Biochimica et Biophysica Acta 965 (1988) 52–59
Elsevier

BBA 22907

Application of the rat thymus receptor in a specific, sensitive and simplified assay of 1,25-dihydroxyvitamin D in blood serum

Iwona Grądzka, Jacek Łukaszkiewicz and Roman Lorenc

Department of Biochemistry and Experimental Medicine, Child's Health Center, Warsaw (Poland)

(Received 29 June 1987)
(Revised manuscript received 18 January 1988)

Key words: 1,25-Dihydroxyvitamin D receptor; Cooperative behavior; Receptor affinity; Receptor specificity; Radioreceptor assay; (Rat thymus)

A simple method has been employed to prepare crude nuclear extract from rat thymus, using hypertonic buffer after previous treatment with hypotonic buffer. The preparation is free from serum vitamin D-binding protein and contains a 3.7 S receptor molecule, which specifically binds 1,25-dihydroxyvitamin D-3 (1,25-$(OH)_2D_3$). The receptor is of high affinity ($K_D = 0.85 \cdot 10^{-11}$ M at 0 °C) and low capacity (260–460 fmol/g tissue). The Scatchard analysis of ligand binding results in a concave downward curve. The Hill analysis of the same data gives good linear fitting ($r = +0.971$) with the Hill coefficient $n_H = 1.63$. These facts indicate positive cooperativity between two ligand binding sites of the rat thymus 1,25-$(OH)_2D_3$ receptor. The preparation was used in a competitive protein binding assay of 1,25-$(OH)_2D$ in serum extracts, purified on Sep-Pak C_{18} followed by silica Sep-Pak cartridges. The method was sensitive to 0.5 pg/tube (2.0 ng/l) when 1 ml of serum was extracted. Intra- and interassay coefficients of variation were 9% and 14%, respectively. The serum 1,25-$(OH)_2D$ concentration estimated in 33 children (mean age 6.5 ± 3 years) was 46.6 ± 18.4 ng/l (mean ± S.D.).

Abbreviations: 1,25-$(OH)_2D$ receptor, 1,25-dihydroxyvitamin D receptor; PMSF, phenylmethylsulfonyl fluoride; TEDMP buffer, 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF and 10 mM sodium molybdate, pH 7.4; TEDMPK-0.3 buffer, 0.3 M KCl in TEDMP buffer; HPLC, high performance liquid chromatography; PPO, 2,5-diphenyloxazole; POPOP, 1,4-di-2-(5-phenyloxazolyl)benzene; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D-3; 1,25-$(OH)_2D_2$, 1α,25-dihydroxyvitamin D-2; 1α-OHD_3, 1α-hydroxyvitamin D-3; 25-OHD_2, 25-hydroxyvitamin D-2; 25-OHD_3, 25-hydroxyvitamin D-3; 24,25-$(OH)_3D_3$, 24(*R*),25-dihydroxyvitamin D-3; 1,24,25-$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D-3; 1,25-$(OH)_2$-24-oxo-D_3, 1α,25-dihydroxy-24-oxovitamin D-3; 1,23,25-$(OH)_3$-24-oxo-D_3, 1α,23ζ,25-trihydroxy-24-oxovitamin D-3. DBP, vitamin D-binding protein. DCC, dextran-coated charcoal.

Correspondence: I. Grądzka, Department of Biochemistry and Experimental Medicine, Child's Health Center, Al. Dzieci Polskich 20, 04 736 Warsaw, Poland.

Introduction

1α,25-Dihydroxyvitamin D (1,25-$(OH)_2D$) is the most active form of vitamin D. Estimation of this secosteroid is helpful in diagnosis and management of various diseases connected with vitamin D and mineral metabolism disorders.

Many methods have been developed to estimate 1,25-$(OH)_2D$ levels in blood serum, namely isotope dilution-mass fragmentography [1], bioassay [2], radioimmunoassay [3–7], cytoreceptor assay [8] and radioreceptor assay [9–19]. All of them are very laborious, including extraction of serum with organic solvent and purification of the extract with the use of techniques such as Sephadex LH-20, Extrelut, Celite, high performance liquid

chromatography (HPLC), etc., prior to the final 1,25-$(OH)_2D$ assay. Further improvements of these methods aim for reducing and simplifying the preparatory work. It seems that the methods using 1,25-$(OH)_2D$ receptors may be the most successful in this regard. They provide high specificity, so that the long prepurification procedure can be shortened and the HPLC step omitted. In addition, high sensitivity of the receptors allows a reduction of the serum volume used, which is of great importance, especially in the case of paediatric patients.

The chick intestinal cytosol was the first, and till now the most commonly used preparation of the receptor for 1,25-$(OH)_2D$ estimations [9–15]. Next, other reports appeared recommending 1,25-$(OH)_2D$ receptors from rabbit intestine [16], pig intestine [18], and bovine mammary gland [19].

In 1984 Reinhardt et al. [17] used calf thymus as a source of binding protein for 1,25-$(OH)_2D$ assay. In contrast to the chick intestinal receptor, the thymus receptor was found to be insensitive to lipid interference [13,17] and reacted equally with both 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ [17].

Considering these advantages, we have investigated the possibility of utilization of rat thymuses, which are freely available in our laboratory.

In this paper we propose a simple and efficient method for obtaining 1,25-$(OH)_2D$ receptor from rat thymus. Rat thymus 1,25-$(OH)_2D$ receptor has been characterized with respect to its binding and specificity. We have demonstrated that the preparation is suitable for the radioreceptor assay of 1,25-$(OH)_2D$ in blood serum.

Materials and Methods

Chemicals. 1,25-$(OH)_2$-[23,24(n)-^{3}H]D_3 (spec. act. 80–120 Ci/mm and 130–180 Ci/mmol; > 95% purity by HPLC analysis) was purchased from Amersham (Amersham, U.K.). 1α-OHD_3 was a gift of Dr. W.O. Godtfredsen from Leo Farmaceutical Products (Denmark). 1,24,25-$(OH)_3D_3$, 1,25-$(OH)_2$-24-oxo-D_3 and 1,23,25-$(OH)_3$-24-oxo-D_3 were synthesized in the laboratory of Dr. M. Uskokovic of Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.) and kindly supplied by Dr. E. Mayer (Heidelberg, F.R.G.). Vitamin D-2, 25-OHD_2, vitamin D-3, 25-OHD_3, 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were from Merck Co. (Darmstadt, F.R.G.).

Toluene for scintillation fluid (Scintran) was from BDH Chemicals Ltd. (Poole, U.K.), and all other solvents were from Merck Co. (Darmstadt, F.R.G.).

Charcoal-activated Dextran-80, dithiothreitol and PMSF were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). PPO, POPOP and Tris were purchased from Fluka A.G. and Busch S.G. (Switzerland). Triton X-100, human γ-globulin, ovalbumin and chymotrypsinogen were from Serva Co. (Heidelberg, F.R.G.), bovine serum albumin fraction V from Miles Laboratories Inc. (U.S.A.), and sucrose for ultracentrifuge work from Carl Roth KG Chemische Fabrik (Karlsruhe, F.R.G.).

Other reagents, all of analytical grade, were from POCH (Gliwice, Poland). In all preparations we used water distilled twice from glass.

Animals. 1–1.5-month-old Wistar rats, both male and female were obtained from our laboratory breeding unit. They were kept on standard diet.

Preparation of a crude nuclear extract from rat thymus glands. Rats were killed by etherization, and thymus glands were removed and washed several times with ice-cold 0.9% NaCl, then immediately used for 1,25-$(OH)_2D$ receptor preparation, or frozen in liquid nitrogen and stored at $-30\,^\circ$C until use. The preparation was carried out at 0–4 °C. Fresh or frozen thymus tissue was homogenized in a hypotonic TEDMP buffer, pH 7.4 (2.5% w/v) with three to five strokes of a Potter-Elvehjem Teflon glass homogenizer. The homogenate was centrifuged for 1 h at 100 000 × g in a Beckman L8-80 ultracentrifuge and the supernatant (the low-ionic-strength extract) was separated together with the lipid layer. The pellet was then resuspended in a hypertonic TEDMPK-0.3 buffer, pH 7.4 (10%, w/v) with the use of a Potter-Elvehjem homogenizer. The suspension was centrifuged again as above to give the crude nuclear extract (the high-ionic-strength extract). Aliquots of the two successive thymus extracts were analyzed on sucrose density grdients, as described below. The high-ionic-strength extract, which revealed 1,25-$(OH)_2D$ receptor activity, was frozen in liquid nitrogen and stored at $-60\,^\circ$C

until use (at least 6 months without any loss of receptor activity). For the 1,25-$(OH)_2D$ assay a dilution of the preparation was used, which specifically bound 20% of total radioactivity added. Protein concentration in the preparation was estimated by the method of Lowry et al. [20].

Time and temperature dependence of receptor binding. 0.5 ml of the crude nuclear extract from rat thymuses was incubated with 0.1 nM [^{3}H]-1,25-$(OH)_2D_3$ with (nonspecific binding) or without (total binding) a 100-fold excess of 1,25-$(OH)_2D_3$ at 0, 20 or 37 °C over a period of time from 0 to 20 h.

Subsequently, 0.2 ml of ice-cold dextran-coated charcoal (DCC) was added for 20 min to adsorb the unbound 1,25-$(OH)_2D_3$. The tubes were then centrifuged at 2500 rpm for 10 min in a K26 D centrifuge (Janetzki, G.D.R.) and radioactivity of supernatants was counted in a Beckman LS-7500 counter with the use of 5 ml scintillation fluid consisting of 5 g of PPO, 0.1 g of POPOP, 0.3 l of Triton X-100 and 0.7 l of toluene.

Sucrose density gradients. Linear 4–20% (w/v) gradients (3.55 ml) were prepared in TEDMPK-0.3 buffer (pH 7.4) as described [21]. 0.2 ml aliquots of either the low-ionic-strength extract (TEDMP extract plus KCl to 0.3 M) or the crude nuclear extract (TEDMPK-0.3 extract) were incubated for 5 h at 0 °C with 0.5 nM [^{3}H]-1,25-$(OH)_2D_3$ (spec. act. 94 Ci/mmol) in the absence or presence of a 100-fold excess of nonradioactive 1,25-$(OH)_2D_3$ or 25-OHD_3 and then applied to the gradients. After centrifugation at 50000 rpm for 20 h in a Beckman SW-60 rotor (Beckman L8-80 ultracentrifuge) they were fractionated into three-drop fractions and radioactivity was measured as above. Sedimentation markers were chymotrypsinogen (2.5 S), ovalbumin (3.7 S) and human γ-globulin (7.3 S) run in parallel gradients. After fractionation, protein was determined by the method of Lowry et al. [20].

Saturation study. 0.5 ml of the crude nuclear extract was incubated at 0 °C for 18 h with 0.01–1 nM [^{3}H]-1,24-$(OH)_2D_3$ with (nonspecific binding) or without (total bindng) a 100-fold excess of 1,25-$(OH)_2D_3$. The unbound ligand was removed by incubation with DCC and radioactivity measured as above. The experimental data were calculated by the method of Scatchard [22]. The Scatchard plot demonstrated a 'hook' effect, which is evidence of the cooperativity within binding sites of the receptor. Thus, better linear fitting of the data could be achieved by using the Hill equation [23], adapted by Rodbard and Bertino [24] to describe allosteric cooperativity in binding of a ligand to only two sites. The maximum content of binding sites (B_{max}) was indicated by the intercept of the Scatchard plot with the x axis. The dissociation constant of the second ligand binding site (K_{D2}) was calculated from the asymptote to the second portion of the Scatchard curve. The average dissociation constant of the two binding sites (K_D) as well as the Hill coefficient (n_H) were calculated from the Hill plot.

Preparation of serum samples. For each batch of samples at least three samples of pooled serum were added to monitor efficiency of the purification procedure. Before extraction they were incubated for 10 min with [^{3}H]1,25-$(OH)_2D_3$, 3000 dpm per sample (spec. act. 180 Ci/nmol) in 20 μl ethanol. Serum samples were extracted and purified according to the method of Reinhardt et al. [17]: to 1 ml serum sample (if less, it was filled up to 1 ml with 0.9% NaCl) 1 ml of acetonitrile was added, the tube was vortexed for 20 s and the protein precipitate was removed by centrifugation at 2000 rpm for 10 min at 4 °C in a K26D centrifuge (Janetzki, G.D.R.).

The supernatant was then transferred to a tube containing 0.5 ml of 0.4 M K_2HPO_4 solution (pH 10.6), vortexed and immediately applied to a Sep-Pak C_{18} cartridge (Waters Associated Inc., Milford, MA), prewashed with 3 ml of acetonitrile and 5 ml of water. After application of the sample, the cartridge was washed with 5 ml of water, 3 ml of methanol/water (70 : 30) and 3 ml of acetonitrile. The acetonitrile fraction, containing vitamin D metabolites, was dried under a stream of nitrogen. The fraction was subsequently applied to a silica Sep-Pak cartridge in hexane/isopropanol (96 : 4), twice in 0.5 ml. The cartridge was washed with 11 ml of hexane/isopropanol (96 : 4), 8 ml hexane/isopropanol (94 : 6), and 1,25-$(OH)_2D$ was eluted with 8 ml of hexane/isopropanol (85 : 15).

The final eluate was dried under N_2 and redissolved in 300 μl ethanol. From this volume, one 50 μl sample was used to determine recovery of

the tracer and two 50 µl aliquots were used for the assay.

Nonequilibrium radioreceptor assay. 50 µl of 1,25-$(OH)_2D_3$ standard solutions (1–32 pg/tube and 4 ng/tube for nonspecific binding) or 50 µl purified serum extracts were pipetted into glass test tubes in an ice-water bath. 500 µl of the 1,25-$(OH)_2D$ receptor preparation was added to each tube and vortexed. The samples were then incubated for 18 h at 0 °C. Subsequently, 6000 dpm of [^{3}H]-1,25-$(OH)_2D_3$ (spec. act. 90 Ci/mmol) was added and incubated for 2 h at 0 °C. At the end of the second incubation 200 µl of DCC was added, vortexed, and after 20 min at 0 °C bound and free hormones were separated by centrifugation. Radioactivity specifically bound to the receptor (B) was calculated by subtracting nonspecifically bound dpm from totally bound dpm. The maximal specific binding (B_0) was specifically bound radioactivity in the absence of unlabelled steroid. The standard curve was plotted as logit B/B_0 versus the logarithm of 1,25-$(OH)_2D_3$ concentration. 1,25-$(OH)_2D_3$ concentration in individual samples of patient sera was calculated from the standard curve and corrected for the mean recovery of the tracer, as well as for the serum dilution factor.

Specificity of nonequilibrium assay. Several vitamin D-2 and vitamin D-3 metabolites were studied for their ability to displace [^{3}H]-1,25-$(OH)_2D_3$ from the rat thymus receptor. These assays were identical to the nonequilibrium reaction described above, except that a series of dilutions of investigated metabolites, instead of 1,25-$(OH)_2D_3$, were incubated. For each incubation a standard curve with 1,25-$(OH)_2D_3$ was added to make the comparison reliable.

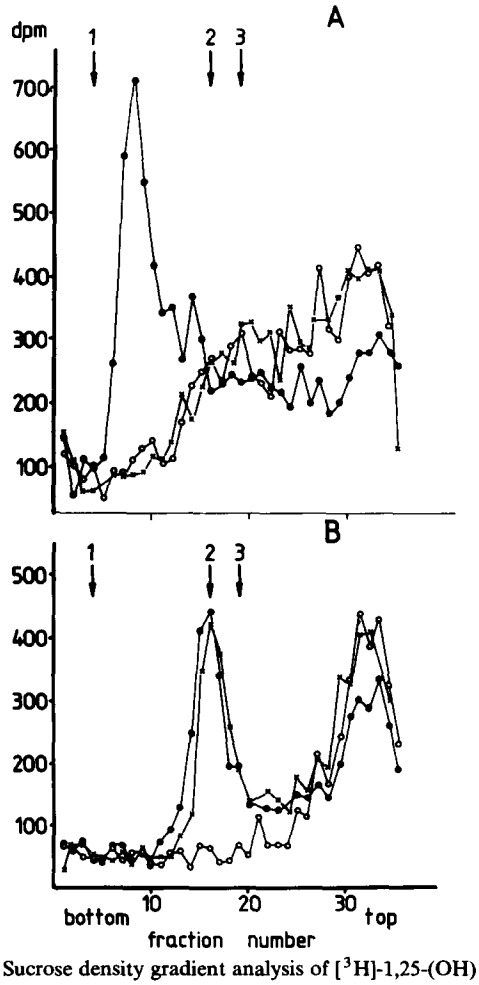

Fig. 1. Sucrose density gradient analysis of [^{3}H]-1,25-$(OH)_2D_3$ binding in two successive extracts from rat thymus tissue. (A) First, low-ionic-strength extract (in TEDMP buffer, plus 0.3 M KCl added just before centrifugation). (B) Second, high-ionic-strength extract (in TEDMPK-0.3 buffer). Aliquots were incubated with 0.5 nM [^{3}H]-1,25-$(OH)_2D_3$ alone (●) or with a 100-fold excess of either 1,25-$(OH)_2D_3$ (○) or 25-OHD_3 (×). Arrows indicate sedimentation positions of external protein standards: 1, human γ-globulin (7.3 S); 2, ovalbumin (3,7 S); 3, chymotrypsinogen (2.5 S).

Results

In the preparation of 1,25-$(OH)_2D$ receptor from rat thymus gland we followed the ability of 1,25-$(OH)_2D$ receptors to bind to chromatin DNA at low ionic strength [25,26]. Two-step extraction of thymus tissue was carried out and two successive extracts were analyzed on sucrose density gradients (Fig. 1). The first extraction, with the use of low-ionic-strength TEDMP buffer, resulted in the elution of a 6 S macromolecule, from which [^{3}H]-1,25-$(OH)_2D_3$ could be easy displaced by a 100-fold excess of both unlabelled 25-OHD_3 and 1,25-$(OH)_2D_3$ (Fig. 1A). This 6 S compound was probably the DBP-actin complex which usually contaminates tissue preparations [27,28]. Subsequent high-ionic-strength extract (after use of TEDMPK-0.3 buffer) contained a single [^{3}H]-1,25-$(OH)_2D_3$ binding macromolecule, which sedimented at 3.7 S (Fig. 1B). This 3.7 S component

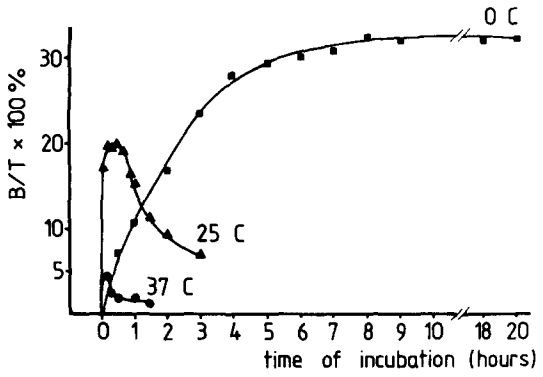

Fig. 2. Time and temperature dependence of specific [^{3}H]-1,25-$(OH)_2D_3$ binding by high-ionic-strength extract from rat thymuses. Samples were incubated with 0.1 nM [^{3}H]-1,25-$(OH)_2D_3$ at 0, 25 or 37 °C for different time periods. Protein concentration in the incubation mixture was 0.25% and the ethanol concentration was 10%.

could be eliminated by the addition of a 100-fold excess of unlabelled 1,25-$(OH)_2D_3$ but not 25-OHD_3 (Fig. 1B), suggesting specificity of the binding macromolecule for the former metabolite. The 3.7 S component of the high-ionic-strength extract (crude nuclear extract) from rat thymus gland is apparently 1,25-$(OH)_2D_3$ receptor previously found in rat thymus lymphocytes by Provvedini et al. [29].

The saturation rate and stability of the 1,25-$(OH)_2D_3$ receptor was examined at three temperatures: 0, 25 and 37 °C (Fig. 2). At 25 and 37 °C, saturation time is in the range of minutes, but the binding macromolecule undergoes fast thermal denaturation before maximum binding is reached. At 0 °C, equilibrium is reached by 5 h and remains stable up to 20 h of incubation. Thus, 0 °C was chosen as the optimal temperature for all subsequent experiments.

To obtain kinetic data for the rat thymus 1,25-$(OH)_2D_3$ receptor at 0 °C, a saturation study was made (Fig. 3A). The results were calculated by the method of Scatchard [22], giving a 'hook' downward curve (Fig. 3B), thus suggesting positive cooperativity in the binding of 1,25-$(OH)_2D_3$ to this receptor preparation. The maximal concentration of binding sites (B_{max}) found at the intercept of the Scatchard plot with the x axis was 24 pM (260 fmol/g tissue) for 1.5-month-old animals (Fig. 3B) and 45 pM (460 fmol/g tissue) for 1-month-old

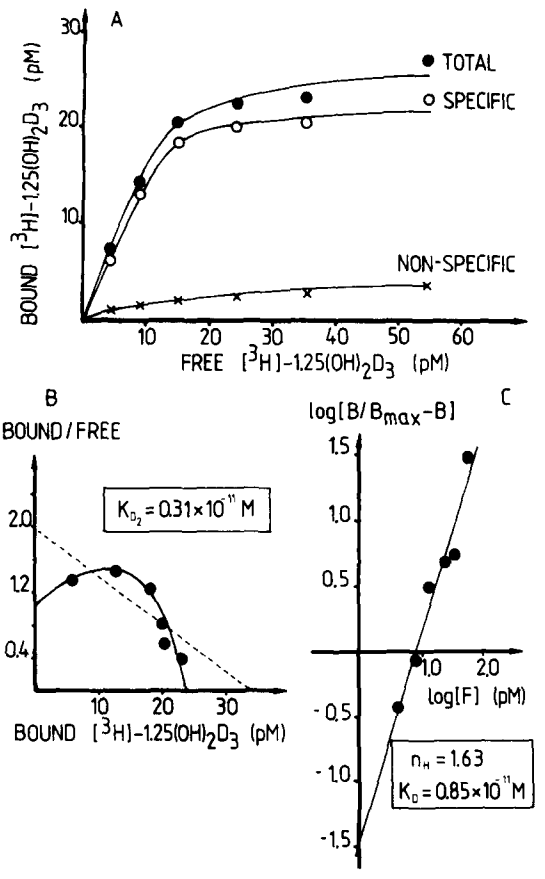

Fig. 3. Kinetic measurements for 1,25-$(OH)_2D_3$ binding to its receptor from rat thymus. (A) Saturation analysis of the high-ionic-strength extract (TEDMPK-0.3 extract) from rat thymuses of 1.5-month-old rats. The preparation was incubated for 18 h at 0 °C with increasing concentrations of [^{3}H]-1,25-$(OH)_2D_3$ in the presence (nonspecific binding) or absence (total binding) of a 100-fold excess of 1,25-$(OH)_2D_3$. Specific binding was calculated by subtracting nonspecific binding from total binding. (B) Scatchard plot of the data from experiment A. The dashed line shows the linear regression slope for all experimental points (correlation coefficient, $r = -0.817$). The 'hook' line represents the best fitting. (C) Hill plot of the data from experiment A. Linear regression analysis gives a correlation coefficient of +0.971.

animals (data not shown). The B_{max} value was then employed in transformation of the data by the Hill method [23,24]. As was to be expected, the Hill plot (Fig. 3C) showed better linear fitting (correlation coefficient, $r = +0.971$), than the Scatchard plot ($r = -0.817$). The Hill coefficient, n_H 1.63, indicated significant positive cooperativity of the two binding sites of the receptor. The

average dissociation constant of the two binding sites, estimated from the Hill plot, was K_D $0.85 \cdot 10^{-11}$ M, and the second binding site dissociation constant, calculated from the limiting slope of the Scatchard curve, was $K_{D2} = 0.31 \cdot 10^{-11}$ M.

Several vitamin D-2 and vitamin D-3 analogues were compared with 1,25-$(OH)_2D_3$ for their ability to displace [^{3}H]-1,25-$(OH)_2D_3$ from the rat thymus receptor, under nonequilibrium conditions at 0 °C (Figs. 4 and 5). The calculations made for 50% displacement of [^{3}H]-1,25-$(OH)_2D_3$ by several vitamin D metabolites in comparison to 1,25-$(OH)_2D_3$ gave the following coefficients of displacement: 1,25-$(OH)_2$-24-oxo-D_3 93%; 1,24,25-$(OH)_3D_3$ 74%; 1,23,25-$(OH)_2$-24-oxo-D_3 44%; 1α-OHD_3 0.79%; 25-OHD_2 0.79%; 25-OHD_3 0.63%; 24,25-$(OH)_2D_3$ 0.063%; vitamin D-2 0.019%; vitamin D-3 0.004%. As in the case of other 1,25-$(OH)_2D$ receptors, 25-OHD competes with 1,25-$(OH)_2D$ for the rat thymus receptor in concentrations normally occurring in human serum (about 1000-fold greater than 1,25-$(OH)_2D$ concentrations) and has to be removed from the serum

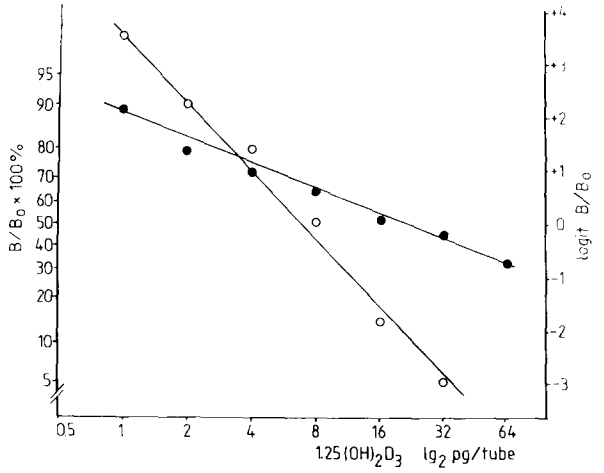

Fig. 4. 1,25-$(OH)_2D_3$ radioreceptor assay standard curves. Rat thymus receptor preparation was incubated with 0–64 pg/tube and 4 ng/tube (nonspecific binding) of 1,25-$(OH)_2D_3$ and 10 000 dpm/tube of [^{3}H]-1,25-$(OH)_2D_3$ (spec. act. 90 Ci/nmol) at 0 °C in two reaction systems: ●, equilibrium system (16–18 h incubation with both unlabelled 1,25-$(OH)_2D_3$ and [^{3}H]-1,25-$(OH)_2D_3$; ○, non-equilibrium system (16–18 h incubation with unlabelled 1,25-$(OH)_2D_3$ and then 2 h incubation with [^{3}H]-1,25-$(OH)_2D_3$). B, specific binding of [^{3}H]-1,25-$(OH)_2D_3$ in the presence of unlabelled 1,25-$(OH)_2D_3$; B_0, specific binding of [^{3}H]-1,25-$(OH)_2D_3$ in the absence of unlabelled 1,25-$(OH)_2D_3$.

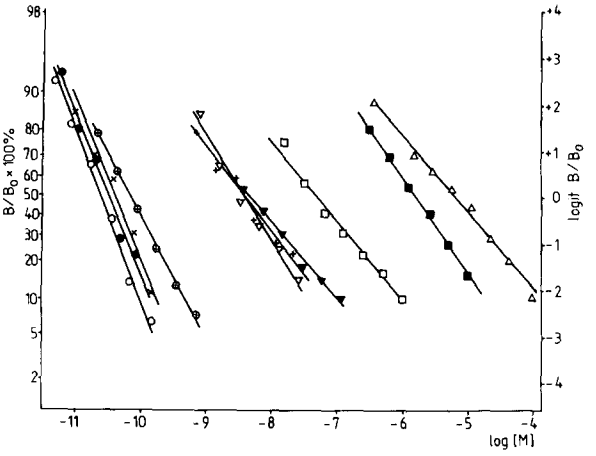

Fig. 5. Competition of vitamin D-2 and vitamin D-3 analogues with [^{3}H]-1,25-$(OH)_2D_3$ for the rat thymus receptor. Incubations were made in the non-equilibrium reaction system: 16–18 h incubation with vitamin D metabolite and then 2 h incubation with [^{3}H]-1,25-$(OH)_2D_3$, at 0 °C. Metabolites were marked as follows: ○, 1,25-$(OH)_2D_3$; ●, 1,25-$(OH)_2$-24-oxo-D_3; ×, 1,24,25-$(OH)_3D_3$; ⊗, 1,23,25-$(OH)_3$-24-oxo-D_3; +, 25-OHD_2; ▽, 1α-OHD_3; ▼, 25-OHD_3; □, 24,25-$(OH)_2D_3$; ■, vitamin D-2; △, vitamin D-3.

extract during the prepurification step.

Recovery of [^{3}H]-1,24-$(OH)_2D_3$ after extraction and purification of the extract on Sep-Pak cartridges was 76 ± 4% (mean ± S.D., n = 50). This good reproducibility led us to omit the addition of labelled steroid to each serum sample in order to monitor recovery.

Sensitivity of the nonequilibrium radioreceptor assay was established for five standard curves and was calculated as 0.5 pg/tube, resulting in a detection limit of 2.0 ng/l when 1 ml of serum was extracted.

To establish reproducibility of the assay, four different plasma pools were processed in single assays (five to ten samples in each) and in ten different assays. The mean intra-assay coefficient of variation was 9% and the inter-assay coefficient of variation was 14%.

The 1,25-$(OH)_2D$ level estimated in 33 apparently healthy children (mean age 6.5 ± 3 years) was 46.6 ± 18.4 pg/ml (mean ± S.D.).

Discussion

In this work we have presented a simple and efficient method for obtaining rat thymus 1,25-

$(OH)_2D$ receptor. The method was based upon the ability of 1,25-$(OH)_2D$ receptors to bind to chromatin DNA at low ionic strength [25,26]. Thus, 2.5% (w/v) homogenization of thymus tissue in a hypotonic TEDMP buffer resulted in washing of a 6 S compound, which was probably DBP-actin complex [28]. Under these conditions, apparently all of the 1,25-$(OH)_2D$ receptor remained bound to the chromatin fraction and after centrifugation could be extracted from the pellet by using a hypertonic TEDMPK-0.3 buffer. Sucrose density gradient analysis demonstrated that no substantial contamination of a 6 S vitamin D binding compound was present in the receptor preparation. The procedure could be applied successfully also to calf and rabbit thymus tissue (data not shown).

1,25-$(OH)_2D$ receptor present in high-ionic-strength rat thymus extract is a 3.7 S macromolecule, similar to other 1,25-$(OH)_2D$ receptors examined. Also, its binding specificity does not differ markedly from that of other 1,25-$(OH)_2D$ receptors. The competition study discovered the following order of affinity of vitamin D analogues for rat thymus 1,25-$(OH)_2D$ receptor: 1,25-$(OH)_2D_3 \geqslant$ 1,25-$(OH)_2$-24-oxo-$D_3 \geqslant$ 1,24,25-$(OH)_3D_3 >$ 1,23,25-$(OH)_3$-24-oxo-$D_3 >$ 1α-$OHD_3 \geqslant$ 25-$OHD_2 \geqslant$ 25-$OHD_3 >$ 24,25-$(OH)_2D_3 >$ vitamin D-2 > vitamin D-3.

Establishing kinetic parameters of the rat thymus 1,25-$(OH)_2D$ receptor, we found that it possesses two classes of positively cooperating ligand binding sites. This was concluded from the 'hook' downward Scatchard plot and high Hill coefficient, n_H 1.63 (data from saturation experiment). It is interesting that dissociation constants of the rat thymus receptor are at least 10-fold lower than dissociation constants of 1,25-$(OH)_2D$ receptors isolated from classical target organs. Thus, for the rat thymus receptor the average dissociation constant, K_D, was $0.85 \cdot 10^{-11}$ M and the second binding site dissociation constant, K_{D2}, was $0.31 \cdot 10^{-11}$ M, while for the rat 1,25-$(OH)_2D$ receptor from intestine, $K_D = 3.8 \cdot 10^{-10}$ M [30], kidney, $K_D = 3.4 \cdot 10^{-10}$ M [31], and bone, $K_D = 2.0 \cdot 10^{-10}$ M [32]. This extremely high affinity is accompanied with relatively low capacity: 260–460 fmol/g tissue. Indeed, Provvedini et al. [29] established that rat thymus 1,25-$(OH)_2D$ receptor was probably exclusively located in one subset of thymocytes – large, mitotically active lymphoblasts. Contrary to our results, these authors have observed only single class of binding site of the receptor. This disagreement could be the result of other preparation conditions [33], as well as of different [^{3}H]1,25-$(OH)_2D_3$ concentrations used in the saturation experiment. However, the apparent dissociation constant calculated by Provvedini et al. [29] (K_D $0.33 \cdot 10^{-11}$ M) corresponded with the second binding site dissociation constant (K_D $0.31 \cdot 10^{-11}$ M) established by us.

Finally, we have demonstrated that the preparation of 1,25-$(OH)_2D$ receptor from rat thymus is suitable for simplified 1,25-$(OH)_2D$ assay in blood serum.

The preparation, after freezing in liquid nitrogen, was stable for at least 6 months at $-60\,^\circ$C without any loss of binding activity. The amount of the receptor prepared from 100 rat thymuses was sufficient for ten 1,25-$(OH)_2D$ assays, with 40 serum samples in each. The rat thymus receptor is highly specific for 1,25-$(OH)_2D$, which allows the use of a simplified serum prepurification procedure omitting HPLC [17]. The assay is sensitive to 0.5 pg/tube, so that practically as little as 0.3 ml serum can be used. Inter- and intra-assay coefficients of variation were 14% and 9%, respectively. The 1,25-$(OH)_2D$ level estimated in sera of 33 apparently healthy children (mean age 6.5 ± 3 years) was 46.6 ± 18.4 ng/l (mean $\pm$ S.D.).

Acknowledgments

We are grateful to Dr. Eberhard Mayer and Dr. W.O. Godtfredsen for kindly providing vitamin D compounds. We also wish to thank Mr. Tomasz Kowalski for his excellent technical assistance. The work was partly supported by grant PL-ARS-99 I-MOA-USDA-4.

References

1 Björkhem, J., Holmberg, H., Kristiansen, T. and Pedersen, J.I. (1979) Clin. Chem. 25, 584–588.
2 Stern, P.H., Phillips, T.E. and Mavreas, T. (1980) Anal. Biochem. 102, 22–30.
3 Clemens, T.L., Hendy, G.N., Papaulos, S.E., Fraher, L.J., Care, A.D. and O'Riordan, J.L.H. (1979) Clin. Endocrinol. 11, 225–234.

4 Bouillon, R., De Moor, P., Baggiolini, E.G. and Uskokovic, M.R. (1980) Clin. Chem. 26, 562–567.

5 Gray, T.K., McAddo, T., Pool, D., Lester, G.E., Williams, M.E. and Jones, G. (1981) Clin. Chem. 27, 458–463.

6 Gray, T.K. and McAdoo, T. (1983) Clin. Chem. 29, 196–200.

7 Faher, L.J., Adami, S., Clemens, T.J., Jones, G. and O'Riordan, J.L.H. (1983) Clin. Endocrinol. 18, 151–165.

8 Manolagas, S.C., Howard, J.E., Abare, J.M., Culler, F.L., Brickman, A.S. and Deftos, J.L. (1982) in Vitamin D, Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism (Norman, A.W. et al., eds.), pp. 769–771, Walter de Gruyter, Berlin.

9 Brumbaugh, P.F., Haussler, D.H., Bursac, K.M. and Haussler, M.R. (1974) Biochemistry 13, 4091–4097.

10 Eisman, J.A., Hamstra, A.J., Kream, B.E. and De Luca, H.F. (1976) Arch. Biochem. Biophys. 176, 235–243.

11 Mallon. J.P., Hamilton, J.G., Nauss-Karol, C., Karol, R.J., Ashley, C.J., Matuszewski, D.S., Tratnyek, C.A., Bryce, G.F. and Miller, O.N. (1980) Arch. Biochem. Biophys. 201, 227–285.

12 Dabek, J.T., Härkönen, M. and Adlerkreutz, H. (1981) Scand. J. Clin. Lab. Invest. 41, 151–158.

13 Dokoh, S., Pike, J.W., Chandler, J.S., Mancini, J.M. and Haussler, M.R. (1981) Anal. Biochem. 116, 211–222.

14 Jongen, M.J.M., Van der Vijgh, W.J.F., Willems, H.J.J. and Netelenbos, J.C. (1981) Clin. Chem. 27, 444–450.

15 Endres, D., Lu, J., Mueller, J., Adams, J., Holick, M. and Broughton, A. (1982) in Vitamin D, Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism (Norman, A.W., et al., eds.), pp. 813–815, Walter de Gruyter, Berlin.

16 Duncan, W.E., Aw, T.C., Walsh, P.G. and Haddad, J.G. (1983) Anal. Biochem. 132, 209–214.

17 Reinhardt, T.A., Horst, R.L., Orf, J.W. and Hollis, B.W. (1984) J. Clin. Endocrinol. Metab. 58, 91–98.

18 Clayton, J., Guilland-Cumming, D.F., Kanis, J.A. and Russel, R.G.G. (1982) in Vitamin D, Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism (Norman, A.W., et al., eds.), pp. 821–823, Watler de Gruyter, Berlin.

19 Blayau, M., Leray, G., Prodhomme, C., David, V. and Peron, P. (1986) Clin. Chim. Acta 158, 199–206.

20 Lowry, O.M., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

21 Kream, B.E., Yamada, S., Schones, H.K. and De Luca, H.F. (1977) J. Biol. Chem. 252, 4501–4505.

22 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672.

23 Hill, A.V. (1910) J. Physiol. 40, 190.

24 Rodbard, D. and Bertino, R.E. (1973) Receptors for Reproductive Hormones, pp. 327–341, Plenum Press, New York.

25 Kream, B.E., Reynolds, R.D., Knutson, J.C., Eisman, J.A. and De Luca, H.F. (1976) Arch. Biochem. Biophys. 176, 779–787.

26 Walters, M.R., Hunziker, W. and Norman, A.W. (1980) J. Biol. Chem. 255, 6799–6805.

27 Van Baelen, H., Bouillon, R. and De Moor, P. (1977) J. Biol. Chem. 252, 2515–2518.

28 Van Baelen, H., Bouillon, R. and De Moor, P. (1980) J. Biol. Chem. 255, 2270–2272.

29 Provvedini, D.M., Deftos, L.J. and Manolagas, S.C. (1984) Biochem. Biophys. Res. Commun. 121, 277–283.

30 Halloran, B. and De Luca, H.F. (1981) J. Biol. Chem. 256, 7338–7342.

31 Chandler, J.S., Pike, J.W. and Haussler, M.R. (1979) Biochem. Biophys. Res. Commun. 90, 1057–1063.

32 Manolagas, S.C., Haussler, M.R. and Deftos, L.J. (1980) J. Biol. Chem. 255, 4414–4417.

33 Wilhelm, F. and Norman, A.W. (1985) J. Biol. Chem. 260, 10087–10092.