Pyridoxal Phosphate Induced Alterations in Glucocorticoid Receptor Metabolism by Proteases†

John A. Cidlowski

ABSTRACT: Previous studies from our laboratory [Cidlowski, J. A., & Thanassi, J. W. (1979) *Biochemistry 18*, 2378–2384] have shown that treatment of cytoplasmic dexamethasone receptor complexes with pyridoxal phosphate and $NaBH_4$ results in the production of a small, 2.9S receptor form which has enhanced stability. We have now examined the mechanisms by which pyridoxal phosphate alters the size of rat thymocyte glucocorticoid receptor by using Sephacryl S-200 molecular sizing columns equilibrated with 0.01 M tris(hydroxymethyl) aminomethane, 0.001 M ethylenediaminetetracetic acid, and 0.012 M α-thioglycerol buffer, pH 7.5. Fresh unactivated (non DNA binding form) or activated (DNA-binding form) cytoplasmic receptors obtained from whole thymocytes incubated with [^{3}H]dexamethasone chromatograph in two forms. The larger species, form A (Stokes radius, 56 Å; 250 000 M_r), accounts for 90% of the receptor-bound steroid in fresh cytosol and the smaller species, form D (Stokes radius, 18 Å; 17 000 M_r), accounts for the remainder of receptor. Extended incubation of cytosol at 0–4 °C or addition of $CaCl_2$ (100 mM) results in the time-dependent conversion of form A to form D without detection of intermediate-sized dexamethasone receptor. Treatment of steroid receptor complexes with 5 mM pyridoxal phosphate for 2 h at 3 °C, followed by reduction with $NaBH_4$, results in conversion of form A to a new smaller species, form B (Stokes radius, 42 Å; 110 000 M_r), which is not detected in control or $NaBH_4$-treated cytosols. Extended incubation of pyridoxal phosphate treated steroid receptor, with or without $NaBH_4$ reduction or with $CaCl_2$ addition, leads to conversion of form B to a new smaller species form C (Stokes radius 32 Å, 55 000 M_r). Cytosols treated with pyridoxal phosphate under any condition do not contain form D receptors (meroreceptors). The conversions of forms A to D and B to C but not A to B are retarded by the addition of 5 mM *N*-ethylmaleimide or iodoacetate. Only the active form of vitamin B_6, pyridoxal phosphate, and not pyridoxal or pyridoxine lead to conversion of form A receptors to forms B and subsequently to form C. Furthermore, it is shown that exogenously added trypsin leads to the conversion of form A receptors to species similar in size to form D. Pyridoxal phosphate produced form B receptors are insensitive to trypsin proteolysis. These studies show that pyridoxal phosphate treatment of rat thymocyte dexamethasone receptors alters the metabolism of these molecules by proteases and renders receptor insensitive to trypsin cleavage. This alteration has led to the detection of two new receptor forms in rat thymocytes. Both unactivated and activated dexamethasone receptors behave similarly with regard to in vitro metabolism.

Steroid hormone receptors have gained acceptance as important proteins in the regulation of phenotypic expression of genes in eukaryotic cells (Vedeckis et al., 1978; Munck & Leung, 1977; Yamamoto & Alberts, 1976). In recent years serious attempts have been made to characterize and purify these protein receptor molecules and analyze their physical properties. Studies on the molecular properties of glucocorticoid receptors have resulted in widespread general variation in published values for glucocorticoid receptor size (Middlebrook & Aronow, 1977; Koblinsky et al., 1972; Beato & Feigelson, 1972; Sherman et al., 1978; Govindan & Sekeris, 1978; Wrange et al., 1979; Kalimi et al., 1975; Carlstedt-Duke et al., 1979; Climent et al., 1976). The reported diversity of receptor properties may be due to species or target tissue differences although recent studies indicate methodological variations, particularly relating to the process of proteolysis (Sherman et al., 1976; Wilson & French, 1979), are a potential source of the heterogenous receptor properties reported (Wrange et al., 1979; Carlstedt-Duke et al., 1979).

In three model systems the process of proteolytic cleavage of receptor molecules have been analyzed in detail. Studying progesterone receptors from chick oviduct, Sherman et al. (1974, 1978) and Vedeckis et al. (1980) have characterized at least five receptor forms. Receptor form I is a large heterodisperse molecule which presumably contains the two A and B subunits (form III and form II, respectively) of the progesterone receptor described by Schrader & O'Malley (1972). Receptor form IV results from proteolytic cleavage of forms II and III (Vedeckis et al., 1980), and form V or meroreceptor is a further proteolytic degradation product of form IV. Analysis of androgen receptor proteins by Wilson & French (1979) from a number of target tissues indicates the presence of at least four different receptor forms, two large peptides similar in size to forms I, II, and III of the progesterone receptor system, and two smaller forms which result from a proteolytic process which is sensitive to inhibition by diisopropyl fluorophosphate. In the glucocorticoid receptor system, studies by Carlstedt-Duke et al. (1979) with rat liver cytosol demonstrate the presence of three receptor forms which have stokes radii of 61, 36, and 19 Å. The conversion of the 61-Å form to the 36-Å form and the 19-Å receptor forms was found to be augmented by trypsin and by preparations of rat liver lysosomes (Carstedt-Duke et al., 1979).

Recent studies from our laboratory (Cidlowski & Thanassi, 1979) indicate that treatment of rat thymocyte dexamethasone receptor complexes with pyridoxal phosphate, followed by reduction with $NaBH_4$, leads to the formation of a "small" 2.9S receptor form when the starting reactants are either 8S unactivated or 5S activated precursor steroid receptor complexes. Dexamethasone receptors can also be extracted from nuclei with millimolar concentrations of pyridoxal phosphate (Cidlowski & Thanassi, 1978), and treatment of rat liver dexamethasone receptors inhibits their binding to DNA[1]–

† From the Department of Biochemistry, University of Vermont, College of Medicine, Burlington, Vermont 05405. *Received April 3, 1980.* This work was supported by Grants AM 20892 and AM 25316 from the National Institutes of Health.

0006-2960/80/0419-6162$01.00/0

cellulose (Cake et al., 1978). The interaction of pyridoxal phosphate with steroid receptor complexes apparently is not restricted to glucocorticoid receptors. Nishigori & Toft (1979) have shown that pyridoxal phosphate most likely binds to and alters the molecular properties (charge) of the avian progesterone receptor, such that decreased binding of progesterone receptor complexes to ATP–Sepharose results. Finally, recent studies in the estrogen–receptor rat uterine system indicate that pyridoxal phosphate alters the sedimentation coefficient of this steroid receptor (Muldoon & Cidlowski, 1980).

In the present paper we have investigated the biochemical requirements necessary for the production of small dexamethasone receptor forms in rat thymocyte cytosol preparations. With the aid of pyridoxal phosphate we have been able to identify four glucocorticoid receptor forms under low-salt conditions. We show data which demonstrate that pyridoxal phosphate interacts with rat thymocyte glucocorticoid receptors. This interaction results in an altered glucocorticoid receptor which is metabolized differently by endogenous proteases and is not susceptable to trypsin proteolysis.

Methods

General. Male Sprague–Dawley rats were adrenalectomized 6–8 days prior to sacrifice and maintained on normal rat chow (Purina) and 0.85% NaCl drinking water. One or two animals were sacrificed by decapitation, thymus tissue was removed, and suspensions of thymus cells (thymocytes) were prepared in Krebs–Ringer bicarbonate buffer with 10 mM glucose (KRBG) (Munck, 1968). These cell suspensions consist of 98% small lymphocytes which remain 95% viable for at least 5 h in culture. The formation of [^{3}H]dexamethasone receptor complexes was accomplished by incubating 1–2 mL of whole cell suspension (1×10^9 cells/mL) with 2×10^{-8} M [6,7-$^3H_2$]dexamethasone (33.0 Ci/mmol) obtained from New England Nuclear for 1.5–2.5 h at 0 °C. Since the ^{3}H-labeled steroid is stored in benzene–ethanol (9:1 v/v), prechilled cells were added to chilled 15-mL glass Erlenmeyer flasks in which ^{3}H-labeled steroid solutions were previously evaporated to dryness.

Pyridoxal phosphate was obtained from Aldrich Chemical Co., and the other vitamin B_6 analogues used in this study, i.e., pyridoxal, pyridoxine, and pyridoxamine phosphate, were obtained from Sigma Chemical Co. Stock solutions of these compounds were prepared in 1.5 mM $MgCl_2$, and the pH of each was adjusted to 7.0 with NaOH. The solutions were stored protected from light and frozen until use. All manipulations involving vitamin B_6 forms were performed in subdued light. *N*-Ethylmaleimide (NEM) was purchased from Kodak, and stock solutions (100 mM) were prepared fresh daily in 1.5 mM $MgCl_2$. Other standard chemicals were reagent grade; these were purchased from Sigma, Fisher Scientific, or Scientific products. Radioactivity was measured in a Beckman LS-100C scintillation spectrometer having an efficiency of 45% for tritium, and all samples were counted for sufficient times to obtain a maximum of 3% error. Routinely 300-μL samples of column effluent were counted in 3.0 mL of Phase Combining System (PCS; Amersham Searle)–xylenes (2:1 v/v). Little or no variation in counting efficiency occurred among the samples. Scintillation counting background in our laboratory is consistly low (12–15 cpm), and these values were not subtracted from experimental data.

Preparation of Cytoplasmic Dexamethasone Receptors. Unless noted otherwise, all steps were conducted at 0–4 °C. Unactivated [^{3}H]dexamethasone receptor complexes (those which bind minimally to nuclei or DNA–cellulose at 0 °C) were prepared from whole cells incubated with 2×10^{-8} M [^{3}H]dexamethasone as described above. Following this incubation the cell suspensions were centrifuged at 1000*g* for 5 min and the supernatant containing the free steroid was decanted. To each cell pellet (0.1–0.2-mL packed cell volume) 3–4 mL of ice-cold 1.5 mM $MgCl_2$ was added, vortexed, and allowed to sit on ice for 30 min. DNA and broken nuclei were then removed by centrifugation at 1500 *g* for 5 min, and the clear supernatant was recovered for subsequent treatment. Under these conditions >95% of the macromolecular bound steroid appears to be associated with saturable receptor proteins. Dexamethasone receptors complexes prepared by these techniques are "unactivated" and will not bind to DNA–cellulose or isolated nuclei at 0 °C (Cidlowski & Munck, 1978). Activated receptors, 60–70% of which will bind to nuclei or DNA cellulose under low ionic strength conditions, were prepared by incubating the preparation described above for 15 min at 25 °C and then cooling the solution to 0–4 °C. This treatment results in an ~33% loss in receptor activity, probably due to steroid dissociation (Cidlowski & Munck, 1978). Under the conditions of our assays, inclusion of a 100–1000-fold excess of unlabeled dexamethasone during the initial incubation of the whole cells with tritiated steroid results in a loss of 90% of the macromolecular bound steroid indicating that we are measuring saturable receptor binding.

Treatment of Dexamethasone Receptors prior to Gel Chromatography. In general, 1–2 mL of "cytosol" solution was placed in aluminum foil covered polyethylene tubes, and 20–100-μL aliquots of concentrated stock solutions of B_6 vitimers, protease inhibitors, or $CaCl_2$ were subsequently added. The final concentrations achieved are shown in the figure legends. All incubations took place with the tubes on ice. Reactions involving pyridoxal phosphate were stopped at the times indicated by the addition of small amounts of solid $NaBH_4$ to reduce pyridoxal phosphate to pyridoxine phosphate and protein-bound pyridoxal phosphate, presumably phosphopyridoxyllysine. The appropriate controls were treated with $NaBH_4$ alone. This agent appears to be without effect in nonpyridoxal phosphate treated cytosols. Prior to gel filtration chromatography, 1.0-mL samples of the various treated cytosols were mixed for 10 min with the dextran-coated charcoal pellet from 1.0-mL suspension of a 1% Norit A–0.1% dextran solution prepared in 1.5 mM $MgCl_2$. The dextran-coated charcoal containing free steroid was removed by two 1-min centrifugations at 10000*g* in a Beckman microfuge B.

Gel Filtration Studies. A Sephacryl S-200 column (45 × 1.5 cm) was prepared and equilibrated with 0.01 M Tris, 0.001 M Na_2ETA, and 0.012 M α-thioglycerol buffer at pH 7.5. The column had a void volume (V_0) of 28.0 mL and a total volume (V_t) of 80.0 mL. Molecular size calibration was performed by using the purified proteins catalase (M_r, 243 000; Stokes radius, 51 Å), aldolase (M_r, 156 000; Stokes radius, 45 Å), albumin (M_r, 67 000; Stokes radius, 36 Å), ovalbumin (M_r, 45 000; Stokes radius, 29 Å), and myoglobin (M_r, 18 000; Stokes radius, 20 Å) purchased from Boehringer-Mannheim or Sigma. The Stokes radii were determined graphically from a plot of *K* average [$K_{av} = (V_e - V_0)/(V_t - V_0)$] vs. Stokes radius in angstroms. Molecular weights were derived from a combination of Stokes radius data and sedimentation data by using the standard equation $M_r = 6\,nNas/(1 - Vp)$ where *n* is the viscosity of medium (0.019 s^{-1} cm^{-1}), *N* is Avagadro's

[1] Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; ATP, adenosine 5′-triphosphate.

number, a is the Stokes radius in 10^8 cm, s is svedberg unit in 10^{13} S, V is the partial specific volume of 734 cm^3/g, and p is the density of the medium (1.03 g/cm^3). Sedimentation coefficients were determined from a linear standard curve of sedimentation coefficient vs. migration by using the following standards: equine myoglobin (2.0 S), bovine hemoglobin (4.2 S), bovine serum albumin (4.6 S), yeast alcohol dehydrogenase (6.7 S), and rabbit muscle aldolase (7.9 S). Sedimentation coefficients were determined as described by Martin & Ames (1961). Sucrose density gradient procedures have been described previously (Cidlowski & Thanassi, 1979). The column's flow rate in each experiment was ~300 μL/min. Typically 750–1000 μL of treated cytosol sample was mixed with 300 μL of myoglobin solution and layered on top the column. Five-drop (300-μL) fractions were collected into liquid scintillation vials after exclusion of 27.0 mL. The same column was used in all the experiments described in this paper. The molecular size calibrating purified proteins did not change over the course of these experiments. The myoglobin standards was included in each run to enable superimposition of the data from several different experiments.

Results

Figure 1 provides the Sephacryl S-200 chromatographic patterns of cytoplasmic glucocorticoid receptors obtained following incubation of whole thymocytes with [^{3}H]dexamethasone. Glucocorticoid receptors obtained after lysing cells in 1.5 mM $MgCl_2$ are in the unactivated form, such that binding to DNA–cellulose or nuclei is minimal (Cidlowski & Munck, 1978). Figure 1 shows that over 90% of such a fresh receptor preparation elutes just after this column's void volume, corresponding to a Stokes radius of 56 Å (form A). In addition to this large receptor form, approximately 10–12% of this freshly prepared cytosol chromatographs as a small ~18-Å (form D) steroid-binding macromolecule which is similar in size to meroreceptor molecules identified in other systems (Sherman et al., 1974, 1978). The open circles provide the chromatographic pattern of a receptor preparation which has been activated or transformed into a DNA or nuclei binding protein by warming of the preparation for 15 min at 25 °C. These receptor molecules also chromatograph as only two receptor forms in the system used corresponding to the ~56- and ~18-Å species observed in the unactivated receptor preparation. The absolute amount of steroid receptor present in the small ~18-Å species is similar to that in the unactivated receptor preparation, although its relative proportion or percent of the total is increased because of steroid dissociation which occurred during the activation process (Cidlowski & Munck, 1978). In general, the ratio of large receptor form A to small receptor form D is ~9:1 even when cells are lysed in the presence of protease inhibitors such as *N*-ethylmaleimide or iodoacetate. Interestingly, sucrose density gradient analyses of unactivated and activated dexamethasone receptors under low ionic strength conditions reveal quite different patterns. Unactivated receptor sediments as two distinct ~8S and ~2.6S species whereas activated receptor sediments as an aggregate, ~5S component and ~2.6S moieties. In both cases the smaller molecular form predominates (Cidlowski & Thanassi, 1979). In a previous publication (Cidlowski & Thanassi, 1979) we were tentatively identifying the small species of receptor present in untreated cytosols as a 2.9S species. After comparison of over 16 different experiments, it became apparent that the endogenous small form of receptor was actually slower in sedimenting than the 2.9S species identified following pyridoxal phosphate treatment and $NaBH_4$ reduction. On the basis of the linear standard curve described under Methods, we have corrected our error and reassigned a sedimentation value of 2.6S to the endogenous small glucocorticoid receptor in rat cytosol. Nevertheless, this correction does not account for the discrepancy between the ratios of large or small receptor forms following analysis on Sephacryl S-200 and sucrose density gradients. One striking difference in the two techniques is the duration of the quantiative period, i.e.,

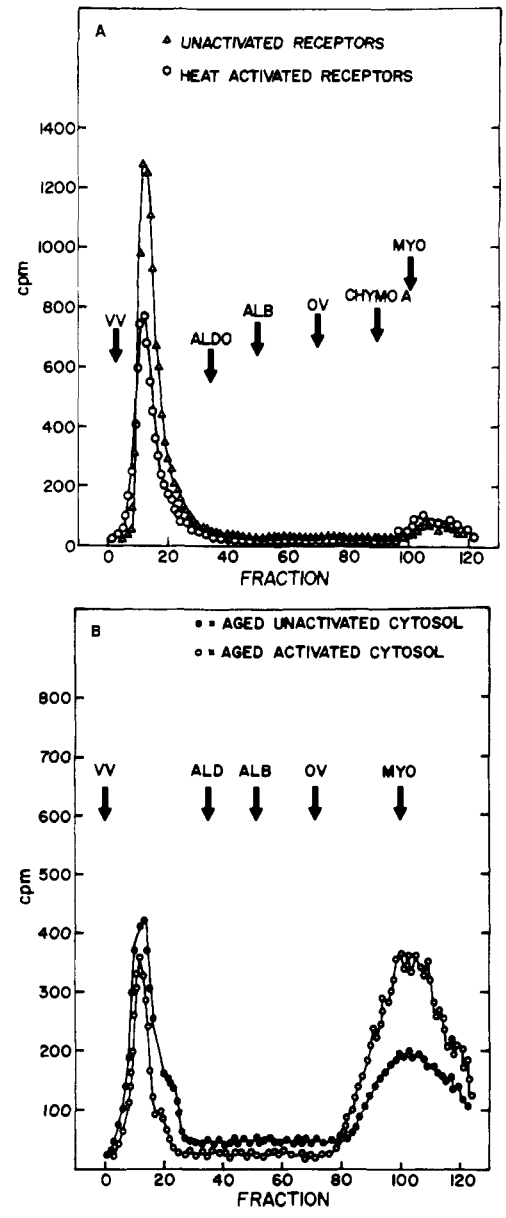

FIGURE 1: (A) Comparison of the Sephacryl S-200 chromatographic analysis of unactivated and activated [^{3}H]dexamethasone receptor complexes from isolated rat thymocytes, "fresh forms". One-milliliter aliquots of unactivated (△) and heat activated (○) dexamethasone receptors were each cochromatographed with 300 μL of myoglobin solution on a 45 × 1.5 cm Sephacryl S-200 column equilibrated with Tris–EDTA–thioglycerol buffer, pH 7.4. The positions of the protein sizing standards were determined in different column runs, each of which contained a myoglobin standard for normalization. Free dexamethasone was removed by dextran-coated charcoal treatment of cytosols prior to chromatography. Free dexamethasone elutes after fraction 130. Five-drop fractions (300 μL) were collected into scintillation vials, and the samples were counted for radioactivity as described. Inclusion of 2×10^{-6} M unlabeled dexamethasone during the incubation of cells with the 2×10^{-8} M [^{3}H]dexamethasone used in these experiments eliminates the presence of the radioactivity peaks seen between fraction 0 and 40 and 100 and 120. (B) One-milliliter aliquots of unactiviated (●) and heat activated (○) dexamethasone receptors were allowed to age for 16 h at 4 °C prior to chromatography on Sephacryl S-200 size as described.

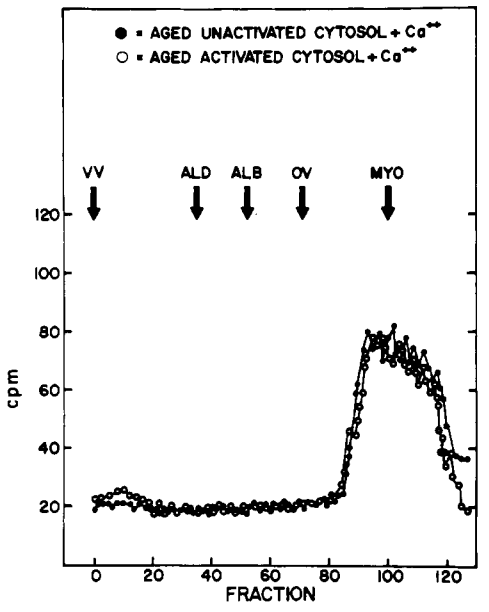

FIGURE 2: Sephacryl S-200 chromatography of "aged", $CaCl_2$-treated cytoplasmic [^{3}H]dexamethasone receptors. Unactivated and activated cytosol fractions from thymocytes incubated with 2×10^{-8} [^{3}H]-dexamethasone were prepared as described under Methods and in Figure 1. These preparations were allowed to sit for 16 h at 0–3 °C with 100 mM $CaCl_2$ prior to treatment with dextran-coated charcoal. One milliliter of each sample was then cochromatographed with myoglobin standard, and 5-drop (300-μL) fractions were collected into scintillation vials and counted for radioactivity. The patterns observed are representative of those seen in three separate experiments.

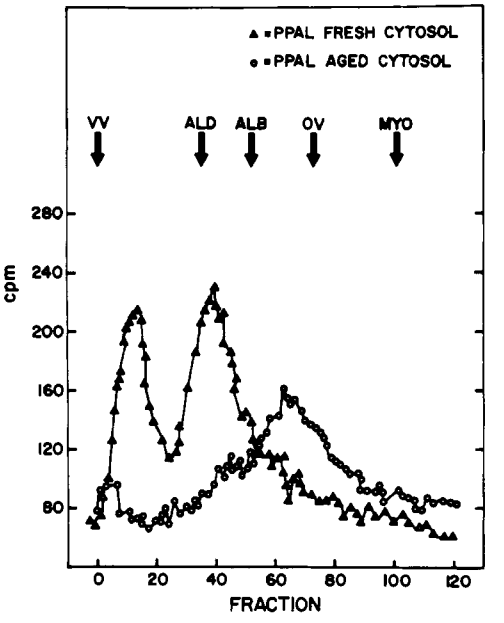

FIGURE 3: Analysis of the influence of pyridoxal phosphate (PPAL) on the Sephacryl S-200 chromatography pattern of "fresh" and "aged" cytoplasmic dexamethasone receptors. Cytosol fractions from thymocytes incubated with 2×10^{-8} M [^{3}H]dexamethasone were prepared as described under Methods. Samples of fresh cytosols were then treated with 5 mM pyridoxal phosphate for 2 or 16 h prior to being reduced with $NaBH_4$. Both samples were treated with dextran-coated charcoal just prior to chromatography on Sephacryl. One milliliter of each cytosol sample was chromatographed with 300 μL of myoglobin standard, and 5-drop (300-μL) samples were collected for determination of radioactivity.

2–3 h for Sephacryl analysis and 16 h for the sucrose gradient studies. Obviously, receptor degradation may occur during this extended incubation.

Thus we next analyzed the properties of the dexamethasone receptor on Sephacryl under conditions comparable to those used in our earlier sucrose density gradient studies. Figure 1B shows the Sephacryl S-200 chromatographic pattern of the 16-h "aged" unactivated and activated dexamethasone receptor complexes. In both types of preparations only the two binding forms A and D described in fresh cytosol preparation (Figure 1A) are present. The relative proportions of these forms differ substantially from fresh cytosol however. Aging of cytosol results in an increased relative proportion of the 18-Å species, which elutes as a relatively broad peak of radioactivity, suggesting heterogeneity. When pooled fractions in the 2.6S region of 5–20% sucrose density gradients (Cidlowski & Thanassi, 1979) are chromatographed on Sephacryl, only the 18-Å species is observed. Similarly, the ~8S fraction on sucrose density gradients elutes on Sephacryl as form A (56 Å). Thus it appears that during centrifugation on sucrose gradients, aging of receptor occurs such that the 2.6S, 18-Å receptor fragment accumulates. On the basis of molecular chromatography studies and sucrose density gradient centrifugation studies, the A and D species have approximate molecular weights of 250 000 and 17 000, respectively.

Studies in other systems (Sherman et al., 1974, 1978; Vedeckis et al., 1980) indicate that the production of small forms of receptor similar to those reported in Figure 1 occurs in response to degradation of receptor by a Ca^{2+}-dependent proteolytic mechanism. Figure 2 shows that the addition of 100 mM Ca^{2+} to preparations of either unactivated or activated receptors leads to production exclusively of 18-Å rat thymocyte dexamethasone receptors. Both unactivated and activated receptors are metabolized to similar products by either aging of cytosol or Ca^{2+} addition. After 16 h of aging which occurs in the absence of Ca^{2+}, 80% of the macromolecular steroid is recovered, whereas only 40% is recovered following Ca^{2+} treatment. This suggests that, although the same sized products are generated, different mechanisms may be involved. Furthermore, these data suggest that the exposure of the DNA or nuclear binding domain during activation does not alter the ability of the receptor to be metabolized by the putative protease, nor is the size of the ultimate products which are produced altered. These studies, however, have no information about the relative affinity of the proteolytic enzymes for activated and unactivated receptor forms.

We next wished to determine if the small 2.9S form of glucocorticoid receptor produced by pyridoxal phosphate treatment and $NaBH_4$ reduction was similar to or identical with the 18-Å species (form D) described in Figures 1B and 2. Figure 3 is a Sephacryl S-200 chromatograph of unactivated dexamethasone receptor cytosol exposed to 5 mM pyridoxal phosphate for 2 h at 0–4 °C and then reduced with $NaBH_4$. Sodium borohydride treatment by itself has no measurable effect on the receptor Sephacryl chromatography pattern or the sucrose density gradient sedimentation coefficient. Pyridoxal phosphate treatment results in the partial conversion of the form A (56-Å) receptor to a new form B (42 Å) and a small shoulder in the 32-Å region of the column. Extended incubation of receptor with pyridoxal phosphate (3 h) results in complete conversion of the 56-Å form to the 42-Å species. The rate of conversion is variable from experiment to experiment and probably reflects the concentration of the cytosol used since available lysines serve as traps for pyridoxal phosphate. Treatment of receptor for three hours at 4 °C with pyridoxal phosphate causes no measurable loss of steroid binding. No receptor form analogous to either the 18-Å species seen in aged cytosol or Ca^{2+}-treated cytosol is present following pyridoxal phosphate exposure. Extended incubation of cytosol in the presence of 5 mM pyridoxal phosphate for 16 h resulted

in an apparent decrease in steroid binding (55% reduction), although similar apparent dissociation of steroid from receptor does not occur following sodium borohydride reduction as measured by dextran-coated charcoal resistance. This suggests that extended pyridoxal phosphate treatment without $NaBH_4$ reduction is deliterious to receptor–steroid interaction. Following 16 h of pyridoxal phosphate treatment virtually all of the macromolecular bound steroid remaining resides in the 32-Å region with only a shoulder remaining in the 42-Å region. Once again, under conditions which produced large quantities of form D receptor in control cytosol (Figure 1B), no form D receptor was observed in pyridoxal phosphate treated cytosols. The presence of primarily form C receptor (32 Å) following 16 h of incubation with this vitamin, whereas form B (42 Å) predominates at 3 h, suggested a time-dependent conversion possibly involving proteolysis. Interestingly, when activated preparations of receptor are used as substrate in a similar series of experiments, virtually identical results are observed (data not shown). Furthermore, we obtain very similar elution profiles from cytosol preparations not exposed to dextran-coated charcoal, suggesting that the forms described are not being generated or lost by differential charcoal stripping of steroid. Thus, treatment of dexamethasone receptor with pyridoxal phosphate appears to alter the metabolism of receptor which occurs during aging of cytosol samples.

The next studies were developed to analyze the influence of pyridoxal phosphate on the metabolism of dexamethasone receptor by the putative Ca^{2+}-activated proteases present in thymocyte cytosol. Figure 4A shows the effect of Ca^{2+} (100 mM) alone and Ca^{2+} plus 5 mM pyridoxal phosphate on rat thymocyte dexamethasone receptor size. Calcium alone at 100 mM for 3 h at 0 °C causes partial conversion of the form A receptor (56 Å) to the form D (18-Å) species. No other intermediate-sized receptors are observed under these conditions of partial conversion, as is seen in the aging experiments in absence of added Ca^{2+} (Figure 1B). This conversion of form A receptor to form D receptor is a time-dependent process. When 5 mM pyridoxal phosphate is included in the incubation mix, a strikingly different pattern of receptor-bound steroid is found on Sephacryl S-200. Under these conditions, form A and form D receptor species are virtually absent, and a large disperse peak of radioactivity occurs between the form B (42-Å) and form C (32-Å) regions of the column suggesting a mixture of the two forms described in the previous figure. Thus Ca^{2+} addition at 100 mM does not override the ability of pyridoxal phosphate to change the metabolism of the rat thymocyte dexamethasone receptor. In addition, the presence of pyridoxal phosphate inhibits the formation of form D (18-Å) receptor even under conditions which accelerate proteolysis in control cytosols (Figure 2). In other studies we have shown that the addition of pyridoxal phosphate after complete Ca^{2+}-induced conversion of form A to form D receptor is ineffective in reversing the process, suggesting that pyridoxal phosphate is not simply causing aggregation of two form D (18-Å) species. These data suggest that pyridoxal phosphate induces the production of form B (42-Å) receptor which was subsequently altered by a Ca^{2+}-activated protease to produce a form C (32-Å) fragment. To test this hypothesis, we treated cytosol with 5 mM pyridoxal phosphate, reduced it with $NaBH_4$, and then treated it with Ca^{2+} (100 mM) for 2 h prior to chromotography. As Figure 4B shows, the addition of Ca^{2+} after the formation of form B (42-Å) receptor by pyridoxal phosphate leads to the conversion of form B receptor to a variety of forms all of which are smaller in size. We are unable to detect significant form D (18-Å) fragments in cytosols

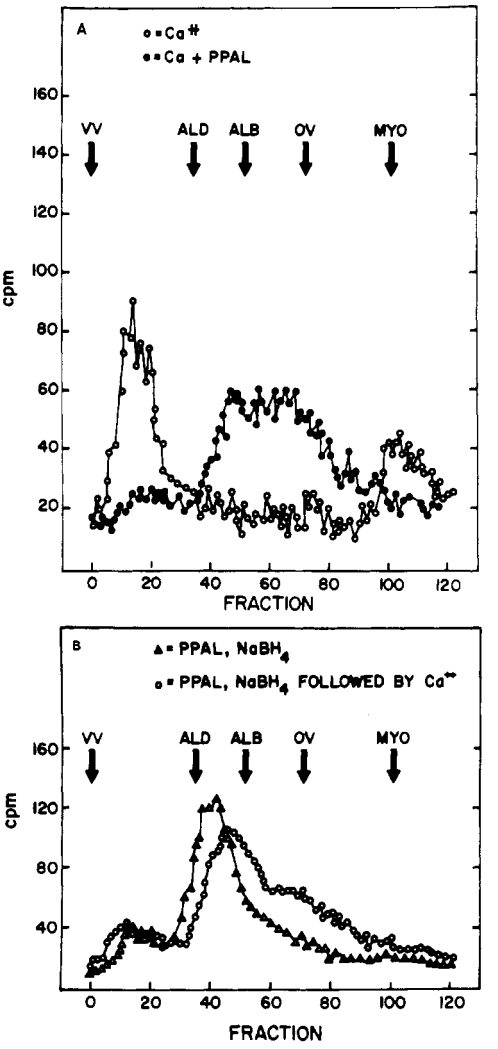

FIGURE 4: Conversion of pyridoxal phosphate (PPAL) induced glucocorticoid receptor to a smaller form by Ca^{2+}. Aliquots of cytosol were obtained from thymocytes incubated with 2×10^{-8} M [^{3}H]-dexamethasone as described under Methods. (A) One-milliliter samples of cytosol were incubated with 100 mM $CaCl_2$ alone or 100 mM $CaCl_2$ plus 5 mM pyridoxal phosphate for 3 h at 0–3 °C. The pyridoxal phosphate samples were reduced with $NaBH_4$, and all the samples were dextran-coated charcoal treated prior to cochromatography of 750 μL of sample with 300 μL of myoglobin standard. Five-drop (300-μL) samples were analysized for radioactivity. The chromatographs are representative of two experiments. (B) One 1-mL sample of cytosol was incubated with 5 mM pyridoxal phosphate for 2 h at 0 °C reduced with $NaBH_4$ and then allowed to age for 2 h. The second sample was exposed to 5 mM pyridoxal phosphate for 2 h at 0–3 °C, reduced with $NaBH_4$, and then treated with 100 mM Ca^{2+} for an additional 2 h. Both were treated with dextran-coated charcoal prior to the chromatography with the myoglobin standard.

treated with pyridoxal phosphate. Similar data were observed when activated receptors served as substrate (data not shown).

In order to more firmly establish that we are dealing with a combination of proteolytic and nonproteolytic mechanisms, with regard to pyridoxal phosphate and glucocorticoid receptor metabolism we have utilized the sulfhydryl protease inhibitor *N*-ethylmaleimide (NEM). Figure 5 shows the Sephacryl S-200 chromatograph patterns for cytosols aged for 16 h in the presence or absence of NEM. The presence of this protease inhibitor blocks the accumulation of form D (18-Å) receptor. *N*-Ethylmaleimide is similarly effective in inhibiting the accumulation of form D (18 Å) even in the presence of exogenous added $CaCl_2$ (100 mM). Interestingly, NEM appears to increase the total radioactivity recovered after 16 h of aging as compared to the nontreated controls. Ca^{2+} addition, however,

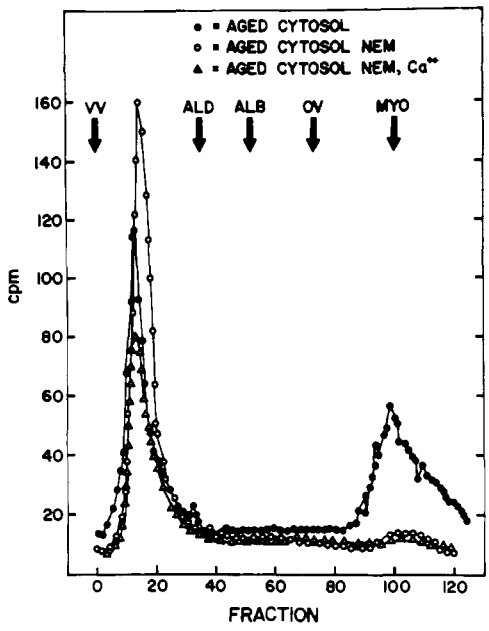

FIGURE 5: Influence of *N*-ethylmaleimide on endogenous and Ca^{2+}-induced proteolytic degradation of thymocyte [^{3}H]dexamethasone receptors. Aliquots of cytosol from thymocytes incubated with [^{3}H]dexamethasone were allowed to sit at 0–3 °C for 16 h prior to chromatography in the presence or absence of 5 mM *N*-ethylmaleimide or with Ca^{2+} (100 mM) plus 5 mM *N*-ethylmaleimide. Samples were then treated with dextran-coated charcoal and chromatographed as described. Patterns are representative of three experiments performed.

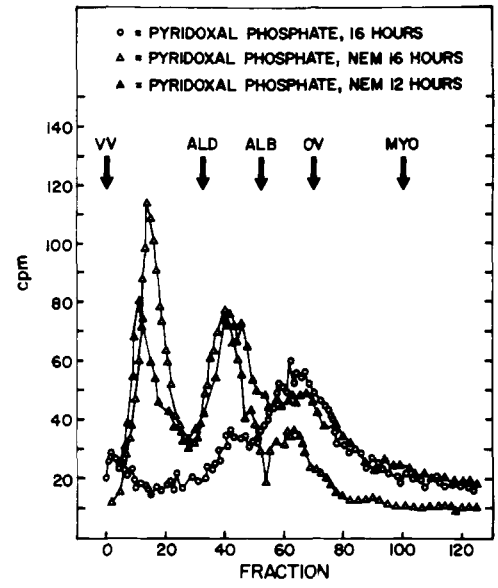

FIGURE 6: Influence of *N*-ethylmaleimide on the metabolism of pyridoxal phosphate treated dexamethasone receptor. Cytosols containing [^{3}H]dexamethasone receptor were incubated for 16 h at 0–3 °C. The samples were treated with 5 mM pyridoxal phosphate, 5 mM pyridoxal phosphate plus 5 mM *N*-ethylmaleimide, or 5 mM pyridoxal phosphate for 16 h plus *N*-ethylmaleimide for the last 13 h only. All samples were reduced with $NaBH_4$ at 16 h and then treated with dextran-coated charcoal prior to chromatography with myoglobin standard.

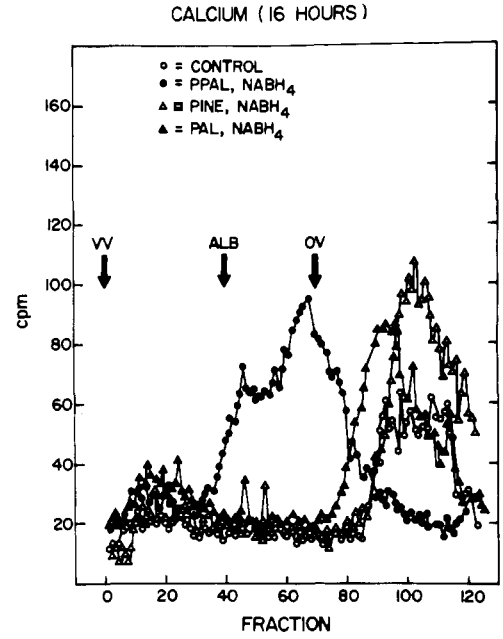

FIGURE 7: Specificity of the interaction of vitamin B_6 forms with rat thymocyte dexamethasone receptors. Cytosols from thymocytes incubated with [^{3}H]dexamethasone were exposed to 100 mM $CaCl_2$ alone or $CaCl_2$ plus 10 mM pyridoxal phosphate (PPAL), pyridoxal (PAL), or pyridoxine (PINE) for 16 h at 0–3 °C. All cytosols were then reduced with $NaBH_4$, treated with dextran-coated charcoal, and cochromatographed with the myoglobin standard. Radioactivity was quantitated as described. Chromatograph patterns are representative of two to three experiments as shown.

appears to stimulate dissociation of steroid from receptor even in the presence of NEM.

Since the data in Figure 4 indicate that the conversion of form B (42-Å) pyridoxal phosphate induced, $NaBH_4$-stabilized dexamethasone receptor to a form C (32-Å) fragment occurred via either an aging-dependent or Ca^{2+}-activated process, we investigated the possible role of NEM in altering this transition. Figure 6 shows the results of an experiment during which cytosols were incubated with pyridoxal phosphate alone or with *N*-ethylmaleimide for various durations. Pyridoxal phosphate treatment alone for 16 h results in the production of predominently form C receptor activity, although some form B remains. All cytosols treated with NEM usually contain some amount of the large molecular weight form A or larger receptor. Simultaneous exposure of cytosol to pyridoxal phosphate and *N*-ethylmaleimide, however, results in a strikingly different pattern of receptor chromatography with distinct peaks of radioactive bound dexamethasone in 56- and 42-Å regions and a small amount of 32-Å material. These data suggest that pyridoxal phosphate induced form B (42-Å) receptor was not subsequently processed to form C material in the presence of NEM. The presence of form A 56-Å material in cytosol samples treated with pyridoxal phosphate and NEM was suprising, particularly in view of the fact that kinetic studies showed that conversion of 7S to 3.5S glucocorticoid receptors from HeLa S_3 cells was complete within 5 h (O'Brien & Cidlowski, 1980). Cytosol treated with NEM or with pyridoxal phosphate consistantly has an increased receptor binding in the 56-Å region of the column. These receptors may represent a different species than that in controls since they sediment to the bottom of 5–20% sucrose gradients, whereas control 56-Å receptor sediments as an ~8S moiety. Furthermore, delayed addition of NEM for 3 h after pyridoxal phosphate results in an increased percentage of receptor in the form C (32-Å) form as compared to cytosol treated with pyridoxal phosphate and *N*-ethylmaleimide simultaneously, suggesting again a time-dependent conversion. No form D (18 Å) is present in any pyridoxal phosphate treated cytosol.

The experiments shown in Figure 7 were designed to assess the specificity of pyridoxal phosphate in inducing formation of the form C (32-Å) receptor fragment and preventing form D (18-Å) receptor formation. Cytosols were incubated with

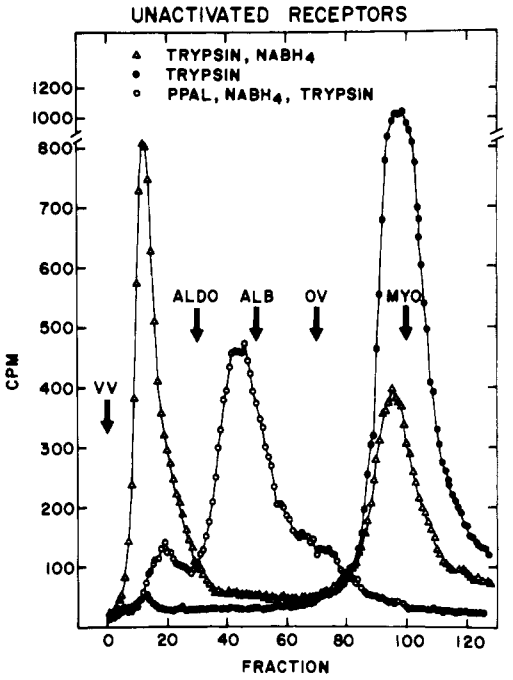

FIGURE 8: The influence of trypsin on the metabolism of rat thymocyte glucocorticoid receptor. Cytosols from thymocytes incubated with ^{3}[H]dexamethasone were treated with Worthington trypsin 10 μg/mL for 30 min at 4 °C. The trypsin reaction was stopped by the addition of 1 mg of soybean trypsin inhibitor (Sigma), and samples were treated with dextran-coated charcoal as described under Methods prior to chromatography on Sephacryl. The closed circles were from untreated (unactivated) cytosol, and the open triangles were from samples to which a small amount of solid sodium borohydryde was added prior to trypsin. The open circles were derived from samples of cytosol treated with 5 mM pyridoxal phosphate for 3 h at 0–4 °C, reduced by the addition of solid sodium borohydryde and then trypsin treated. The patterns are representative of three similar experiments.

5 mM of pyridoxal phosphate, pyridoxine, or pyridoxal for 16 h at 4 °C in the presence of 100 mM Ca^{2+}. Only pyridoxal phosphate, the active form of the vitamin, is capable of preventing form D (18-Å) receptor formation and form C (32-Å) accumulation under the conditions of this experiment. These data suggest that pyridoxal phosphate is a specific agent which probably binds to lysine residues on dexamethasone receptors and alters the subsequent molecular metabolism of receptor by endogenous and Ca^{2+}-stimulated proteases.

One point which deserves further consideration relates to the question of whether the altered steroid receptor metabolism reported is due to an effect of pyridoxal phosphate on the receptor itself or the protease responsible for its degradation. The experiment shown in Figure 8 addresses this question through the use of exogenous trypsin. Treatment of thymocyte cytosol containing [^{3}H]dexamethasone receptors with trypsin (10 μg/mL) for 30 min leads to almost complete conversion of the form A (56-Å) receptor for the form D (18-Å) receptor with no loss of steroid binding. Reduction of cytosol with $NaBH_4$ prior to trypsin treatment slows the rate of trypsin action under these conditions but does not alter the size of the product. Trypsin most likely is not the endogenous cellular or Ca^{2+}-stimulated enzyme responsible for meroreceptor formation in the previous studies, since *N*-ethylmaleimide effectively inhibits the endogenous enzyme but fails to block trypsin-induced degradation (data not shown). We next treated receptor with pyridoxal phosphate for 3 h at 0 °C to induce the formation of the form B (42-Å) form. Following $NaBH_4$ reduction, the cytosol was exposed to trypsin. Strikingly little or no degradation of receptors occurs. These data suggest pyridoxal phosphate interacts with receptor at a trypsin-sensitive site (lysine) and infers that in cytosol a similar situation may exist with regard to the metabolism of receptor by endogenous proteases.

Discussion

Within the past year the potential usefulness of pyridoxal phosphate as either a probe for steroid receptor structure or as a modulator of steroid hormone action has become apparent. The original studies of Cake et al. (1978) with rat liver glucocorticoid receptor showed that pyridoxal phosphate presumably bound to receptor and altered the DNA binding capability of activated dexamethasone receptor preparations. Similarily, Nishigori & Toft (1979) suggested the presence of a pyridoxal phosphate binding site on the chicken oviduct progesterone receptor. The interaction of pyridoxal phosphate with the avian progesterone receptor interestingly once again altered the capability of the receptor to bind to ATP–Sepharose. This group suggests that the altered ATP–Sepharose binding capacity is the result of an alteration in receptor charge. Studies from our laboratory (Cidlowski & Thanassi, 1978, 1979; Muldoon & Cidlowski, 1980) indicated that pyridoxal phosphate interacts with and alters the sedimentation coefficients of rat thymocyte glucocorticoid receptors and rat uterine estrogen receptor complexes. In both cases under a variety of conditions, a substantial reduction of the steroid receptor complex sedimentation coefficient occurred. A number of investigators (Vedeckis et al., 1980; Sherman et al., 1974, 1978; Wilson & French, 1979) have demonstrated that proteolytic mechanisms are involved in the production of small steroid receptor forms, similar in size to that originally observed in our sucrose density gradient centrifugation experiments (Cidlowski & Thanassi 1979). We have therefore studied the mechanism by which pyridoxal phosphate acts on the rat thymocyte dexamethasone receptors. In order to accurately distinguish between small changes in receptor size, we have utilized Sephacryl S-200 to characterize a variety of receptor forms.

Our first action was to mimic the conditions utilized by us previously in sucrose density gradient studies (Cidlowski & Thanassi, 1979); thus, we first investigated the influence of aging of cytosol on thymocyte receptor size. Our data (Figures 1 and 2A) indicate the presence of two steroid receptor forms, one of which is 56 Å and the other 18 Å. The accumulation of the smaller receptor form appears to be the result of "degradation" of the larger receptor which occurred via a time-dependent and Ca^{2+}-stimulated process. These observations suggest that rat thymocytes contain enzymes which metabolize receptors which are similar to those characterized by Vedeckis et al. (1980) in the chick oviduct progesterone receptor system. Furthermore, dexamethasone receptors in both unactivated and activated forms behaved similarly with regard to in vitro metabolism, suggesting that the exposure of DNA or nuclear binding sites on the receptor during activation does not alter its metabolism by endogenous protease or Ca^{2+}-activated proteases. One further point of interest comes from recent studies which indicate the presence of form D (18-Å) receptor when tissues are lysed in the presence of NEM. This finding suggests that receptor turnover may involve similar mechanisms in intact cells. The presence of similar proteolytic receptor fragments has been described for several classes of steroid hormone receptors (Sherman et al., 1978; Vedeckis et al., 1980; Wilson & French, 1979). In the progesterone receptor field there appears to be general agreement that the two receptor's subunits are metabolized to a common initial degradation product (form IV), and then there is subsequent proteolytic conversion to meroreceptor, the

smallest receptor degradation product still retaining steroid. Wilson & French (1979) describe four androgen receptor forms, two of which were large and two of which resulted from proteolysis. These two smaller androgen receptor forms are comparable in size to form IV and meroreceptor described for progesterone receptors (Sherman et al., 1974; Vedeckis et al., 1980). Unlike these results, there has been reported only three receptor forms or proteolytic products which have good precursor product relationships from rat liver system. Carlstedt-Duke et al. (1979) also find the same three products following exposure of glucocorticoid receptors to rat liver lysosomes. The smaller two receptor fragments are again comparable in size to those described for androgen and progesterone receptors. These data suggest that two steps occur in the metabolism of steroid receptors, an initial attack and a subsequent conversion to meroreceptor. From the data presented in this paper, it would appear that rat thymocyte cytosol dexamethasone receptors are metabolized directly from a large 56-Å form A to a small 18-Å form D. The same conclusion is valid when metabolism occurs with or without exogenous Ca^{2+} or by exogenously added trypsin. Whether the apparent different pattern of metabolism of thymocyte glucocorticoid receptor reflects a real difference in receptor structure or simply is a consequence of conducting these studies in crude cytosol preparation remains to be established. It is of interest, however, that all of the receptors analyzed thus far appear to have similar final degradation products (or meroreceptors). This observation alone suggests a great deal of receptor similarity for different steroids.

Having this information, we proceeded with the analysis of the effect of pyridoxal phosphate on glucocorticoid receptor metabolism in order to determine whether the small 2.9S form of receptor which we described evolved from proteolysis. Figure 3 shows that treatment of receptor with pyridoxal phosphate for 2 h at 3 °C results in the partial conversion of form A (56-Å) receptor to a new form B (42-Å) species. Extension of the incubation time or elevation of pyridoxal phosphate concentration can lead to complete conversion of form A receptor to form B. Subsequent conversion of the 42-Å receptor to a smaller form C (32 Å) can be inhibited by the inclusion of *N*-ethylmaleimide during cytosol preparation, whereas the form A to B conversion can not. We have recently shown that the form C (32-Å) receptor recovered from Sephacryl columns sediments as a 2.9S moiety on sucrose density gradient. Thus it appears that our initially observed small 2.9S glucocorticoid receptor following pyridoxal phosphate treatment and $NaBH_4$ reduction differs from the endogenous small receptor degradation product (form D, 18 Å). It would appear that our original 2.9S receptor form was produced via proteolytic-independent and -dependent mechanisms. Interestingly, this receptor fragment is quite similar to the meroreceptor precursor described in the other model systems. Furthermore, the complete absence of form D (18 Å) in any cytosol which was treated with pyridoxal phosphate suggests that the association of pyridoxal phosphate with receptor lysines blocks a protease-sensitive protein of the receptor. This notion is supported by studies using exogenous trypsin which indicate that pyridoxal phosphate can block trypsin-sensitive receptor sites. Whether such as interaction of pyridoxal phosphate with receptor is a specific or simply random association with lysines requires sufficient receptor purification to allow use of physiological poyridoxal phosphate concentrations during experimentation.

To further substantiate the role of proteolytic activity in the process of receptor conversion, we have utilized the sulfydryl attacking reagent *N*-ethylmaleimide. This compound effectively antagonizes purified chicken oviduct, Ca^{2+}-stimulatable receptor protease (Vedeckis et al., 1980). The addition of *N*-ethylmaleimide to cytosol appears to have several effects, some of which in terms of proteolytic mechanisms are explanable and others not. *N*-Ethylmaleimide addition blocks the accumulation of form D (18-Å) receptor resulting from either aging of cytosol or Ca^{2+} addition. The presence of *N*-ethylmaleimide during exposure of cytosol to pyridoxal phosphate has two apparent effects. The first of these is the result that very little form C (32-Å) receptor is produced, whereas delayed addition of NEM results in increased form C receptor formation. A second action of NEM would appear to be on the form A to form B (42 Å) step; in this regard, the data shown may be somewhat misleading. First of all, NEM increases recovery of receptor in the form A (56-Å) region of the column only. These NEM-treated receptors appear to differ from normal form A (56-Å) control receptors since they sediment near the bottom of 5–20% sucrose gradients, whereas controls accumulate as 8S moieties. These observations suggest the possibility that NEM is acting directly with receptor, to produce a molecule with a higher tendency to aggregate. We postulate that this receptor form may be insensitive to the action of pyridoxal phosphate. However, further studies are necessary to determine the mechanism by which the metabolism of the glucocorticoid receptor is being altered by *N*-ethylmaleimide.

A point of interest worth mentioning from the data presented is our observation that only the active form of vitamin B_6, pyridoxal phosphate, alters receptor metabolism. As is illustrated in Figure 7, pyridoxal phosphate is highly specific in preventing form D (18-Å) receptor formation and form C (32-Å) accumulation. These biochemical studies can only suggest that this vitamin known to alter so many physiological processes may be acting in living cells. Definitive answers for this question await, however, accurate determination of receptor biosynthetic and degradative rates in whole cells deficient in pyridoxal phosphate. Whether the receptor forms (B and C) described here following pyridoxal phosphate treatment represent physiologically functional molecules which act to regulate gene function also remains uncertain until such time that each form can be purified to homogeneity and probes can be developed for theor detection in cells. Nevertheless, pyridoxal phosphate appears to be an appropriate probe with which to further elucidate the structure and metabolism of steroid hormone receptors.

One other consideration should be made when comparing our results to those published by Vedeckis et al. (1980), Sherman et al. (1978), Carlstedt-Duke et al. (1977), and Wilson & French (1979). Unlike their reports, our studies have been conducted with low ionic strength conditions, following preparation of dilute rather than concentrated cytosols. We have taken this approach and believe it is valid on the basis of the following reasons: (1) use of dilute cytosol preparations rather than concentrated preparations reduces the likelihood of receptor aggregation; (2) High-salt conditions have been shown to stimulate receptor proteolysis (Sherman et al., 1974); (3) High-salt conditions could easily dissociate loosely associated receptor subunits. Interestingly, even though our conditions differ significantly from those previously used, many common features are beginning to appear about the nature and mechanism of steroid receptor metabolism.

Acknowledgments

I thank Dr. John W. Thanassi for his helpful discussions concerning this work.

References
Beato, M., & Feigelson, P. (1972) *J. Biol. Chem.* 247, 7890-7896.
Cake, M. H., Disorbo, D. M., & Litwack, G. (1978) *J. Biol. Chem.* 253, 4886-4891.
Carlstedt-Duke, J., Gustafsson, J., & Wrange, O. (1977) *Biochim. Biophys. Acta 497*, 507-524.
Carlstedt-Duke, J., Wrange, O., Dahlberg, E., Gustafsson, J., & Hogberg, B. (1979) *J. Biol. Chem. 254*, 1537-1539.
Cidlowski, J. A., & Munck, A. (1978) *Biochim. Biophys. Acta 543*, 545-555.
Cidlowski, J. A., & Thanassi, J. W. (1978) *Biochem. Biophys. Res. Commun. 82*, 1140-1146.
Cidlowski, J. A., & Thanassi, J. W. (1979) *Biochemistry 18*, 2378-2384.
Climent, F., Bugary, H., & Beato, M. (1976) *FEBS Lett. 66*, 317-321.
Govindan, M. V., & Sekeris, C. E. (1978) *Eur. J. Biochem. 89*, 95-104.
Kalimi, M., Colman, P., & Feigelson, P. (1975) *J. Biol. Chem. 250*, 1080-1086.
Koblinsky, M., Beato, M., Kalimi, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7897-7904.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372-1379.
Middlebrook, J. L., & Aronow, L. (1977) *Endocrinology 100*, 271-282.
Muldoon T. G., & Cidlowski, J. A. (1980) *J. Biol. Chem. 255*, 3100-3107.
Munck, A. (1968) *J. Biol. Chem. 243*, 1039-1042.
Munck, A., & Leung, K. (1977) *Mod. Pharmacol.-Toxicol. 8*, 311-397.
Nishigori, H., & Toft, D. (1979) *J. Biol. Chem. 254*, 9155-9161.
O'Brien, J. M., & Cidlowski, J. A. (1980) *J. Steroid Biochem.* (in press).
Schrader, W. T., & O'Malley, B. W. (1972) *J. Biol. Chem. 247*, 51-59.
Sherman, M. R., Atienza, S. B. P., Shansky, J. R., & Hoffman, L. M. (1974) *J. Biol. Chem. 249*, 5351-5363.
Sherman, M. R., Tuazon, F. B., Diaz, S. C., & Miller, L. K. (1976) *Biochemistry 15*, 980-989.
Sherman, M. R., Pickering, L. A., Rollwagen, F. M., & Miller, L. K. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 167-173.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1978) *Biochem. Actions Horm. 5*, 321.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1980) *Biochemistry 19*, 343-349.
Wilson, E. M., & French, F. S. (1979) *J. Biol. Chem. 254*, 6310-6319.
Wrange, O., Carlstedt-Duke, J., & Gustafsson, J. (1979) *J. Biol. Chem. 254*, 9284-9290.
Yamamoto, E. R., & Alberts, B. M. (1976) *Annu. Rev. Biochem. 45*, 721-746.

Conformation of Phosphatidylethanolamine in the Gel Phase As Seen by Neutron Diffraction†

Georg Büldt* and Joachim Seelig

ABSTRACT: For confirmation of some general aspects of phospholipid conformation in membranes and extension of previous neutron diffraction studies on dipalmitoyllecithin, measurements have now been made on 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) in the gel phase by the same method. Three selectively deuterated samples were investigated; in one of the specimens the first methylene segment close to the glycerol backbone in both chains was deuterated, and in the other two samples one of the methylene segments in the phosphoethanolamine group was replaced by CD_2. Together with the undeuterated DPPE, these probes were investigated at very low water content (about 1.5-2 molecules of water per lipid) as oriented samples at 25 °C. The intensities of the first 12 reflections were collected and phased, and the mean positions of the segments were determined. The results confirm the idea that the conformation of a DPPE molecule in the gel state is very similar to the crystal structure of *rac*-1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine. The two main features are (1) the chains remain in all all-trans conformation having an axial displacement of about 3-4 Å, (2) the zwitterionic dipoles in the head groups of both compounds are found to be aligned almost parallel to the bilayer surface. The main advantage of the method results in the fact that the combination of neutron scattering with selectively deuterated probes allows the determination of the mean label position to an accuracy of up to ±1 Å.

When the characteristics of the phospholipids in biological membranes were investigated, it was soon found that many lipids in their pure form exist in several thermodynamic phases, dependent on water content, temperature, pH, and ion concentration (Chapman et al., 1967; Luzzati, 1968; Chapman & Wallach, 1968; Sackmann, 1978). The main structural features of these phases were determined by X-ray diffraction (Tardieu et al., 1973; Levine & Wilkins, 1971; Janiak et al., 1976). At least two of these phases seemed to be particularly important for biological membranes, the gel phase and the liquid-crystalline phase. Moreover, it was found that changes of temperature, pH, or ion concentration can cause a transition between these two states (Träuble & Eibl, 1974). The effect

† From the Department of Biophysical Chemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland. *Received March 6, 1980.* This work was supported by Grant 3.409.78 from the Swiss National Science Foundation and by a European Molecular Biology Organization travel grant (G.B.).

0006-2960/80/0419-6170$01.00/0