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EFFECT OF DITHIOTHREITOL ON THE 1 α, 25-DIHYDROXYVITAMIN D_3 CHICK INTESTINAL RECEPTOR ANALYZED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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This is the first report of the use of non-denaturing polyacrylamide gel electrophoresis (PAGE) to measure the apparent molecular weight of the chick intestinal 1α , 25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ - D_3) receptor and to study the effect of dithiothreitol on it . When prepared in the absence of this factor, chick intestinal cytosol contained one major specific 1,25 - $(OH)_2$ - D_3 binding peak . Its apparent molecular weight was 95,200 ± 1,900 (SD) daltons . Preparation of the cytosol in the presence of 5 mM dithiothreitol resulted in the appearance, besides the 95,000 daltons peak, of an additional 1,25 - $(OH)_2$ - D_3 binding peak, the molecular weight of which was 73,600 ± 3,300 (SD) . This effect of dithiothreitol could be suppressed by the simultaneous addition of 10 mM Nα-p-tosyl-L-arginine methyl ester (TAME), a protease inhibitor .

1,25-$(OH)_2$-D_3 is known as the hormonally active form of vitamin D_3, capable of stimulating calcium and phosphate absorption in the intestine and an action mechanism similar to that described for classic steroid hormones has been proposed for it (1) . This mechanism involves the presence of specific receptor proteins characterized by high affinity and low capacity located in the target tissues . 1,25 - $(OH)_2$-D_3 receptors have been detected in tissues implicated in the handling of calcium including intestinal mucosa, bone, parathyroid glands, pancreas and kidney (2,3,4) . Among them the chick intestinal mucosa 1,25-$(OH)_2$-D_3 receptor has been the most thoroughly studied and its apparent molecular weight has been determined many times via gel filtration resulting in various values ranging from 47,000 to 96,000 (5, 6, 7, 8, 9, 10) . As PAGE used in non-denaturing conditions had been shown to be suitable for analysis of progesterone and glucocorticosteroid receptors (11, 12) , we decided to use it to measure the 1,25-$(OH)_2$-D_3 receptor mole-

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cular weight and to study the effects of dithiothreitol, a stabilizing factor usually added to the receptor preparation buffers .

MATERIALS AND METHODS

Animals

One-day old Warren cockerels were supplied by SFPA, Lorris, France . They were put on a vitamin D-free diet containing 0.15 % Ca and 0,3 % P (CNRZ, La Minière, France) and water ad libitum . They were maintained under UV-deprived constant lighting until the age of twelve days .

Chemicals

Steroids . [^{3}H] - 25-OH-D_3 (9.6 Ci/mmol) and [^{3}H]- 1,25 -$(OH)_2$-D_3 (110 Ci/mmol) were obtained from the Radiochemical Center, Amersham, U.K. Non-radioactive 25-OH-D_3 was a gift from Dr. Six of Roussel-Uclaf Laboratories, France, and non-radioactive 1,25-$(OH)_2$-D_3 was kindly donated by Dr Uskokovic of Hoffmann-La Roche, Nutley, N.J., U.S.A.

Cytosol preparation

The procedure described by Eisman et al. (13) for the preparation of binding protein was followed, including some modifications .
Chicks were killed by decapitation and their duodenal loops were removed and rinsed twice with 10 ml ice-cold Tris buffer (50 mM Tris/HCl, 25 mM KCL, 5 mM $MgCl_2$ pH 7.4)in the presence or absence of dithiothreitol (5 mM).Throughout the preparation the temperature was maintained at 4° C .The mucosa was centrifuged at 800 x g for 10 min. and the supernatant discarded . The pellet was resuspended twice in 5 volumes of buffer, centrifuged and the supernatant discarded . The final pellet was resuspended and homogenized in 2 volumes of buffer with three passes of Potter-Elvejhem teflon-glass homogenizer . The homogenate was centrifuged at 21,000 x g for 30 min. The supernatant was then centrifuged at 105,000 x g for 60 min in a Beckman L3 50 ultracentrifuge . After removing the lipid layer the clear cytosol was collected, sampled in 1 ml fractions and either used immediatly or stored frozen at - 80°C until used . Cytosol preparations contained a final protein concentration of 10-13 mg/ml .

Binding reactions

Appropriate concentrations of [^{3}H] - 1,25- $(OH)_2$-D_3 in absolute ethanol were pipetted into glass conic tubes (1,5 ml), the solvant removed under nitrogen and the labelled compound redissolved in 10 µl ethanol and mixed with 100 µl Tris-buffer prior to cytosol addition . Non-saturable 1,25 -$(OH)_2$-D_3 binding was assessed by parallel incubation with 100-fold excess unlabelled 1,25-$(OH)_2$-D_3 . The binding reaction was initiated by the addition of 4 mg protein in a volume of 400 µl . Equilibrium was attained by incubating either at 25°C for 60 min or at 0°C for 3 hr .

Sucrose gradient centrifugation

Linear 4 to 20% sucrose in Tris-buffer containing 150 mM KCl gradients (4,5 ml) were prepared in polyallomer tubes with a gradient former (Buchler Instruments, U.S.A) . A 400 µl sample of [^{3}H]- 1,25-$(OH)_2$-D_3-cytosol mixture was layered on the top of the gradient. Centrifugation was carried out for 18 hr at 200,000 xg at 4° C with a SW 50 rotor in a Beckman model L3 50 ultracentrifuge. Gradients were fractionated from the top by displacement with a 40 % sucrose 20 % glycerol solution into the bottom. Fractions of 150 µl were collected into scintillation vials and 4 ml of scintillation fluid (Biofluor, NEN C°, U.S.A.) added for radioactivity measurement. Bovine serum albumin (Miles Laboratories) was used as a sedimentation marker .

Polyacrylamide gel electrophoresis

All experiments were performed in a Biorad model 150 A apparatus maintained at 4° C by circulation through the jacket of the chamber. Cylindrical

(5 x 130 mm) separation gels without stacking gels were used. Bromophenol blue was added as tracking dye to samples of 1,25-$(OH)_2$-D_3-cytosol receptor complex 100 µl, each containing about 1 mg protein, which were layered on polyacrylamide gels. Electrophoresis was conducted for 2 hr 30 min at 1 mA/gel. When electrophoresis was completed each gel was removed and sectioned transversally into slides, 1.2 mm thick, which were allowed to stand overnight with 1 ml toluene to extract the labelled steroid. The extract was mixed with 4 ml scintillation solution (Biofluor, NEN C°, U.S.A.) before radioactivity was counted. Standard proteins were run in parallel gels. The protein bands were localized by staining with Coomassie brilliant blue R 250 according to Vesterberg's method (14). PAGE was performed in a multiphasic buffer system (Tris/glycine/HCl) operative at pH 10.2 as described by Rodbard and Chrambach (15) except that stacking gels were omitted. The total concentration (T) of acrylamide monomer (Fluka A.G.) plus the cross-linking agent N, N-methylene-bisacrylamide (Sigma) was varied from 7 to 12 %. The degree of crosslinking (C) i.e. the bisacrylamide/monomer ratio was held constant at 2 % . Electrophoretic relative mobilities (Rfs) were determined according to Rodbard and Chrambach (15) .

Logarithms of Rfs for 1,25-$(OH)_2$-D_3 receptors and for standard proteins were plotted versus the gel concentrations T (Ferguson plots). The results were computed according to the least-square method and the retardation coefficient, K_R was calculated from the slope of each Ferguson plot. Calibration curves were constructed by plotting known molecular radii ($\bar{R}$) of standard proteins versus (K_R) 1/2 . The following proteins, purchased from Boehringer (Mannheim, West-Germany) were used as standards : trypsin inhibitor from soybean ($\bar{R}$ = 18.8 Å), ovalbumin ($\bar{R}$ = 23.3 Å), bovine serum albumin ($\bar{R}$ = 26.9 Å) and aldolase from rabbit muscle ($\bar{R}$ = 35.8 Å) . Values of $\bar{R}$ for the 1,25-$(OH)_2$-D_3 receptor were obtained by interpolation from experimentally determined K_R . The results for $\bar{R}$ were used to calculate molecular weights (M) by rearrangement of the equation $\bar{R} = \left(\frac{3\,M\bar{v}}{4\pi N}\right)$ 1/3 eq. 1 .

The value of partial specific volume ($\bar{v}$) was estimated to be 0.74 by analogy with other steroid receptors (16). N = Avogadro's number .

RESULTS

Chick intestinal cytosol prepared in the absence of dithiothreitol and labelled in vitro with (^{3}H)-1,25-$(OH)_2$-D_3 was run in PAGE : a major specific radioactive peak was found but as it comigrated in PAGE with the tissue vitamin D binding protein (DBP), which was present in cytosol, we had first to separate it from DBP by sucrose density gradient ultracentrifugation in high salt buffer (0.15 M KCl). In these conditions DBP sedimented at 5.8 S and the 1,25-$(OH)_2$-D_3 receptor at 3.6 S (figure 1 A). Then the 3.6 S 1,25-$(OH)_2$-D_3 binding peak was sampled, dialyzed and run in PAGE. A single radioactive peak migrating with the same mobility was obtained (Figure 1 B) and its molecular weight was determined : replicate gels (8 assays on average) were run with concentrations of T varying from 7 to 12 % and Ferguson plots were calculated for the 1,25-$(OH)_2$-D_3 receptor and for standard proteins respectively. As indicated in Methods a calibration curve was constructed by

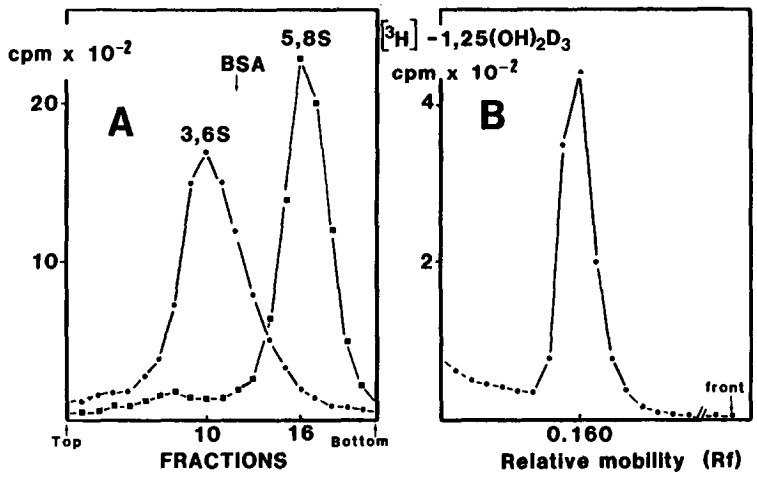

Figure 1. PAGE analysis of the 1,25-$(OH)_2$-D_3 receptor after separating it from tissue DBP in sucrose densitiy gradient ultracentrifugation. A) Cytosol protein (4 mg) labelled with 3 nM (^{3}H)-1,25-$(OH)_2$-D_3 (—•—) was analyzed by sucrose density gradient (4 to 20 %) in Tris buffer containing 0.15 M KCl . Cytosol labelled with 7 nM (^{3}H)-25-OH-D_3 (—▪—) was analyzed in the same run. Position of marker protein is indicated : (bovine serum albumin, 4.4. S). B) Aliquots of the 3.6 S radio-active peak (fraction 10) were sampled, dialyzed against Tris buffer and run in PAGE . Each sample was analyzed in 110 mm gels containing 7 to 12 % total acrylamide (T) ; data are shown for 10 % T gels.

plotting the known values of $\overline{R}$ versus (K_R) 1/2 for the standard proteins. The value of $\overline{R}$ for the 1,25-$(OH)_2$-D_3 receptor was obtained by interpolation from the calibration curve . The molecular weight was calculated using equation 1 and assuming an asymmetry of the receptor similar to that of the standard proteins. The value obtained for the apparent molecular weight was 95,200 $\pm$ 1,900 (SD) daltons .

Effect of dithiothreitol .

Addition of dithiothreitol (5 mM) to the preparation buffer resulted in striking alterations of the electrophoretic pattern. Cytosol prepared in the presence of dithiothreitol and in vitro occupied with (^{3}H)-1,25-$(OH)_2$-D_3 exhibited, besides the 95,000 daltons peak I, an additional fast moving 1,25-$(OH)_2$-D_3 binding peak, designated as peak II. A simultaneous decrease in the height of peak I was observed, suggesting a partial transformation of peak I into peak II. A typical electrophoretic pattern of cytosol prepared in the presence of dithiothreitol is presented in Figure 2. The two peaks could be displaced by a 100-fold excess of unlabelled 1,25-$(OH)_2$-D_3 . By

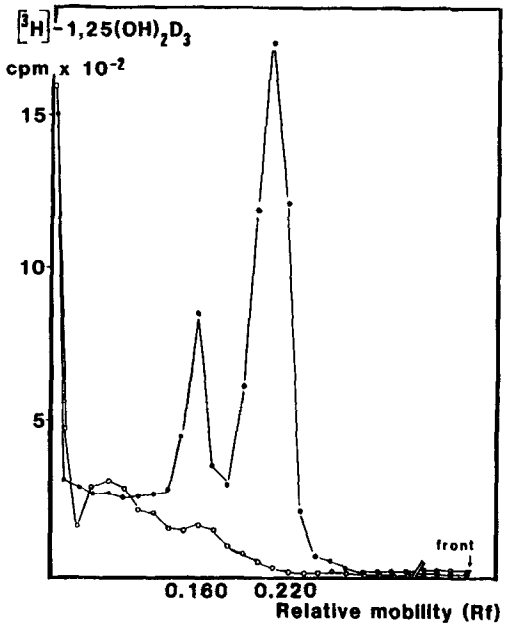

Figure 2. PAGE analysis of the binding of (^{3}H)-1,25-$(OH)_2$-D_3 by chick intestine mucosa cytosol prepared in the presence of dithiothreitol : 1 mg cytosol protein prepared in Tris buffer (50 mM Tris-HCl), 25 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, pH 7.4) was incubated in the same buffer for 3 hr at 4° C with 3 nM (^{3}H)-1,25-$(OH)_2$-D_3 alone (—•—) or with 300 nM of unlabelled 1,25-$(OH)_2$-D_3 (—•—). Electrophoresis was conducted for 2 hr 30 min at 1 mA/gel. Relative mobilities (Rfs) are : migration of protein/migration of tracking dye (Bromophenol blue). Data presented are for 10 % T gels .

running replicate assays at concentrations of T varying from 8 to 12 % the molecular radii and the molecular weights of these binding fractions could be estimated. The results are presented in Table 1 .

Table 1 . Biophysical characteristics of the two 1,25-$(OH)_2$-D_3 binding peaks obtained in presence of dithiothreitol

1,25 $(OH)_2$ D_3 RECEPTOR	MOLECULAR RADII (Å) (mean ± sd)	MOLECULAR WEIGHTS (mean ± sd)
PEAK I	29.7 ± 0.5	90,300 ± 4,800
PEAK II	27.6 ± 0.6	73 600 ± 3 300

Effect of protease inhibitors .

Protease inhibitors, leupeptin (1 mM) and TAME (10 mM) were added to the preparation buffer . In the absence of dithiothreitol, the addition of either leupeptin or TAME did not modify the electrophoretic pattern showing a major peak I . By contrast, when cytosol was prepared with dithiothreitol, the addition of TAME resulted in the disappearance of peak II . Only one peak migrating like peak I was evident .

DISCUSSION

PAGE analysis provided us with an apparent molecular weight for the chick intestinal 1,25-$(OH)_2$-D_3 receptor which was larger than most of the values previously published (5,6,7,8,9) . However, it agrees well with a recently reported large value (96,000) determined using a chick intestinal cytosol fraction prepared in the presence of phenylmethylsulfonyl fluoride, a protease inhibitor (10) . As, until now, the published values have been obtained via gel filtration it could be questioned whether the PAGE method can give comparable results . Nevertheless, the validity of PAGE-determined molecular weights is attested by the good correlation between the two methods, which was found for the tissue vitamin D binding protein : 102,000 by PAGE (personal results) vs 95,000 by gel filtration (17). Steroid hormone receptors are known to form very large molecular weight aggregates in low ionic strength buffers, like those used in PAGE, and highly crosslinked gels with 15 % C have therefore been proposed for the migration of such large molecules (18) . However, in the present study, the relatively low molecular weights ($\leq$ 100,000) of the 1,25-$(OH)_2$-D_3 receptor forms allowed the use of 2 % gels . Although some radioactivity stayed at the depot, it was not displaced by non-radioactive 1,25-$(OH)_2$-D_3 added in excess and could therefore be considered as a non-specific binding activity . By contrast, all the migrating radioactive peaks were specifically suppressed by a 100-fold excess of unlabelled 1,25-$(OH)_2$-D_3 .

The 1,25-$(OH)_2$-D_3 receptor is known to be stabilized by dithiothreitol like other steroid hormone receptors which require sulfhydryls for ligand binding

(19). The unexpected alteration in size and electrophoretic mobility of the 1,25-$(OH)_2$-D_3 receptor, prepared in the presence of dithiothreitol and observed by us is yet not inconsistent with a stabilizing effect of dithiothreitol since the low molecular weight form II possesses a binding activity . The evidence of a decrease in peak I, concommitant with a proportionate increase in peak II, strongly suggests a transformation of peak I into peak II and therefore makes the hypothesis of an inactivated form being unmasked by the addition of dithiothreitol very unlikely . The possibility that the smaller form II may result from partial proteolysis of the larger form I is supported by the suppression of the effect of dithiothreitol by the addition of TAME, a protease inhibitor . Whatever the mechanism by which dithiothreitol modifies the structure of the 1,25-$(OH)_2$-D_3 receptor, the present results point out the necessity to re-examine the systematic use of dithiothreitol in the preparation of the 1,25-$(OH)_2$-D_3receptor . On the other hand, the efficiency of PAGE as a tool for the study of the 1,25-$(OH)_2$-D_3 receptor under various conditions should be emphasized .

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