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A RADIOIMMUNOASSAY PROCEDURE FOR QUANTITATING PAROTID HORMONE

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SUMMARY: The methodology and characterization of a double antibody radioimmunoassay (RIA) for quantitating parotid hormone (PH) in biological fluids are reported. A specific antiserum against PH was raised in rabbits using PH conjugated to human serum albumin. Its binding capacity and association constant were 22 μg/ml and Ka = 1.01 x 10^{-12} M^{-1}, respectively. The sensitivity of the RIA was 1.5 pg PH when a sequential incubation schedule was used. This RIA makes possible the quantitation of PH in biological fluids and tissue extracts.

For the past 12 years, evidence has accumulated which suggests that parotid glands possess an endocrine function. Parotid hormone (PH) stimulates a fluid transport (FT) mechanism in teeth, which is essential for maintaining dental health (1-4). A bioassay procedure for detecting parotid hormone activity was developed (5) and subsequently modified (6). However, the bioassay is primarily qualitative; consequently, quantification of PH in biological fluids is difficult. A parotid hormone fraction (PH-A_β) was isolated from porcine parotid glands and partially characterized (6). The availability of essentially pure PH-A_β allowed the development of a RIA procedure, the methodology of which is presented.

Materials and Methods

Preparation of PH-A_β conjugates. PH-A_β was isolated as previously described (6). The preparation used for immunization was a pool of several batches of purified hormone that showed a slight contamination by electrophoresis (estimated at less than 5%). We conjugated PH-A_β to human serum albumin (HSA) (Sigma, St. Louis, MO) or to porcine thyroglobulin (Sigma) by the method of Tager (7). The first incubation was carried out at 22°C for 15 minutes at a concentration of 2 mg PH-A_β/ml reagent. The second incubation took place in darkness at 22°C for 22 hours, with HSA or thyroglobulin added at a concentration of 3 mg/mg PH-A_β/0.5 ml borate buffer. We purified the conjugate from unreacted reagents by passing the incubation mixture through a 1.1 x 50 cm column of Sephadex G-50 (fine) equilibrated with saline containing 0.02% NaN_3. The first two peaks of eluate were pooled for immunization.

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Immunization. Over a period of 3 years, we immunized 27 male New Zealand rabbits, each weighing 2.5 kg. With the exception of 5 animals, all received a subcutaneous injection of 0.5 ml pertussis vaccine (Lilly) 2 days prior to immunization. One hundred µg PH-A_β unconjugated, or 200 µg PH-A_β equivalent of PH conjugate, were dissolved in 1 ml 0.05 M phosphate buffer saline (PBS), 0.09% NaCl, pH 7.0, and emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI) before being injected intradermally at 20 sites on the rabbits' backs. All animals were boosted 8 weeks later with half the first immunization dose of antigen emulsified in incomplete Freund's adjuvant and administered subcutaneously in the four axillary areas. Subsequent boosters were administered at 5 week intervals. The animals were bled every 7 to 10 days through the auricular artery. Antiserum titer is expressed as the final dilution necessary for binding 25% of 10,000 cpm of [^{125}I] iodo-PH-A_β (i.e., about 30 pg labelled hormone).

Preparation of labelled PH-A_β. The preparation of PH-A_β used for iodination was a pool of several batches of hormone of highest purity showing no contamination by electrophoresis. Aliquots of 15 µg were freeze-dried and kept for subsequent iodinations. We found no evidence of damage by such treatment. Since PH-A_β does not contain tyrosin (6), we carried out the hormone iodination with diiodinated [^{125}I] Bolton-Hunter reagent (New England Nuclear). We added 15 µg PH-A_β dissolved in 15 µl of 0.1M borate buffer, pH 8.5, to 1 mCi of nitrogen-dried Bolton-Hunter reagent, 4,000 Ci/mM. The reaction was allowed to proceed for 2 hours at 0°C, with agitation every 30 minutes. The labelled hormone was purified by gel filtration through a column of Sephadex G-50 (fine) (1.1 x 50 cm) equilibrated with a PBS-gelatin buffer, 0.2% swine skin gelatin (Sigma), 0.02% NaN_3, pH 7.5. Half ml fractions were collected and counted with a gamma counter (Searle Analytic, Inc.) at 70% efficiency. We tested fractions corresponding to the second peak of elution for maximum binding to PH-A_β antiserum and for competitive binding with addition of 25 pg of unlabelled PH-A_β. The fraction displaying the highest avidity for the antiserum was diluted 1:1 with incubation buffer (see "binding assays") and stored at 4°C.

Binding assays. We used a double antibody procedure. One incubation buffer was used for diluting PH-A_β standard, unknown samples, labelled antigen, and antiserum. Its composition was 0.01 M PBS, 0.01 M EDTA, 0.02% NaN_3, and 2% (vol/vol) normal rabbit serum, at pH 7.5. Assays were carried out in 12 x 75 mm disposable polystyrene tubes, with a total working volume of 0.9 ml made up as follows: 0.7 ml incubation buffer, 0.1 ml antiserum (at a dilution to bind 20-25% of the labelled antigen without unlabelled hormone), 0.1 ml [^{125}I] iodo-PH-A_β (usually 10,000 cpm). Incubation took place at 4°C for 16 to 88 hours, depending on the type of assay. We separated bound from free antigen by adding 0.1 ml of a titered solution of anti-rabbit gamma globulin goat serum (Second Antibodies, Inc., David, CA) in normal saline, incubating at 4°C for 2 more hours, centrifuging at 1,700 x g for 15 min. at 4°C, and removing the supernatant by aspiration.

Competitive binding assay. Unknowns (up to 100 µl) or PH-A_β standards (0.5 µl-16 µl), diluted antiserum and incubation buffer added in quantity sufficient to make 0.8 ml were preincubated for 6 hours at room temperature. After adding [^{125}I] iodo-PH-A_β, the incubation was carried out at 4°C for 16 hours. This was followed by separating bound from free antigen with a second antibody as described above. For Scatchard plot data, we added incubation buffer, standards, antiserum and tracer at the same time, then allowing incubation to proceed to equilibrium for 88 hours at 4°C. We studied the binding capacity of rabbit antiserum for PH-A_β by incubating different quantities of [^{125}I] iodo-PH-A_β with a fixed amount of antiserum using the same conditions as for the Scatchard plot data assay, but without addition of unlabelled PH-A_β. Statistical analysis and reduction of the RIA data were done according to Rodbard (8).

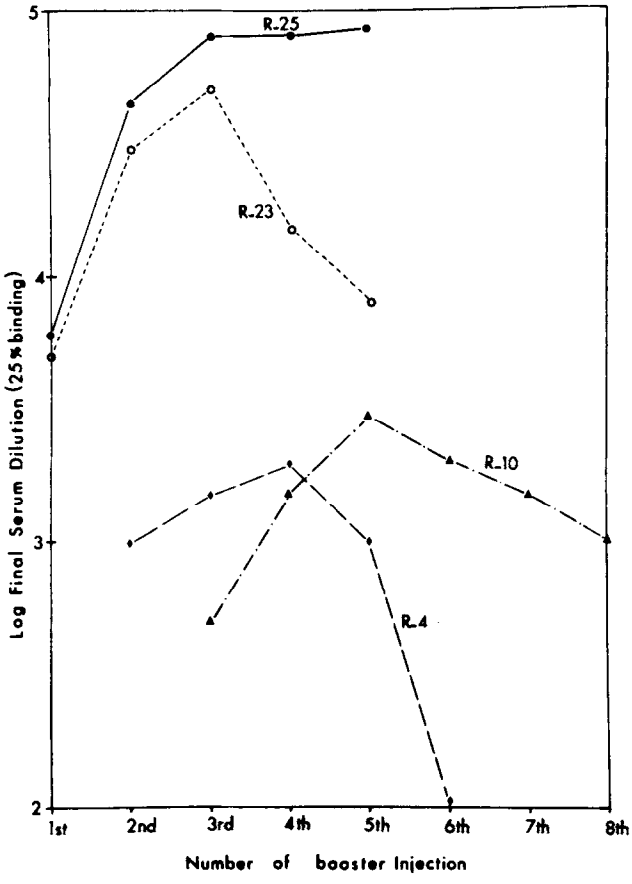

Figure 1: PH-A_β antibody titers of four rabbits as a function of time after immunizations. Booster injections were spaced 5 weeks apart. R-4 and R-10 were immunized with PH-A_β alone; R-23 and R-25 received PH-A_β-HSA conjugate.

Results

PH-A_β alone is poorly immunogenic. Only 2 out of 19 animals immunized with PH-A_β generated acceptable antisera, but of low titer (antisera R-4 and R-10, Fig. 1). When PH-A_β was conjugated to HSA or thyroglobulin, its immunogenicity significantly increased. Eight rabbits thus immunized yielded antiserum titers exceeding 1:20,000 with variable levels of avidity. Two animals immunized with the PH-A_β-HSA conjugate produced antisera of good titer and avidity suitable for RIA (R-23 and R-25, Fig. 1). Only two animals were immunized with the PH-A_β-thyroglobulin conjugate. The resulting antibodies were of fair titer (dilution 1:40,000) and avidity but of lesser quality than

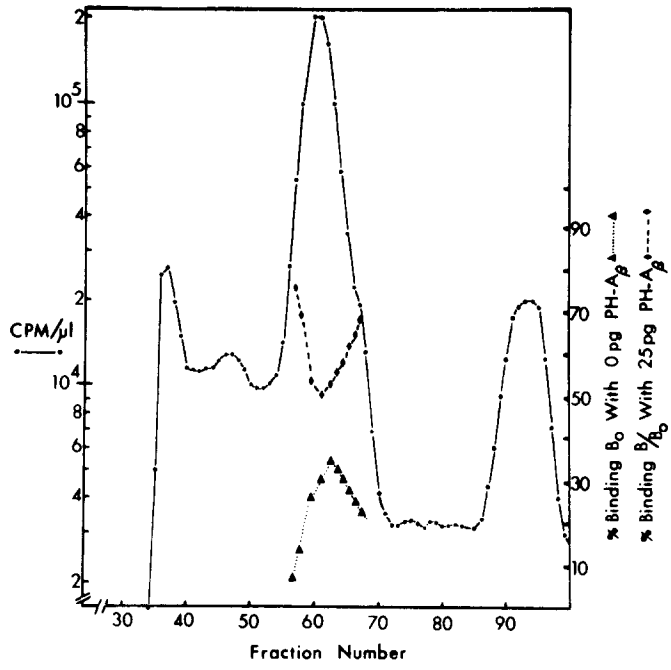

Figure 2: Purification of [^{125}I] iodo-PH-A_β through Sephadex G-50 (fine) 0.5 ml fractions were assayed for maximum binding (B_o) and displacement (B/B_o) with 25 pg unlabelled PH-A_β (detailed procedure described in "materials and methods" section). The first peak (F-34-52) is composed of labelled hormone polymerized during iodination. Fractions containing [^{125}I[iodo-PH-A_β that display the highest maximum binding (B_o) and displacement (B/B_o) are generally found on the descending portion of the second peak (F-54-70). To the 3rd peak (F-86-100) corresponds the unreacted labelled reagent.

R-23 and R-25. All animals showed a rise in titer with time and booster immunizations. With the exception of R-25, the rise was followed by a rapid drop (Fig. 1). Antiserum R-25-3-28 (Rabbit No. 25 bled on the 28th day after the 3rd booster) was the best obtained and is currently used for all RIAs.

Fig. 2 shows the elution pattern of [^{125}I] iodo-PH-A_β during purification through Sephadex G-50 immediately following iodination. On the average, a 40-60% incorporation of the iodinated reagent was obtained following a "normal" iodination (see "discussion"). The [^{125}I] iodo-PH-A_β fraction used for RIA could be kept for 4-6 weeks at 4°C diluted 1:1 with incubation buffer, with minimal loss in binding avidity. Storing of the labelled hormone in a frozen state, as compared to storing at 4°C, was not found to be more beneficial in

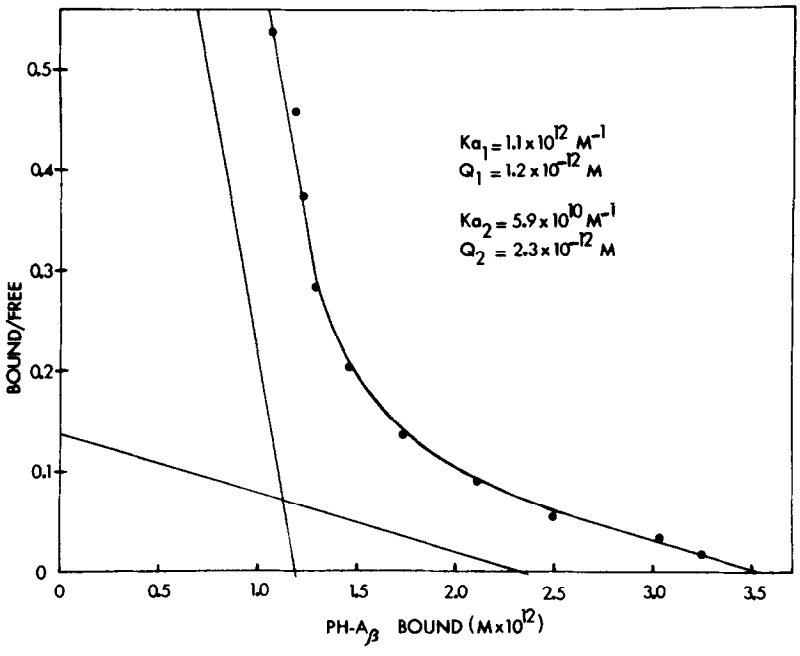

Figure 3: Scatchard plot binding data of PH-A_β to antiserum R-25-3-28 from one typical assay. High and low equilibrium association constants (Ka_1, Ka_2) and binding site concentrations (Q_1, Q_2) were computed from the asymptotes to the curvelinear best fit of the Scatchard plot.

terms of its binding activity. Specific activities ranging between 160 and 350 μCi/μg-PH-A_β were obtained from over 20 iodinations.

Antiserum R-25-3-28 has been partially characterized. Its binding capacity was 22 μg PH-A_β/ml of serum and was routinely used at a final dilution of 1:100,000. A Scatchard plot of the competitive binding assay data of PH-A_β with R-25-3-28 showed a biphasic response indicating the presence of a high affinity, low capacity class of binding sites with a high equilibrium association constant (Ka) of $1.01 \pm 0.47 \times 10^{-12}\ M^{-1}$, and lower affinity, higher capacity binding sites with a low K_a of $4.6 \pm 0.9 \times 10^{10}\ M^{-1}$ (averages of 4 runs). Fig. 3 illustrates one of these binding assays. We investigated the binding specificity of R-25-3-28 for PH-A_β by testing several hormones, proteins, and neurotransmitters for possible competitive binding (Fig. 4). None showed significant crossreactivity. The highest level was obtained with

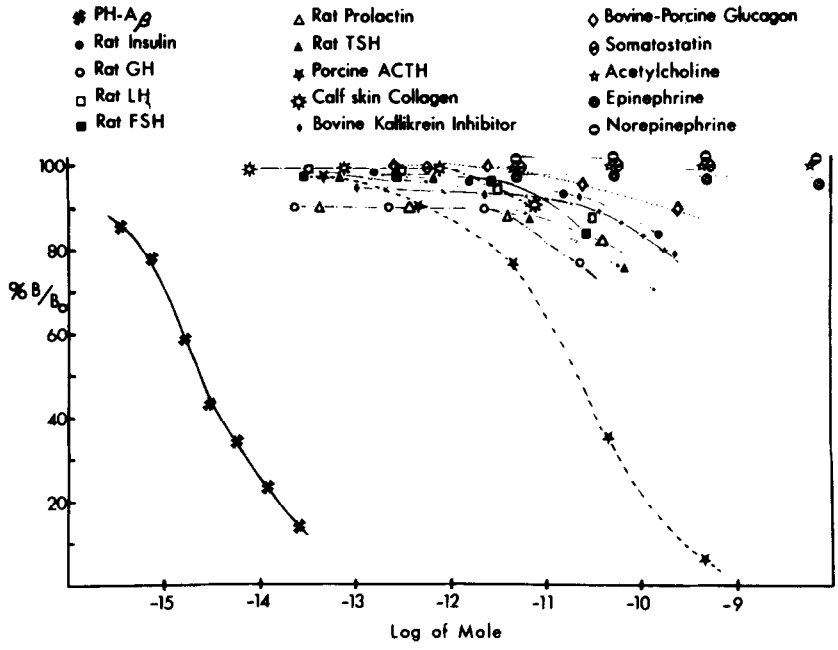

Figure 4: Immunocrossreactivity study of several hormones, proteins, and neurotransmitters with PH-A_β antiserum R-25-3-28.

porcine ACTH, the relative affinity of which amounted to 0.015%, at 50% binding, compared to PH-A_β.

The coefficients of variation "within assay" (CV_w) and "between assay" (CV_b) have been computed, according to Rodbard (8), for PH-A_β standard and rat serum each at 3 dose-levels. Table 1 summarizes these data computed from 12 different assays, with each dose tested in duplicate. We further ascertained the validity of the PH RIA by comparing the parallelism of the competitive binding responses to dilution, using PH-A_β standard, pooled rat serum, and crude porcine parotid gland aqueous extract (Fig. 5). Linearity of the responses to dilution was tested by regression analysis. The coefficients of correlation for PH-A_β, rat serum, and parotid gland extract were 0.996, 0.990, and 0.995, respectively. All 3 samples "dosed out" in a parallel fashion. (Parallelism of the curve slopes was tested at the p level of 0.01 using a t-test.)

TABLE I

Coefficients of variation of PH potency estimates by RIA

PH-A_β Standard			Rat Serum		
$\bar{X}$ (a)	% CV_w (b)	% CV_b (c)	$\bar{X}$ (a)	% CV_w (b)	% CV_b c)
3.1 pg	4.8%	2.6%	3.6 pg	17.8%	25.5%
12.5 pg	3.3%	4.7%	11.1 pg	6.5%	6.2%
100 pg	6.8%	6.1%	109 pg	9.8%	19%

(a) : potency estimate average of 12 assays run in duplicates.
(b) : "within assay" coefficient of variation.
(c) : "between assay" coefficient of variation.

Discussion

Because of numerous parameters involved in the immune response to antigens, we decided to follow only one schedule of immunization and one route of injection. Pretreatment of the animals with pertussis vaccine did not seem to affect the immune response. We have not tried different doses of immunogen, particularly higher doses, because of the scarcity of PH-A_β; however, this is planned. It should be noted that production of antibodies both of good titer and avidity occurred within a very limited time period (2-6 days on the average), after which it dropped to lower levels. Consequently, it was necessary to screen blood samples frequently after each booster immunization. To circumvent this problem, studies are planned to use different immunization schedules and conditions.

Iodination of PH-A_β with [^{125}I] Bolton-Hunter reagent is a reliable method provided that one extremely critical condition is satisfied. Before introducing the hormone solution into the reagent vial, the reagent must be kept in a totally anhydrous state. We have found that evaporation of benzene prior to iodination with a gentle stream of N_2 from commercial tank is not sufficient. A moisture trap must be placed in the gas line. In our experience, failure to follow this requirement resulted in a drop in incorporation from 40-60% on the average for a normal iodination to less than 1%.

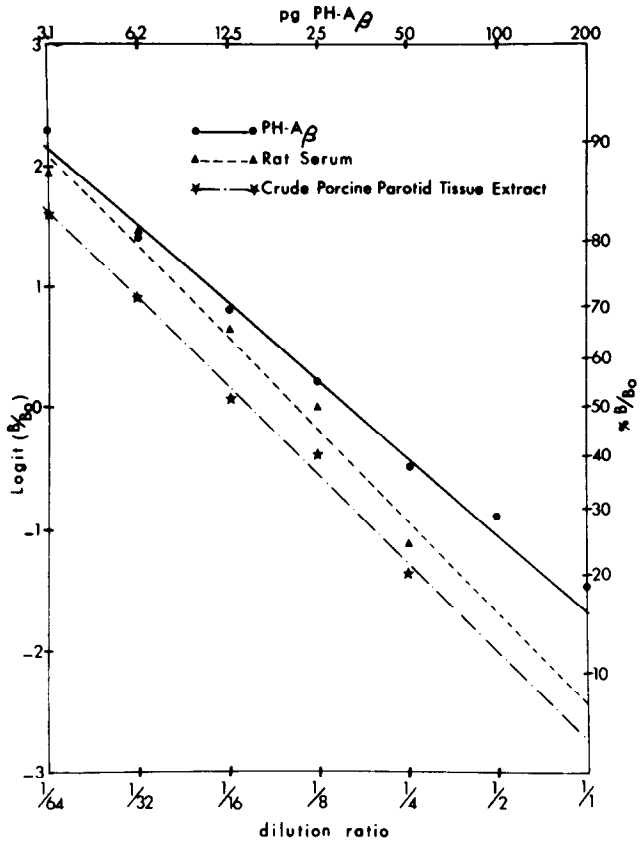

Figure 5: Competitive binding assay data using a Logit-Log transformation of the coordinates. Serial dilutions of PH-A_β standard, "high pool" rat serum, and crude porcine parotid gland tissue extract were incubated sequentially with antiserum R-25-3-28 then with 30 pg [^{125}I] iodo-PH-A_β for 6h and 15h respectively. Each point is the average of triplicates.

One area of improvement for this RIA procedure is to minimize the response variability when blood samples are quantitated for PH at levels corresponding to the low concentrations of the standard curve (see Table 1). This problem appears to be primarily limited to blood samples and not to other biological fluids. Extraction of PH from, or treatment of the serum prior to, the assay might be possible solutions to the problem.

The availability of a specific antiserum against PH-A_β is significant because it permits the quantitation of PH. Although linearity of the dose-response using the pure PH-A_β has been demonstrated with the bioassay (6), the assay is considered to be qualitative rather than quantitative because

linearity could not readily be shown with other less purified hormone preparations. Therefore, it was not possible to establish a direct correlation between bioassay and RIA.

The PH RIA makes possible a wide spectrum of investigations, particularly the exploration of the hypothalamic-parotid gland endocrine axis and its relationship to other hormones and to metabolism in general. It will also allow indepth investigation of the systemic factors that are essential for maintaining dental health, as related to DFT activity.

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