Development of a radioligand immunoassay for 1,25-dihydroxycholecalciferol receptors utilizing monoclonal antibody

Shigeharu DOKOH,* Mark R. HAUSSLER and J. Wesley PIKE†

Department of Biochemistry, University of Arizona Health Sciences Center, Tucson, AZ 85724, U.S.A.

(Received 12 January 1984/Accepted 8 March 1984)

A radioligand immunoassay for 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$] receptors was developed utilizing a specific, high-affinity ($K_d = 1.8 \times 10^{-11} \text{ M}$) monoclonal antibody (9A7 γ) obtained from suspension cultures of rat spleen $\times$ mouse myeloma hybrid SP2/0-9A7. A standard curve was established, based on the competition between $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor (18 fmol/tube) and increasing concentrations of radioinert $1,25(\text{OH})_2\text{D}_3$ -receptor (0–240 fmol/tube) for the binding site on 9A7 γ . Samples, prepared in identical buffer, contained 0–100 fmol of receptor/tube. After an equilibrium incubation of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor with either standard or sample (16 h at 4°C), antibody-bound receptor was immunoprecipitated with rabbit anti-(rat immunoglobulin) prelinked to *Staphylococcus aureus* and quantified. The assay is statistically sensitive to 2 fmol of receptor/tube, with intra- and inter-assay variations of 7 and 12% respectively. Occupied, unoccupied and denatured receptor were observed to compete equally in the assay. This quantitative technique has been successfully applied to the characterization of receptors after fractionation by sedimentation analysis and DNA–cellulose chromatography. Finally, the measurement of total receptor by this assay, in conjunction with $1,25(\text{OH})_2\text{D}_3$ binding assays, has revealed that rachitic, normal and $1,25(\text{OH})_2\text{D}_3$ -injected chicks have respectively 13, 20, and 56% of receptor in the occupied form. From these results we consider that this radioligand immunoassay will be a useful tool in further research focusing on quantifying $1,25(\text{OH})_2\text{D}_3$ receptors in tissue and cell extracts.

$1,25(\text{OH})_2\text{D}_3$, the active metabolite of the secosteroid cholecalciferol (vitamin D_3), is a steroid hormone by classical criteria (DeLuca & Schnoes, 1976; Haussler & McCain, 1977). It has been shown to localize in the nucleus of target cells by both biochemical (Haussler *et al.*, 1968; Brumbaugh & Haussler, 1974; Pike & Haussler, 1983) and autoradiographic (Zile *et al.*, 1978; Stumpf *et al.*, 1979) means. Further, receptor proteins for this sterol have been detected by biochemical fractionation and $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ binding in a host of tissues, including intestine

(Brumbaugh & Haussler, 1975), bone (Kream *et al.*, 1979) and kidney (Christakos & Norman, 1981; Chandler *et al.*, 1979), and current evidence suggests that this protein mediates the biological action of $1,25(\text{OH})_2\text{D}_3$ at the level of the nucleus (Zerwekh *et al.*, 1976; Pike, 1982).

$1,25(\text{OH})_2\text{D}_3$ receptors, as well as other steroid-receptor proteins, have been traditionally measured in classic radioactive-ligand-binding assays. However, these assays are limited to the measurement of unoccupied receptors, involve non-covalent interactions subject to dissociation, and measure a receptor species that is exceedingly labile even after the complex is formed. Fairly recently, several exchange assays have been devised (Hunziker *et al.*, 1980; Massaro *et al.*, 1983) to measure $1,25(\text{OH})_2\text{D}_3$ receptors under experimental or physiological conditions in which both endogenously occupied and unoccupied receptors are present. However, the conditions under which both assays are carried out favour considerable loss of hormone-binding sites, i.e. high temperature or

Abbreviations used: $1,25(\text{OH})_2\text{D}_3$, dihydroxycholecalciferol; DBP(s), cholecalciferol (vitamin D_3)-binding protein(s); Ig, immunoglobulin; r.l.i.a., radioligand immunoassay; R_0 , R_s , R_T and $R_{\text{denat.}}$, unoccupied, occupied, total and denatured receptor respectively.

* Present address: Code No. T 603, 63 Okamoto-guchi Cho, Kamigamo, Kita Ku, Kyoto City, Japan.

† To whom correspondence and reprint requests should be sent.

low concentrations of reducing agents, and the extent of this loss is difficult to assess.

We recently developed hybridomas that secrete monoclonal antibodies against the chick receptor for $1,25(\text{OH})_2\text{D}_3$ (Pike *et al.*, 1982, 1983). We report here the successful development of a radioligand immunoassay for $1,25(\text{OH})_2\text{D}_3$ receptors utilizing one of those antibodies. This assay is the first ligand-independent technique devised for $1,25(\text{OH})_2\text{D}_3$ receptors, and is not sensitive to receptor lability, ligand dissociation or interference with DBPs. The technique has been applied to the characterization and quantification of total receptor, and has enabled us to quantify physiological levels of occupied receptor in chicks under several dietary and experimental manipulations.

Materials and methods

Animals

Rhode Island Red cockerel chicks (1 day old) were obtained from Red Wing Hatcheries (Irvine, CA, U.S.A.) and maintained on a diet deficient in cholecalciferol (McNutt & Haussler, 1973), containing either 0.5 or 2.7% CaCO_3 , for 3 weeks before they were killed. Supplementation of cholecalciferol in one experiment was achieved by adding 2 i.u./g of rachitogenic diet. Injection of $1,25(\text{OH})_2\text{D}_3$ was achieved by subcutaneous administration of 6 nmol of the hormone in 0.1 ml of propylene glycol to a single bird 2, 24 and 48 h before killing.

Compounds

25-Hydroxy[26,27- ^3H]cholecalciferol (sp. radioactivity 148 Ci/mmol) was obtained from Amersham International (Arlington Heights, IL, U.S.A.) and converted into $1,25(\text{OH})_2[26,27-^3\text{H}]\text{D}_3$ as described by Haussler *et al.* (1980). Unlabelled $1,25(\text{OH})_2\text{D}_3$ was generously supplied by Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ, U.S.A.).

Monoclonal antibodies to $1,25(\text{OH})_2\text{D}_3$ receptors

We previously described the development of these anti-receptor antibodies (Pike *et al.*, 1982, 1983). Antibodies derived from the SP2/0-9A7 hybridoma were obtained by precipitation with $(\text{NH}_4)_2\text{SO}_4$ as described previously (Pike, 1984). Working dilutions of the concentrated antibodies were 1:10000 and were maintained at -20°C as portions freeze-dried in 20 mM-sodium phosphate, pH 7.2.

Rabbit anti-(rat Ig) (Sigma Chemical Co., St. Louis, MO, U.S.A.), utilized as second antibody, was coupled to *Staphylococcus aureus* (IgG sorb;

Enzyme Center, Boston, MA, U.S.A.) as previously described (Pike, 1984). This immunoprecipitating reagent was stored at 4°C as a 10% (w/v) suspension in 20 mM- Na_2HPO_4 , pH 7.2, containing 0.05% NaN_3 , and exhibited a shelf life of at least 4 months.

Preparation of chick intestinal $1,25(\text{OH})_2\text{D}_3$ receptors

Duodenal mucosa from chick intestines was scraped free of the underlying serosa and a 10% (w/v) homogenate was prepared with a Potter-Elvehjem glass/Teflon tissue grinder in KETD-0.3 buffer [10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/0.3 M-KCl/5 mM-dithiothreitol] at 4°C . After brief centrifugation at 17000 g for 10 min, the supernatant was ultracentrifuged at 160000 g for 45 min to obtain the buffer-soluble fraction of the cell. Liver was prepared by identical means. Formation of $1,25(\text{OH})_2\text{D}_3$ -receptor complexes was routinely achieved by incubating the cell extracts for 6–8 h at 4°C with $4\text{ nM}-1,25(\text{OH})_2[^3\text{H}]\text{D}_3 \pm 0.4\text{ }\mu\text{M}$ $1,25(\text{OH})_2\text{D}_3$ [in 9% (v/v) ethanol], and macromolecular-bound $1,25(\text{OH})_2\text{D}_3$ was determined with charcoal (Dokoh *et al.*, 1981) or by adsorption to DE-81 filters (Pike & Haussler, 1979), both of which provided identical results. Specific binding was determined by the difference between total binding and that obtained with excess radioinert $1,25(\text{OH})_2\text{D}_3$. In certain situations, mucosal extracts were incubated with $4\text{ nM}-1,25(\text{OH})_2\text{D}_3$ (in 9% ethanol) as described above, or left free of hormone.

In order to prepare a uniform supply of $1,25(\text{OH})_2\text{D}_3$ -receptor-containing mucosal extracts for radioligand immunoassay, we freeze-dried an extensive number of samples (6 mg of protein/ml) whose receptor content [2.4 pmol of $1,25(\text{OH})_2\text{D}_3$ -binding activity/ml] was determined by saturation analysis (Dokoh *et al.*, 1983). These samples, on solubilization and labelling with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ as described above, yielded preparations with identical receptor concentrations from assay to assay, and almost identical $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -binding activities over a period of at least 6 months.

Radioligand immunoassay for $1,25(\text{OH})_2\text{D}_3$ receptor

The immunoassay standard curve was constructed as follows. $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor complexes (18 fmol) were mixed with increasing concentrations of competing $1,25(\text{OH})_2\text{D}_3$ -receptor complexes (0–240 fmol) and incubated with diluted 9A7 γ monoclonal antibody for 16–18 h at 4°C . Both radiolabelled and radioinert hormone-receptor complexes, created as above, were formed from identical freeze-dried samples of mucosal

extract. Thus their concentrations remained identical, whether liganded with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ or $1,25(\text{OH})_2\text{D}_3$. Incubations were carried out in triplicate in 0.25 ml of KETD-0.3 buffer containing 1% foetal-bovine serum, until maximal binding was achieved (16–18 h), whereupon receptor-antibody complexes were immunoprecipitated with anti-(rat Ig)-coated *S. aureus*. The latter manipulation was performed by incubating 0.01 ml of this reagent in each tube for 5 h, adding 1 ml of KETD-0.5 [10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/0.5 M-KCl] containing 0.5% Tween 20, and then centrifuging at 4000g for 10 min. The pellet was washed a second time in the same buffer, and then the $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ extracted for 1 h with acetone (1 ml), decanted, dried, and quantified in ACS scintillation cocktail (Amersham International) in a Beckman 7500 liquid-scintillation counter.

Receptor-containing samples were similarly prepared in KETD-0.3 buffer, and quantified directly by r.l.i.a., or after either sedimentation through 10–30% sucrose (Pike *et al.*, 1982) or DNA-cellulose chromatography (Pike & Haussler, 1979). Although the pH optimum for the immunoassay is broad (pH 7–9), both high sucrose and high ionic strength interfere with receptor-antibody interactions. However, these effects are seen only in samples quantified by immunoassay after fractionation as described above, and are generally minimal. The high KCl concentration (0.3 M) in the incubation mixtures, though decreasing the total $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor binding to antibody, was found to be necessary to decrease the non-specific binding in the assay. Furthermore, it was determined in mock immunoassays (receptor estimated without immunoprecipitation) that the labelled $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor was not subject to proteolysis or dissociated significantly during the course of the assay. Finally, we routinely preincubated all immunoassay components except the $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor complex for 2 h before the addition of the latter. This manipulation significantly improved the level of sensitivity of the assay.

General techniques

Sedimentation analyses of $1,25(\text{OH})_2\text{D}_3$ receptors were carried out in KETD-0.3 containing 10–30% (w/v) sucrose (Pike *et al.*, 1982) and fractionated from the top. DNA-cellulose chromatography of receptor was performed as previously described (Pike & Haussler, 1979; Pike, 1982; Pike *et al.*, 1983) utilizing immobilized calf thymus DNA (Sigma). The protein concentration was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard. The measurement of $1,25(\text{OH})_2\text{D}_3$ in chick serum was performed as described by Dokoh *et al.* (1981).

Results

Saturation analysis of 9A7 monoclonal antibody

Before development of an immunoassay, we determined the equilibrium dissociation constant (K_d) of our individual monoclonal antibodies for $1,25(\text{OH})_2\text{D}_3$ receptor. The highest-affinity interaction was observed with 9A7 γ , which is shown in Fig. 1. Increasing concentrations of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ receptors were incubated with a fixed concentration of 9A7 γ to determine total binding. Non-specific binding was determined with the inclusion of 20–50-fold molar excess of $1,25(\text{OH})_2\text{D}_3$ -receptor, and the difference represents specific receptor bound (Fig. 1a). Scatchard analysis of representative specific binding is seen in Fig. 1(b), where the K_d was determined to be $1.8(\pm 1.13) \times 10^{-11}$ M for five independent analyses and 3.0×10^{-11} M for chick kidney (results not shown). This high-affinity property, coupled with the generally cross-reactive nature of 9A7 γ (Pike *et al.*, 1983), enabled us to devise a sensitive r.l.i.a.

Immunoassay standard curve

A typical competition curve, which is achieved after incubation of the components described in

Fig. 1. Saturation analysis of monoclonal antibody 9A7 γ with chick intestinal $1,25(\text{OH})_2\text{D}_3$ receptors

(a) Increasing concentrations of chick $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor complexes ($\circ$) $\pm$ 50–20-fold molar excess of $1,25(\text{OH})_2\text{D}_3$ -receptor complexes (Δ) were incubated with 9A7 γ for 20 h at 4°C, and then antibody-bound receptor immunoprecipitated with anti-(rat Ig)-coated *S. aureus* and quantified as described in the Materials and methods section. Specific binding ($\bullet$) was determined by the difference between total ($\circ$) and non-specific (Δ) binding. (b) Scatchard analysis of specific binding in (a). The line was derived by linear-regression analysis with an r (correlation coefficient) value of -0.98 . K_d , as determined by the average of five separate experiments was $[1.8 \pm 1.13 (\text{s.d.})] \times 10^{-11}$ M.

the Materials and methods section, is illustrated in Fig. 2. Approx. 50% of added $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -receptor complex is bound to antibody in the absence of competitor under these conditions. The assay is statistically sensitive to 2 fmol of receptor/tube, has a range of competition from 2 to 200 fmol of measurable receptor, and an intra- and inter-assay variation of 7 to 12% respectively. Non-specific binding, as determined in the absence of 9A7 γ , is less than 7% and is independent of total receptor concentration in each tube. Furthermore, DBP prelabelled with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ does not interact with 9A7 γ , and thus it neither substitutes for receptor in the assay nor interferes with the critical competition reaction (results not shown). Occupied, unoccupied and denatured receptor [37°C for 1 h, which eliminates $1,25(\text{OH})_2\text{D}_3$ -binding activity] all compete equally with radiolabelled receptor for 9A7 γ (results not shown), suggesting that conversion of one receptor form into another, either experimentally or technically, is unimportant to the final measurement of receptor content in a sample. However, it is important to note that, owing to the design of this r.l.i.a., the receptor concentrations used to construct the standard curve are based on radioligand binding estimation. Thus an unknown background of competition may exist that is attributable to any significant proportion of receptor existing in the standard cytosol which was incapable of binding

Fig. 2. R.l.i.a. standard curve for measurement of $1,25(\text{OH})_2\text{D}_3$ receptors

The competition curve was obtained as described in the Materials and methods section. O, Contained 9A7 γ ; Δ , no 9A7 γ . Each point represents the mean ($\pm$ S.D.) of triplicate determinations.

$1,25(\text{OH})_2\text{D}_3$ at the time its activity was quantified. This cannot be accurately determined at present.

Fig. 3 illustrates the results of an experiment showing that the immunoassay is capable of predicting the concentration of occupied receptor (R_S) in a sample that contains both R_S and unoccupied receptor (R_0). R_0 and R_S [formed by incubation with $1,25(\text{OH})_2\text{D}_3$] were first mixed in various proportions, and then R_0 determined by radioligand-binding activity. Total receptor (R_T) was determined by immunoassay measurements, and the concentration of R_S thus estimated by the difference between the two values, R_0 and R_T . The accuracy and linearity of the determined and estimated values support the validity of this approach to measuring various forms of the $1,25(\text{OH})_2\text{D}_3$ receptor. Taken together with the observed linearity of assayable-receptor values after sample dilution (Fig. 4), it suggests that the assay should be useful in detecting and character-

Fig. 3. Quantification of occupied and unoccupied intestinal $1,25(\text{OH})_2\text{D}_3$ receptors by both immunoassay and hormone-binding assay

Portions of freeze-dried receptor were dissolved in KETD-0.3 and incubated for 6 h at 4°C with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ (to quantify receptor concentration), $1,25(\text{OH})_2\text{D}_3$ (R_S), or left unlabelled (R_0). R_S was mixed with R_0 in proportions of 5, 25, 50, 75%, and 95%, and then R_0 (○) was determined by incubation of portions with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ (4 nM) $\pm$ 0.4 μM $1,25(\text{OH})_2\text{D}_3$. R_T (□) was determined in each mixture by immunoassay. The difference between R_T and R_0 represents the value of R_S (△) obtained in this experiment. All values represent the averages of triplicate determinations.

Fig. 4. Effect of sample volume on the measurement of $1,25(\text{OH})_2\text{D}_3$ receptor

Freshly prepared intestinal-mucosal extracts from four chicks were prepared, appropriately diluted, and then used to determine receptor competition by immunoassay. Each point represents the mean of triplicate determinations.

izing $1,25(\text{OH})_2\text{D}_3$ receptors, as well as in determining occupied receptors under physiological and experimental conditions.

Characterization of $1,25(\text{OH})_2\text{D}_3$ receptor by sedimentation analysis and DNA-cellulose chromatography

We tested the applicability of the immunoassay in receptor characterization first by utilizing it to determine the sedimentation coefficient of the protein. Chick intestinal extracts were sedimented on 10–30% (w/v) sucrose gradients, and then each fraction examined for competition by immunoassay. As illustrated in the profile seen in Fig. 5, the competing molecule sediments less rapidly than ovalbumin, at an $s_{20,w}$ value of approx. 3.3S, a value consistent with that observed for receptor characterized by $1,25(\text{OH})_2\text{D}_3$ binding (also see Fig. 5). Coincidentally, liver cell extracts, examined in an identical fashion, reveal no such competing molecule, verifying both the negative $1,25(\text{OH})_2\text{D}_3$ -binding activity seen in liver (results not shown) as well as the negative competition obtained in liver without gradient fractionation. Note here the increasing background in this Figure due to the interference observed with increasing concentrations of sucrose.

Likewise, we also attempted to characterize the competing molecule by DNA-cellulose chromatography, since previous work has demonstrated that the $1,25(\text{OH})_2\text{D}_3$ receptor is a DNA-binding protein (Pike & Haussler, 1979; Pike, 1982). Chick intestinal extracts were applied to DNA-cellulose under conditions that permitted adsorption ($<0.15\text{M-KCl}$), and then the column was exposed to a linear salt gradient between 0.1 and 0.4M-KCl.

Fig. 5. Sedimentation analysis of chick intestinal $1,25(\text{OH})_2\text{D}_3$ receptors

Mucosal extracts (0.4 ml, 2.4 mg of protein) were incubated with either $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ or $1,25(\text{OH})_2\text{D}_3$ and then sedimented through 10–30% (w/v) sucrose as described in the Materials and methods section. Receptor was detected either directly by radiolabelled $1,25(\text{OH})_2\text{D}_3$ (□), or by immunoassay (○). Control chick liver cell extracts were similarly fractionated and evaluated for receptor by immunoassay (●). The external standard ovalbumin (3.7S) sedimented in fraction 11.

Fractions collected during the gradient were then examined in the immunoassay. Again, as observed in Fig. 6, we find that the molecule that was eluted from DNA-cellulose, and was reactive in the assay, co-migrated with authentic $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ receptor eluted under identical conditions, supporting the general applicability of the assay in detecting and characterizing $1,25(\text{OH})_2\text{D}_3$ receptor molecules.

Analyses of occupied-receptor concentration in chicks under experimental conditions

Secondly, we applied the immunoassay to quantifying the level of occupied receptor in chicks exposed to various dietary and experimental conditions. This problem has been addressed by previous investigators in chicks (Hunziker *et al.*, 1980, 1982) and rats (Massaro *et al.*, 1983) through independent exchange-assay methodology. We studied rachitic chicks fed low- and high-calcium vitamin D-deficient diets, a normal chick, and a chick made vitamin D-replete by repeated injection of pharmacological doses (6 nmol) of

Table 1. Measurement of endogenous 1,25(OH)₂D₃-receptor complexes and comparison with serum 1,25(OH)₂D₃ in chicks under various experimental and physiological conditions

Group*	1,25(OH) ₂ D ₃ receptor				Serum [1,25(OH) ₂ D ₃] (pg/ml)†
	Concn. (pmol/g)†			Ratio R _S /R _T (%)‡	
	R _T	R ₀	R _S		
A	26.7	21.2	5.5	20.5	183
B	28.3	24.5	3.8	13.4	36
C	27.5	23.9	3.6	13.1	31
D	21.2	9.2	12.0	56.7	714

* A, normal diet; B, vitamin D-deficient diet (0.55% calcium); C, vitamin D-deficient diet (2.7% calcium); D, diet as in C and injected with 6nmol of 1,25(OH)₂D₃ 2, 24, and 48 h before killing.

† Values are the averages of triplicate determinations.

‡ A comparison of R_S/R_T (%) with serum 1,25(OH)₂D₃ concentration yields a linear plot with y-intercept at 10.4% and a correlation coefficient of 0.998.

Fig. 6. DNA-cellulose chromatography of chick intestinal 1,25(OH)₂D₃ receptors

Mucosal extracts were incubated with 1,25(OH)₂D₃ or 1,25(OH)₂[³H]D₃ and then chromatographed on DNA-cellulose as described in the Materials and methods section. Fractions eluted from the column during an applied linear gradient between 0.15 and 0.40 M-KCl were either examined for 1,25(OH)₂-[³H]D₃ (●) or evaluated by immunoassay (○) for 1,25(OH)₂D₃ receptors.

1,25(OH)₂D₃. The immunoassay results, coupled with 1,25(OH)₂D₃-binding assays, permit a determination of R_S in these animals, and are summarized in Table 1. They are also contrasted with the circulating 1,25(OH)₂D₃ concentration measured in these animals at the time they were killed. As is observed, rachitic-chick intestine, whether exposed to low- or high-calcium diets, exhibits approx. 13% of the 26pmol of receptor/g in the occupied state. However, the proportion of

R_S increased to 20.5% in the normal animal, concomitant with a significant rise in circulating 1,25(OH)₂D₃. This observation suggests that, under normal physiological conditions, the cell contains an abundance of receptor (80%) capable of mediating the 1,25(OH)₂D₃ signal. Finally, under conditions of hormone excess, achieved by acute pharmacological dose of 1,25(OH)₂D₃ (6nmol/injection), the amount of R_S rises to 56.7%, coincident with the very high 1,25(OH)₂D₃ levels detected in the serum. Interestingly, a plot of 1,25(OH)₂D₃ concentration against occupied receptor yields a line that intercepts at 10.4% R_S (probably denatured receptor, R_{denat.}) and has a correlation coefficient of 0.998, confirming the hypothesis presented previously (Hunziker *et al.*, 1982) that occupied receptors are in a simple equilibrium with the prevailing level of the hormone. Also apparent is a decrease in R_T in the dosed chick, a possible function of either biological variation or the inability of the cell to synthesize new receptor protein as rapidly as it is being processed. Therefore, although these results are limited to single animals, they confirm prior observations by an independent methodology. Thus they validate the immunological approach to measuring the various receptor forms in animals under physiological conditions.

Discussion

The utility of radioimmunoassay in quantifying the levels of various biological components has long been established. Their application to the measurement of steroid receptors, however, has been very recent, primarily owing to the difficulty that has been encountered in purifying steroid receptors, and generating both polyclonal and monoclonal antibodies. Nevertheless, this has now been accomplished for glucocorticoid (Eisen,

1980; Okret *et al.*, 1981), progesterone (Logeat *et al.*, 1981; Renoir *et al.*, 1982), oestrogen (Greene *et al.*, 1980a,b; Monchamont *et al.*, 1982) and 1,25(OH)₂D₃ (Pike *et al.*, 1982, 1983) receptors. Depending on the availability of purified receptor, the approach to the development of an immunoassay has varied. For example, since glucocorticoid receptors can be readily and extensively purified, Okret *et al.* (1981) devised a competitive enzyme-linked immunosorbent assay. In contrast, Greene & Jensen (1982) created an immunoradiometric assay utilizing two monoclonal antibodies that bind to different epitopes on the oestrogen receptor. Our r.l.i.a. represents an initial attempt to devise procedures that do not require purified 1,25(OH)₂D₃ receptor. The result is an assay that is sensitive and accurate, useful over a wide range of receptor concentrations, and applicable to the measurement of any 1,25(OH)₂D₃ receptor for which the K_d for its interaction with antibody can be determined or assumed.

The advantages of this assay over ligand-binding techniques are considerable: (1) quantification is not based on sample binding to 1,25(OH)₂[³H]D₃ and is therefore not directly sensitive to differential dissociation of hormone in the sample; (2) since the antibody also recognizes receptor that no longer can bind hormone ($R_{denat.}$), rigorous preparation of the sample at low temperature, to minimize the formation of this species from functional receptor, is not required if total receptor content is the measurement of interest; (3) since the immunoassay measures R_T , it is useful, combined with 1,25(OH)₂D₃-binding assays, in quantifying endogenously occupied receptor under physiological conditions; (4) it is not influenced by the presence of DBP, which often compromises measurement of receptor owing to its capacity both to bind 1,25(OH)₂D₃ and to interfere with 1,25(OH)₂D₃ binding to receptor; and (5) the assay can detect variant receptor molecules that do not show 1,25(OH)₂D₃-binding activities (see below). The primary disadvantages of the assay are that: (1) it is more time-consuming than the ligand-binding approach, and (2) since $R_{denat.}$ comprises an unknown proportion of immunoassayable R_T , one must be rigorous and consistent about conditions of preparation to minimize the appearance of this form if the intent of the experiment is to estimate R_S .

The assay, as applied here, was capable of identifying both physical and functional characteristics of the 1,25(OH)₂D₃ receptor. First, in comparison with sedimentation analysis, the immunoassay revealed the receptor to be a 3.3S macromolecule (Fig. 5), confirming previous estimates by ligand-binding procedures (Brumbaugh & Haussler, 1975; Pike & Haussler, 1979). Second-

ly, in conjunction with DNA-cellulose chromatography, the immunoassay identified the propensity of the 1,25(OH)₂D₃ receptor to bind to DNA, and to dissociate from this ligand under conditions of increasing ionic strength (Fig. 6). Finally, the assay was essential in the calculation of R_S in chicks exposed to various dietary and hormonal manipulations (Table 1). The results clearly confirm observations made by exchange-assay techniques (Hunziker *et al.*, 1980, 1982; Massaro *et al.*, 1983), that the occupied receptor represents a small proportion of the total receptor content of cells under normal conditions, and is probably in simple dynamic equilibrium with circulating 1,25(OH)₂D₃ (Hunziker *et al.*, 1982). Although occupied-receptor levels in rachitic chicks measured here are slightly higher than that observed by exchange assay (4.5–11%) (Hunziker *et al.*, 1980), they may reflect either the incompleteness of vitamin D-depletion [1,25(OH)₂D₃ = 31–36 pg/ml] or, more likely, the fact that the immunoassay also detects denatured receptor that may arise from R_0 during mucosal-extract preparation. In any event, the assay will probably be useful in examining the potential regulation of the 1,25(OH)₂D₃ receptor by principles such as glucocorticoids and oestrogens, for which positive preliminary data already exist (Hirst & Feldman, 1982; Coty *et al.*, 1982), as well as in the study of receptor ontogenesis, as described initially for its 1,25(OH)₂D₃-binding activity in rats (Halloran & DeLuca, 1981).

The monoclonal antibody 9A7 γ cross-reacts extensively with 1,25(OH)₂D₃ receptors from mammalian tissues and cells, including those from humans (Pike *et al.*, 1982), and saturation analysis has revealed similarities in K_d for these forms (Pike, 1983). Thus the r.l.i.a. will be useful in quantifying receptor from a variety of non-chick sources. In fact, we have recently applied this assay to the quantification of receptors in a series of human fibroblasts, derived in part from patients exhibiting tissue resistance to 1,25(OH)₂D₃ (Liberman *et al.*, 1983). The results have revealed that these cells, many of which exhibit hormone-binding negative phenotypes, actually contain normal concentrations of the receptor by immunoassay (Pike *et al.*, 1984).

This work was supported by National Institutes of Health grants AM-15781 (to M. R. H.) and AM-32313 (to J. W. P.). We thank Ms. Carol A. Donaldson for technical assistance in our cell-culture facility.

References

- Brumbaugh, P. F. & Haussler, M. R. (1974) *J. Biol. Chem.* **249**, 1251–1257
- Brumbaugh, P. F. & Haussler, M. R. (1975) *J. Biol. Chem.* **250**, 1588–1594

- Chandler, J. S., Pike, J. W. & Haussler, M. R. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1057-1063
- Christakos, S. & Norman, A. W. (1981) *Endocrinology* **108**, 140-149
- Coty, W. A., Scordato, J. C. & McConkey, C. L., Jr. (1982) in *Vitamin D, Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism* (Norman, A. W., Schaefer, K., Herrath, D. v. & Grigoleit, M.-G., eds.), pp. 121-123, Walter de Gruyter, New York
- DeLuca, H. F. & Schnoes, H. K. (1976) *Annu. Rev. Biochem.* **45**, 631-666
- Dokoh, S., Pike, J. W., Chandler, J. S., Mancini, J. M. & Haussler, M. R. (1981) *Anal. Biochem.* **116**, 211-222
- Dokoh, S., Donaldson, C. A., Marion, S. L., Pike, J. W. & Haussler, M. R. (1983) *Endocrinology* **112**, 200-206
- Eisen, H. F. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3893-3897
- Greene, G. L. & Jensen, E. V. (1982) *J. Steroid Biochem.* **16**, 353-359
- Greene, G. L., Fitch, F. W. & Jensen, E. V. (1980a) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 157-161
- Greene, G. L., Nolan, C., Engler, J. P. & Jensen, J. R. (1980b) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5115-5119
- Halloran, B. P. & DeLuca, H. F. (1981) *J. Biol. Chem.* **256**, 7338-7342
- Haussler, M. R. & McCain, T. A. (1977) *N. Engl. J. Med.* **297**, 974-983, 1041-1050
- Haussler, M. R., Myrtle, J. F. & Norman, A. W. (1968) *J. Biol. Chem.* **243**, 4055-4064
- Haussler, M. R., Chandler, J. S., Hagan, L. A. & Pike, J. W. (1980) *Methods Enzymol.* **67**, 529-542
- Hirst, M. & Feldman, D. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1590-1596
- Hunziker, W., Walters, M. R. & Norman, A. W. (1980) *J. Biol. Chem.* **255**, 9534-9537
- Hunziker, W., Walters, M. R., Bishop, J. E. & Norman, A. W. (1982) *J. Clin. Invest.* **69**, 826-834
- Kream, B. E., Jose, M., Yamada, S. & DeLuca, H. F. (1977) *Science* **197**, 1086-1088
- Liberman, U. S., Eil, C. & Marx, S. J. (1983) *J. Clin. Invest.* **71**, 192-200
- Logeat, F., Hai, M. T. V. & Milgrom, E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1426-1430
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Massaro, E. R., Simpson, R. U. & DeLuca, H. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2549-2553
- McNutt, K. W. & Haussler, M. R. (1973) *J. Nutr.* **13**, 681-689
- Moncharmont, B., Su, J. & Parikh, I. (1982) *Biochemistry* **21**, 6916-6921
- Okret, S., Carlstedt-Duke, J., Wrangé, Ö., Carlström, K. & Gustafsson, J.-Å. (1981) *Biochim. Biophys. Acta* **677**, 205-219
- Pike, J. W. (1982) *J. Biol. Chem.* **257**, 6766-6775
- Pike, J. W. (1983) *Meet. Endocrine Soc. 65th, San Antonio, TX*, p. 89
- Pike, J. W. (1984) *J. Biol. Chem.* **259**, 1167-1173
- Pike, J. W. & Haussler, M. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5485-5489
- Pike, J. W. & Haussler, M. R. (1983) *J. Biol. Chem.* **258**, 8554-8560
- Pike, J. W., Donaldson, C. A., Marion, S. L. & Haussler, M. R. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7719-7723
- Pike, M. W., Marion, S. L., Donaldson, C. A. & Haussler, M. R. (1983) *J. Biol. Chem.* **258**, 1289-1296
- Pike, J. W., Dokoh, S., Haussler, M. R., Liberman, U. A., Marx, S. J. & Eil, C. (1984) *Science* in the press
- Renoir, J., Radanyi, C., Yang, C. & Baulieu, E. (1982) *Eur. J. Biochem.* **127**, 81-86
- Stumpf, W. E., Sar, M., Reid, F. A., Tanaka, Y. & DeLuca, H. F. (1979) *Science* **206**, 1188-1190
- Zerwekh, J. E., Lindell, T. J. & Haussler, M. R. (1976) *J. Biol. Chem.* **251**, 2388-2394
- Zile, M., Bunge, E. C., Barsness, L., Yamada, S., Schnoes, H. K. & DeLuca, H. F. (1978) *Arch. Biochem. Biophys.* **186**, 15-24