PRODUCTION OF AN ANTIBODY TO BOVINE PARATHYROID HORMONE[1, 2]

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The preliminary experiments reported in this communication provide the first published evidence of the production of a specific antibody to parathyroid calcium mobilizing hormone, indicated by Rasmussen and Craig (1961, 1962) to be a polypeptide with a molecular weight of the order of 9000.

MATERIALS AND METHODS

The parathyroid hormone preparations were made from acetone powder of bovine parathyroid tissue (Wilson Laboratories, Chicago). "Partially purified" hormone, carried through the last step prior to countercurrent distribution in the method of Aurbach (1959), was used for immunization. Two "highly purified" preparations were used in complement fixation experiments. One was obtained as the central peptide band after countercurrent distribution (CCD) for 56 transfers in the salt system of Aurbach (1959); the other was obtained as a fraction from the Sephadex gel filtration procedure of Rasmussen and Craig (1962). Biological assay in parathyroidectomized rats by the method of Munson (1955, 1961) indicated that the activity of the highly purified products (21,000 $\overset{x}{\div}$ 1.22 units/mg N for the CCD preparation;

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22,000 $\overset{x}{\div}$ 1.18 units/mg N for the Sephadex product) was similar to that obtained by others (Aurbach, 1959; Rasmussen and Craig, 1961) and that the partially purified product was approximately one-fifth as active (4300 $\overset{x}{\div}$ 1.25 units/mg N).

Following unsuccessful attempts at immunization of two rabbits with relatively crude parathyroid extracts, a third albino rabbit, weighing 2.5 kg, was given 42 mg of the partially purified hormone dissolved in 1.0 ml of 0.005 M acetic acid and emulsified with 1.0 ml of complete Freund's adjuvant. One half ml of this mixture was injected into the hind toe pads and the remaining 1.5 ml into the thigh muscles bilaterally. Beginning 33 days after the initial injection, the rabbit was given 4 consecutive daily intravenous injections of 3.0 mg of the same partially purified hormone in 1.0 ml of dilute acetic acid. The rabbit was bled from an ear vein one week after the last intravenous injection. The serum obtained was stored in the frozen state without preservatives. Rabbit serum containing a specific anti-bovine serum albumin (BSA) and serum from normal nonimmunized rabbits were also used.

Quantitative complement fixation procedures followed the techniques of Wasserman and Levine (1961), using heat inactivated antiserum diluted 1:100.

Statistical treatment of the data was by analysis of variance.

RESULTS

Highly purified parathyroid hormone (CCD) dissolved in 0.5 ml of 0.85% sodium chloride solution was mixed with 7.6 ml of undiluted serum from the hormone-immunized rabbit; a control mixture consisted of an equal amount of hormone and normal rabbit serum. The final concentration of hormone in each of two experiments was 0.80 and 0.88 ug N/ml, respectively. Almost immediately following mixing of the hormone and the antiserum a fine

white precipitate formed; there was no visible reaction in the control mixture. Both mixtures were kept at 4°C for 12 hours, then centrifuged in the cold at 4500 RPM for 20 minutes. A small white button was recovered from the tube containing hormone and antiserum, but there was no visible precipitate in the control tube.

In a third experiment a similar amount of hormone solution was mixed with anti-BSA rabbit serum. BSA dissolved in 0.85% sodium chloride solution was added, yielding a precipitate comparable in bulk to that obtained in the hormone-antihormone system. The mixture was kept at 4°C for 12 hours and the precipitate was separated by centrifugation as before. This experiment was included to test for adsorption of the hormone on or occlusion in a nonspecific immune precipitate.

Equivalent amounts of each supernatant fluid were injected subcutaneously into young male Holtzman rats immediately after parathyroidectomy as in the biological assay method of Munson (1955, 1961). Control groups of rats were injected with 0.85% sodium chloride solution and normal rabbit serum, respectively. The rats were bled by cardiac puncture 5 hours later and the serum was analyzed for calcium.

Experiments I and II in Table 1 demonstrated that the supernatant fluid remaining after precipitation of the hormone-antiserum mixture had no significant effect on the serum calcium of the test rats. They also showed that the amount of hormone originally present in the mixture, when injected with normal rabbit serum, was adequate to maintain a level of serum calcium significantly ($P<.001$) higher than that of the control groups. A separate experiment not shown indicated that rabbit antiserum alone did not decrease the serum calcium level of parathyroidectomized rats.

Table 1

Treatment	Experiment I No. rats	Experiment I Serum calcium* mg/100ml	Experiment II No. rats	Experiment II Serum calcium* mg/100ml	Experiment III No. rats	Experiment III Serum calcium* mg/100ml
0.85% NaCl solution	7	5.4 ± .40	7	5.7 ± .26	7	6.1 ± .36
Normal rabbit serum (NRS)	6	5.8 ± .43	7	6.3 ± .26		
Hormone + NRS	6	7.8 ± .43	6	8.1 ± .28	5	8.2 ± .44
Hormone + hormone antiserum	5	6.5 ± .47	5	6.0 ± .31		
Hormone + BSA antiserum					5	7.4 ± .44

*Mean ± standard error

Amount of hormone injected per rat: Expt. I - 0.88ug N; Expt. II - 1.12ug N
Expt. III - 0.90 ug N

In Experiment III the small difference in effect between hormone + normal rabbit serum and hormone + BSA antiserum was not statistically significant. Therefore, no loss of hormone by adsorption on the BSA immune precipitate was demonstrated. The presence of a significant ($P<.05$) amount of hormone in solution in the BSA antiserum supernatant fluid after precipitation with BSA, in contrast to the absence of soluble hormone after precipitation with hormone antiserum, is a further indication of the specificity of the hormone antiserum.

The precipitate obtained from the mixture of hormone and hormone-antiserum in two experiments was dissolved in 2.0 ml of 0.01 N hydrochloric acid (pH 2.1). After 14 hours at $4^{\circ}C$, cysteine hydrochloride in 0.01 N hydrochloric acid was added to a final concentration of 20 mg/ml and the mixture was incubated an additional 9 hours in the cold. Cysteine was added because of its known stabilizing or enhancing effect on parathyroid hormone

preparations (Munson, 1961); it also may have contributed to denaturation of the antibody. At the end of the incubation period, the mixture was centrifuged and appropriate aliquots of the clear supernatant fluid (a slight precipitate separated) were tested in parathyroidectomized rats as described for the experiments of Table 1.

The results of two essentially identical experiments are shown in Table 2. The dose of dissociated hormone per rat was 1.5-1.6 times that of the experiments in Table 1, assuming that all the hormone was in the precipitate. The resulting partial maintenance of the serum calcium level, in comparison with the control values, was statistically significant ($P<.001$), demonstrating that the hormone had been precipitated by the antiserum and had not merely been inactivated in a nonspecific manner.

Table 2

Treatment	Experiment II		Experiment IV	
	No. rats	Serum calcium* mg/100ml	No. rats	Serum calcium* mg/100ml
0.85% NaCl solution	7	6.1 ± .32	5	6.5 ± .25
Dissociated hormone from precipitate	4	7.8 ± .42	5	7.8 ± .25

*Mean ± standard error

Both control and hormone solutions contained cysteine hydrochloride, 20mg/ml

Using the rabbit-antiparathyroid serum and two highly purified parathyroid preparations, two closely similar quantitative complement (C') fixation curves were observed (Figure 1). A third hormone sample prepared by Dr. Gerald D. Aurbach by Sephadex gel filtration and generously contributed by him gave similar results.

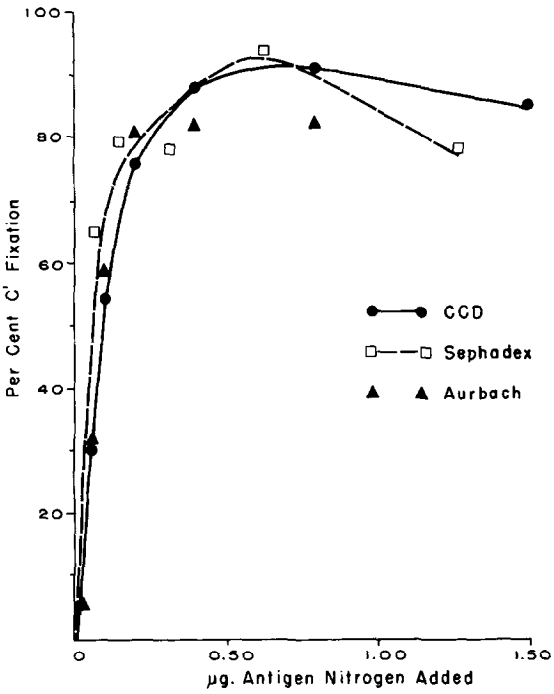

Figure 1. Fixation of complement (C') by three highly purified preparations of bovine parathyroid hormone

The uniformity of the C' fixation curves obtained with three hormone preparations purified by two different procedures in two different laboratories favors the conclusion that the hormone is the antigen that is being measured by C' fixation. As an additional test of this conclusion, three parathyroid hormone preparations ranging in specific activity from 350 to 4300 units/mg N, according to bioassay in parathyroidectomized rats, were evaluated immunochemically by C' fixation in comparison with highly purified hormone. The estimation of hormone content determined by the two methods was in good agreement, again supporting the specificity of the antibody. In the immunochemical assays the comparisons were made in the region of antibody excess. In antigen excess a second shoulder or peak of C' fixation was observed with the less purified preparations. This apparent heterogeneity might be explained by the presence of a contaminating immune system (Vaughan and

Kabat, 1954) or, alternatively, by differences in reactivity of free and protein bound hormone.

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