtained by the two methods (CCD *vs.* Sephadex). There is one more proline residue and approximately one-half less alanine residue per molecule of the material obtained by gel filtration as compared to that obtained from distribution. In all likelihood the true values are three proline and eight aspartic acid residues per molecule.

As noted previously[2], the yield of threonine was thought to be abnormally low in the material obtained by countercurrent distribution. The difference is apparently due to the fact that the hormone obtained by distribution contains traces of salts, which lead to a decreased yield of threonine and proline during acid hydrolysis, because hormone obtained by countercurrent distribution when passed through a Sephadex column and then subjected to acid hydrolysis, had an amino acid content similar to that of material obtained by gel filtration, alone. No accurate estimate of the amide groups could be made from the hormone purified on Sephadex because the buffers contained ammonium acetate and the ammonia was not completely removed by lyophilization. The yield of serine from preparation to preparation was quite variable and the estimate given must be considered a tentative one. The number of serine residues may be six to eight.

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