Egge, H., Peter-Katalinić, J., Reuter, G., Schauer, R., Ghidoni, R., Sonnino, S., & Tettamanti, G. (1985) *Chem. Phys. Lipids 37*, 127–141.
Folch, J., Lees, M., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497–509.
Gruner, K. R., van Eijk, R. V. W., & Mühlradt, P. F. (1981) *Biochemistry 20*, 4518–4522.
Hashimoto, Y., Otsuka, H., Sudo, K., Suzuki, K., Suzuki, A., & Yamakawa, T. (1983) *J. Biochem. 93*, 895–901.
Hirabayashi, Y., Koketsu, K., Higashi, H., Suzuki, Y., Matsumoto, M., Sugimoto, M., & Ogawa, T. (1986) *Biochim. Biophys. Acta 876*, 178–182.
Itoh, T., Li, Y.-T., & Yu, R. K. (1981) *J. Biol. Chem. 256*, 165–169.
IUPAC–IUB Commission on Biochemical Nomenclature (1977) *Eur. J. Biochem. 79*, 11–21.
Kasai, M., Iwamori, M., Nagai, Y., Okumura, K., & Tada, T. (1980) *Eur. J. Immunol. 10*, 175–180.
Kniep, B., Hünig, T. R., Fitch, F. W., Heuer, J., Kölsch, E., & Mühlradt, P. F. (1983) *Biochemistry 22*, 251–255.
Kniep, E. M., Kniep, B., Grote, W., Conradt, H. S., Monner, D. A., & Mühlradt, P. F. (1984) *Eur. J. Biochem. 143*, 199–203.
Lafertē, S., Fukuda, M. N., Fukuda, M., Dell, A., & Dennis, J. W. (1987) *Cancer Res. 47*, 150–159.
Mercurio, A. M., Schwarting, G. A., & Robbins, P. W. (1984) *J. Exp. Med. 160*, 1114–1125.
Momoi, T., Ando, S., & Nagai, Y. (1976) *Biochim. Biophys. Acta 441*, 488–497.
Mühlradt, P. F., Bethke, U., Monner, D. A., & Petzoldt, K. (1984) *Eur. J. Immunol. 14*, 852–858.
Murayama, K., Levery, S. B., Schirrmacher, V., & Hakomori, S. I. (1986) *Cancer Res. 46*, 1395–1402.
Müthing, J., & Mühlradt, P. F. (1984) *Hoppe-Seyler's Z. Physiol. Chem. 365*, 1036–1037.
Müthing, J., & Mühlradt, P. F. (1988a) in *Sialic Acids 1988, Proceedings of the Japanese–German Symposium on Sialic Acids* (Schauer, R., & Yamakawa, T., Eds.) pp 248–249, Kieler Verlag Wissenschaft + Bildung, Kiel.
Müthing, J., & Mühlradt, P. F. (1988b) *Anal. Biochem. 173*, 10–17.
Müthing, J., Egge, H., Kniep, B., & Mühlradt, P. F. (1987) *Eur. J. Biochem. 163*, 407–416.
Nakamura, K., Hashimoto, Y., Suzuki, M., Suzuki, A., & Yamakawa, T. (1984) *J. Biochem. 96*, 949–957.
Nakamura, K., Suzuki, M., Inagaki, F., Yamakawa, T., & Suzuki, A. (1987) *J. Biochem. 101*, 825–835.
Nakamura, K., Hashimoto, Y., Yamakawa, T., & Suzuki, A. (1988) *J. Biochem. 103*, 201–208.
Rokukawa, C., Nakamura, K., & Handa, S. (1988) *J. Biochem. 103*, 36–42.
Schwarting, G. A., & Gajewski, A. (1983) *J. Biol. Chem. 258*, 5893–5898.
Schwartz, R., Kniep, B., Müthing, J., & Mühlradt, P. F. (1985) *Int. J. Cancer 36*, 601–607.
Stout, R. D., Schwarting, G. A., & Suttles, J. (1987) *J. Immunol. 139*, 2123–2129.
Weiss, J., Schwinzer, B., Kirchner, H., Gemsa, D., & Resch, K. (1986) *Immunobiology 171*, 234–251.
Williams, M. A., & McCluer, R. H. (1980) *J. Neurochem. 35*, 266–269.
Young, W. W., MacDonald, E. M. S., Nowinski, R. C., & Hakomori, S.-I. (1979) *J. Exp. Med. 150*, 1008–1019.
Yu, R. K., & Ledeen, R. W. (1972) *J. Lipid Res. 13*, 680–686.

Transformed Glucocorticoid Receptors Consist of Multiple Subspecies with Differing Capacities To Bind DNA–Cellulose†

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ABSTRACT: The glucocorticoid receptor can be transformed into a DNA-binding protein by a process that is both hormone and temperature dependent. We have used a modification of the conventional method of anion-exchange chromatography to separate and analyze a variety of receptor subspecies that result from this transition. One receptor form (peak A) was found to have a capacity to bind DNA–cellulose which was significantly greater than that of the other species. Under conditions of mild heating (15 °C), the relative abundance of peak A in the receptor population and the rate of receptor transformation were both increased as a result of incubating samples with alkaline phosphatase. The mechanism appears to involve the conversion of the more "acidic" forms into that of peak A. The results indicate that receptor transformation is a multistep process which may be promoted by the removal of phosphate from either the receptor or a receptor-bound regulatory factor.

The glucocorticoid receptor is a hormone-dependent regulatory protein possessing the ability to enhance transcription of individual genes through its interaction with specific DNA sequences (Ringold, 1985; Yamamoto, 1985; Majors & Varmus, 1983; Chandler et al., 1983; Payvar et al., 1983). The capacity to bind hormone, transform into a DNA-binding protein, translocate to the nucleus, and eventually undergo productive interactions within a circumscribed set of genes could all potentially be controlled by modifications of the receptor's structure. For instance, there is considerable evidence that receptor sulfhydryl groups may play an important role in determining both steroid- and DNA-binding capacities (Granberg & Ballard, 1977; Sando et al., 1979; Bodwell et al., 1984a,b; Tienrungroj et al., 1987). Covalent modification at these sites by sulfhydryl oxidizing reagents causes a loss

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of receptor function. Conversely, disulfide reducing agents such as dithiothreitol promote ligand binding in vitro; the protein thioredoxin has been shown to have effects similar to dithiothreitol and has been proposed as the cellular component responsible for regulating the oxidation state of groups important for hormone binding in vivo (Grippo et al., 1985).

Phosphorylation has also been implicated in the control of the glucocorticoid receptor's ability to bind steroid. Manipulations which promote dephosphorylation of the unoccupied receptor lead to a loss of hormone-binding capacity (Nielsen et al., 1977). Recent evidence indicates that the glucocorticoid receptor itself contains phosphorus (Housley & Pratt, 1983; Mendel et al., 1986); however, its exact role in regulating receptor function has not been delineated. Relatively little is known about changes that may occur in the structure of the steroid-binding protein during its transition to the DNA-binding state or its residence within the nucleus. Both Mendel et al. (1986) and Tienrungroj et al. (1987) have reported that there was no change in receptor ^{32}P content resulting from receptor transformation.

Prior to transformation, the glucocorticoid receptor exists within a high molecular weight complex (Holbrook et al., 1983; Vedeckis, 1983; Raaka & Samuels, 1983) containing a 90-kDa heat shock protein (Sanchez et al., 1985, 1987). Upon binding hormone, the complex undergoes a temperature-dependent dissociation, releasing the steroid-binding component which subsequently gains the capacity to bind DNA. Both sucrose gradient centrifugation and anion-exchange chromatography (Sakaue & Thompson, 1977; Parchman & Litwack, 1977) have been used to monitor the transition between the high and low molecular weight states. However, regulation of receptor function by covalent modification such as phosphorylation implies the existence of species whose difference would be too subtle to be resolved by these methods. Evidence for receptor charge heterogeneity has been demonstrated using 2-D PAGE (Smith & Harmon, 1985; Cidlowski & Richon, 1984), where several isoforms of the steroid-binding protein were observed. The most basic of the species reported by Smith et al. (1986) was shown to have the greatest capacity to bind DNA, but the basis for the differences in charge between them was not determined.

We have developed a simple modification of the current methods of anion-exchange chromatography which allows high-resolution separation of at least five species of transformed glucocorticoid receptor (Gruol et al., 1988). The method relies upon the use of DEAE Fast Flow Sepharose (Pharmacia) deployed in narrow-bore columns and elution of receptor with shallow KCl gradients. This paper reports our attempts to determine the basis for the different forms observed in the receptor profile. The results indicate that receptor transformation is a multistep process, and experiments with alkaline phosphatase implicate a role for dephosphorylation in the conversion of glucocorticoid receptors into DNA-binding proteins. It is not known if the putative phosphate groups reside on the receptor or a receptor-bound regulatory factor.

Materials and Methods

Cell Culture. WEHI 7.1 (W7) is a thymoma cell line obtained from a female Balb/c mouse after X irradiation (Harris et al., 1973). A derivative of this line resistant to thioguanine (W7TG) was used in all of the experiments. The cells were grown as previously described (Gruol & Dalton, 1984).

Preparation of Cytoplasmic Extracts. Cells were removed from the culture medium by centrifugation and washed twice in phosphate-buffered saline. All further manipulations were carried out at 0 °C unless otherwise noted. The cell pellet was resuspended in a buffer containing 10 mM Pipes (pH 7.1), 2.5 mM EDTA, 2 mM KCl, 1 mM dithiothreitol, and protease inhibitors (23 μg/mL leupeptin, 32 μg/mL antipain, and 33 μg/mL pepstatin). The cell suspension was then subjected to 30 strokes in a Dounce homogenizer equipped with a loose-fitting pestle. The lysate was spun for 20 min at 17000g, and the pellet was discarded. The supernatant was subjected to further centrifugation, 60000g for 30 min, and the resulting supernatant was retained. The sample was passed through a Millex-GV (0.22 μm) filter to remove any remaining particulate matter. Protein concentrations in the extracts were determined by using a Bio-Rad protein assay kit and ranged from 10 to 30 mg/mL.

Labeling of Receptors with Hormone. (*1*) *Intact Cells.* Receptors contained within intact cells were labeled with hormone by first concentrating the cells to 2×10^7 cells/mL in a 1:1 mixture of culture medium and phosphate-buffered saline. The synthetic glucocorticoid [^{3}H]triamcinolone acetonide was added to a final concentration of 3×10^{-8} M. Incubations carried out in the cold were kept on ice, and incubations at 37 °C were carried out in a CO_2 incubator. After 1 h of incubation, the cells were washed 3 times in cold (0 °C) phosphate-buffered saline to remove unbound hormone.

(*2*) *Cytoplasmic Extracts.* Cell extracts were prepared as described above and incubated with [^{3}H]triamcinolone acetonide (3×10^{-8} M) for 30 min at 0 °C. Samples whose receptors were to be transformed by heating were incubated for an additional 20 min at 27 °C and then chilled back to 0 °C for 30 min. After the incubation, the extracts were passed through PD-10 (Pharmacia) sieving columns to remove free hormone. The sieving columns were preequilibrated with 10 mM Pipes (pH 7.1) and 2 mM KCl.

Anion-Exchange Chromatography. Chromatography was carried out using DEAE Fast Flow Sepharose (Pharmacia) which was preequilibrated with sample loading buffer: 10 mM Pipes (pH 7.1), 1 mM dithiothreitol, and 2 mM KCl. Glass columns with an inner diameter of 4 mm and a length of 30 cm were used. After the DEAE-Sepharose had initially settled in the column, the column was pressurized by using a 20-mL syringe in order to ensure complete packing of the matrix. Approximately 40 mg of protein was slowly loaded onto the columns at a rate of 0.2 mL/min. The amount of protein loaded (between 10 and 60 mg) had no noticeable effect upon the elution patterns that were observed. After application of the sample, the column was washed with 50 mL of loading buffer and then with 20 mL of buffer containing 25 mM KCl. Except for the experiment shown in Figure 1, the samples were eluted with a linear gradient of KCl ranging from 25 to 140 mM in a buffer of 10 mM Pipes (pH 7.1) and 1 mM dithiothreitol. The total elution volume was 300 mL, and 1.5-mL fractions were collected. The elution flow rate was approximately 0.45 mL/min. KCl concentrations were determined by using a CDM-80 conductivity meter (Radio-meter Copenhagen). Bound hormone was measured by using a Beckman liquid scintillation counter. Samples incubated in the presence of an additional excess of unlabeled hormone exhibited no peaks of bound tritiated hormone. Therefore, the patterns of [^{3}H]triamcinolone acetonide seen in Figures 1–6 represent hormone specifically bound to receptor.

Receptor Binding to DNA–Cellulose. DNA–cellulose was prepared as described by Litman (1968) and was a gift from Ronald Newby (The Salk Institute). Prior to use, it was washed extensively in DNA-binding buffer [10 mM Pipes (pH 7.1) and 70 mM KCl]. Typically, 0.3 mL of sample was

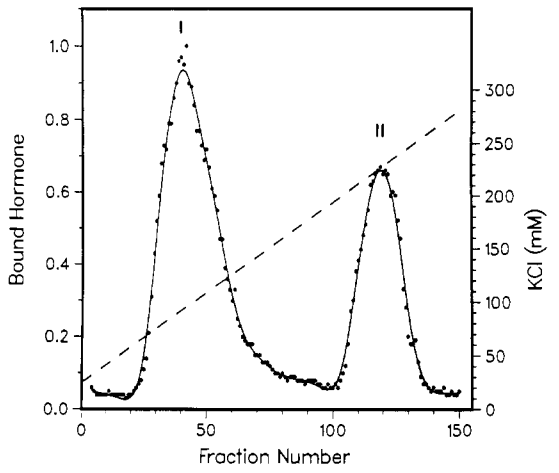

FIGURE 1: DEAE elution profile the cytosolic glucocorticoid receptors from cells incubated with hormone at 0 °C. An exponentially growing culture of WEHI-7 cells was incubated with [^{3}H]triamcinolone acetonide at 0 °C for 1 h as described under Materials and Methods. After washing the cells free of unbound hormone, a cytosol extract was prepared and loaded onto a column of DEAE Fast Flow Sepharose. The bound receptor was eluted with a linear gradient of KCl (25–280 mM). 1.5-mL fractions were collected. The bound hormone is expressed normalized to the fraction with the most radioactivity (7124 cpm).

incubated with 25 mg of DNA–cellulose for 1 h at 0 °C in DNA-binding buffer. The DNA–cellulose was pelleted by centrifugation and washed once in the same buffer. The final pellet was removed and placed into a scintillation vial for measurement of the bound hormone. Measurement of the nonspecific binding was made with samples which had been incubated with an additional excess of unlabeled hormone.

Reagents. [1,2,4-^{3}H(N)]Triamcinolone acetonide (specific activity 29 ^{Ci}/mmol) and ^{14}C-methylated proteins were purchased from Amersham Corp. DEAE Fast Flow Sepharose along with PD-10 Sephadex G-25M columns were obtained from Pharmacia. Alkaline phosphatase (calf intestine, molecular biology grade) and dithiothreitol were purchased from Calbiochem. Chemicals from Sigma Chemical Co. were leupeptin, antipain, pepstatin, a triamcinolone acetonide, glucose 1-phosphate, and Pipes. Protein assay dye was from Bio-Rad.

Results

Multiple Forms of Receptor Have Different DNA-Binding Capacities. Anion-exchange chromatography has proven to be a useful tool for monitoring the hormone-induced conversion of glucocorticoid receptors into DNA-binding proteins. The approach is based upon the observation that nontransformed receptors, contained within high molecular weight heteromeric complexes, elute from DEAE columns at much higher salt concentrations than do their smaller dissociated counterparts (Holbrook et al., 1983; Vedeckis, 1983; Raaka & Samuels, 1983; Sakaue & Thompson, 1977; Parchman & Litwack, 1977). The existence of both forms is illustrated in the DEAE-elution profile shown in Figure 1. A cytoplasmic extract was prepared from WEHI-7 cells that had been incubated intact with [^{3}H]triamcinolone acetonide for 60 min at 0 °C. The lower temperature was chosen to inhibit the complete conversion of receptor into the dissociated form. The sample was loaded onto a column of DEAE-Sepharose, and the proteins eluted with a KCl gradient that ranged from 25 to 280 mM. A peak of bound hormone was released at 250 mM KCl (peak II), indicative of the high molecular weight (sedimenting at approximately 9 S) complex containing the nontransformed receptor. A second peak (peak I) of bound hormone was seen at a lower salt concentration (approximately 90 mM KCl) characteristic of the steroid-binding protein after dissociation from the 9S complex. A similar profile to that shown in Figure 1 was obtained when a cell extract was incubated in vitro with hormone at 0 °C (Gruol et al., 1988). When intact cells were heated to 37 °C during the incubation with hormone, all of the cytoplasmic receptor was found to elute within the low-salt (peak I) form (data not shown) which is conventionally considered to be transformed receptor.

Table I: DNA-Binding Capacity of Glucocorticoid Receptors

(A) Hormone Binding Carried Out in Intact Cells[a]

conditions	total receptor (cpm)	DNA–cellulose-bound receptor (cpm)	% bound to DNA–cellulose
0 °C, 1 h			
expt 1	26404	1561	5.9
expt 2	35385	2805	7.9
37 °C, 1 h			
expt 1	20090	2893	14.4
expt 2	4725	844	18.0

(B) Hormone Binding Carried Out in Cell Extracts[b]

conditions	av bound to DNA–cellulose
0 °C, 1 h	9.1 ± 3.7 (n = 7)
27 °C, 20 min	43.4 ± 13.5 (n = 7)
15 °C, 1 h	18.7 ± 9.8 (n = 4)
15 °C, 1 h, plus alkaline phosphatase	33.2 ± 13.4 (n = 4)
27 °C, 20 min, then 15 °C, 1 h, plus alkaline phosphatase	42.0 ± 10.8 (n = 4)

[a] Cell cultures were incubated with [^{3}H]triamcinolone acetonide (±200× excess of unlabeled hormone) under the conditions indicated. Cytosol extracts were prepared and tested for the capacity of bound hormone to be retained on DNA–cellulose. Nonspecific binding was measured in the samples which were incubated with the excess of unlabeled hormone. Nonspecific binding was subtracted from the totals, and the differences are listed. [b] Cytosol extracts were incubated with triamcinolone acetonide for 30 min at 0 °C and then under the conditions that are listed. Free hormone was removed with dextran–charcoal. The incubations with alkaline phosphatase were carried out at 50 units/mL. The samples were tested for the capacity of bound hormone to bind to DNA–cellulose, and the averages standard deviations are listed for the n experiments that were carried out for each.

The conversion of glucocorticoid receptors into DNA-binding proteins is an incomplete process both in vitro and also in vivo. In intact WEHI-7 cells, only 50% of the receptor translocates to the nucleus (Gruol et al., 1984). In vitro, DNA–cellulose-binding assays detect what appears to be a similar phenomenon. This is illustrated below. Extracts prepared from intact cells that had been incubated with hormone at either 0 °C or 37 °C were tested for the capacity of receptor to bind DNA–cellulose. The results are shown in Table IA. An average of 7% of the receptor in extracts from chilled cells had the ability to be retained on the DNA while approximately 16% of the receptor from the heated cells was bound. The seemingly low value of 16% probably reflects that much of the transformed receptor translocated to the nucleus during the 37 °C incubation period. For comparison, cell extracts were also incubated with hormone in vitro under a variety of conditions and tested for the receptor's ability to bind to DNA (Table IB). In extracts kept at 0 °C, only 9.1% of the receptor bound to DNA. Extracts heated to 27 °C for 20 min contained a larger percentage (43.4%) of receptor which could bind DNA (longer periods of heating did not significantly increase the amount of receptor bound to DNA). All of the receptor was converted to the low-salt chromatographic form (peak I) under these conditions (data not shown). Thus, it would appear that a majority of the receptor which had become dissociated from the 9S complex and which eluted

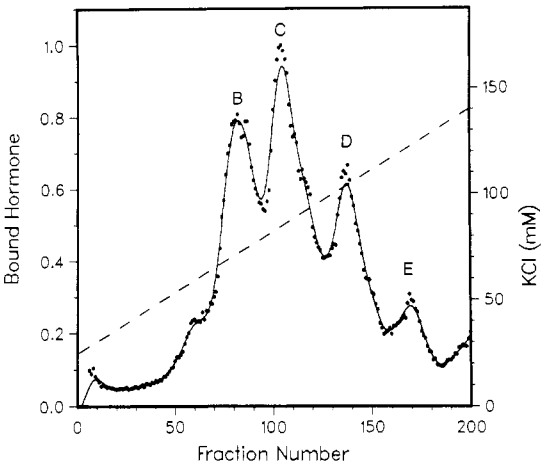

FIGURE 2: High-resolution DEAE elution profile of peak I receptors from cells incubated with hormone at 0 °C. A cytosol sample was prepared and loaded onto a DEAE-Sepharose column as described in Figure 1. The bound receptor was eluted with a linear gradient of KCl confined to the range of peak I (25–140 mM). The bound hormone is expressed normalized to the fraction with the most radioactivity (3500 cpm).

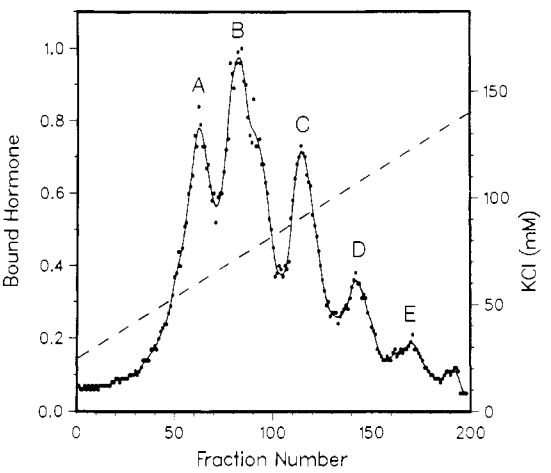

FIGURE 3: DEAE elution profile of cytosolic glucocorticoid receptors from cell extracts incubated at 27 °C. A cytosol sample was prepared and incubated with hormone for 20 min at 27 °C. The sample was analyzed using DEAE-Sepharose as in Figure 2. The bound hormone is expressed normalized to the fraction with the most radioactivity (2138 cpm).

from DEAE within peak I did not have the capacity to bind DNA–cellulose.

The relatively limited capacity of receptors to bind DNA described above could be due either to the inefficiency of our assay system or to an inherent property of the receptors found within peak I. Recent work from this laboratory (Gruol et al., 1988) had shown that the glucocorticoid receptors comprising the low-salt peak represent a heterogeneous population of molecules, possibly reflecting differences in covalent modification and/or protein-bound factors. The data were obtained by using a simple adaptation of the conventional methods of anion-exchange chromatography, employing elution with very shallow salt gradients, which was found to produce resolution of multiple receptor species. Figure 2 illustrates the pattern of bound hormone obtained from a sample prepared similarly to that shown in Figure 1 but eluted with a salt gradient restricted to the range of peak I. Under these conditions, instead of a single peak, there were four distinct species evident (labeled B, C, D, and E). It should be noted that in the earlier publication (Gruol et al., 1988) Roman numerals were used to designate the different species. We have changed to capital letters in order to avoid confusion with an earlier convention established by other authors. The four peaks of bound hormone eluted at 68, 82, 103, and 124 mM KCl, respectively. While the differences in salt concentrations were relatively small, the peaks themselves were separated by 20–30 fractions (1.5 mL each, approximately equal to a column volume). Therefore, each fraction represents the receptor released from the entire column during a change in salt concentration of less than 0.4 mM KCl. This provided for separation of the different forms which would remain unresolved using smaller elution volumes and/or larger increments in ionic strength per fraction. Our experience suggests that the basis of the chromatography is more complex than ion exchange alone and may involve another type of interaction, possibly with the Sepharose matrix.

The existence of multiple species of glucocorticoid receptor in the cytoplasm of WEHI-7 cells is further demonstrated by the experiment shown in Figure 3. In this instance, the sample was prepared by heating a cytoplasmic extract in vitro with hormone at 27 °C. The two patterns are very similar, with all of the peaks occurring at nearly the same salt concentrations as in Figure 2. There was an additional peak evident (labeled A), which eluted at 60 mM KCl coincident with a relative decline in peaks C, D, and E. Table II lists the average KCl concentrations of the different peak fractions from 35 similar experiments carried out over more than a 2-year period. Also listed are the average fraction numbers corresponding to each peak. We have found that the different peaks elute at their characteristic positions as long as consistency is maintained in the column preparation, washing, and elution procedures. The pH of the elution buffers can affect the peak positions (not shown) and was rigorously maintained at 7.1. The separation between peaks A and B was typically less than that between the other peaks. The separations between peaks B, C, D, and E were found to be nearly equal, possibly reflecting a progressive modification. The relative amount of peak A has varied considerably in the experiments that have been carried out so far. It should be noted that in other experiments, the measured ability of receptor in an extract to bind DNA has also varied significantly.

Table II: Summary of Receptor Peak Positions of DEAE Elution Profiles

peak	KCl concn (mM)	KCl increment (mM)	fraction
A	61.3 ± 3.5[a]	12.0	68
B	73.3 ± 3.8	16.6	90
C	89.9 ± 4.3	16.8	120
D	106.7 ± 5.5	15.9	151
E	127.6 ± 6.8		179

[a] Mean ± standard deviation of receptor peaks from 35 elution profiles obtained with cell extracts heated to 27 °C in the presence of triamcinolone acetonide.

The presence of multiple forms of the glucocorticoid receptor in a sample which contained only a limited capacity to bind DNA suggested that the individual species might possess differential capacities for DNA binding. To test this possibility, a cytoplasmic sample was incubated with hormone in vitro (at 27 °C), chilled to 0 °C, and mixed with DNA–cellulose for 1 h. The DNA–cellulose was separated by centrifugation and the supernatant removed. The pellet was washed 1 time, and the two supernatants were combined to form the "unbound" sample. The DNA-bound receptor was eluted from the pellet with 75 mM $MgCl_2$ and separated by centrifugation. After the $MgCl_2$ was removed from the "bound" sample by sieving, both samples were loaded onto separate DEAE-Sepharose columns. Figure 4 shows the re-

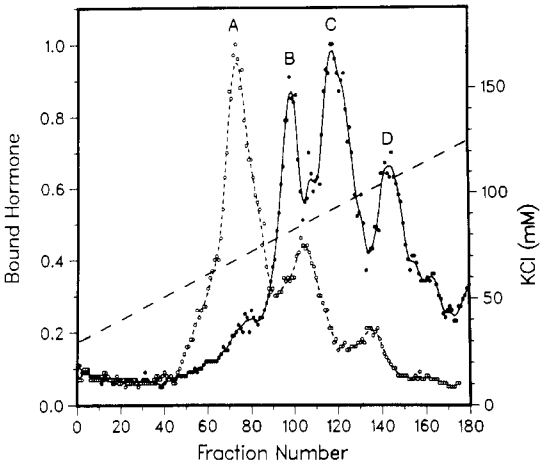

FIGURE 4: DEAE elution profiles of cytosolic glucocorticoid receptors from cell extracts incubated first with hormone at 27 °C and then with DNA–cellulose at 0 °C. A cytosol sample was prepared and incubated at 27 °C with [^{3}H]triamcinolone acetonide for 20 min as described under Materials and Methods. The extract was brought to 70 mM KCl and incubated with DNA–cellulose for 1 h at 0 °C. The DNA-bound portion of the sample was then separated by centrifugation. The remaining supernatant portion was passed through a PD-10 sieving column equilibrated with column loading buffer and analyzed as in Figure 2 (solid line). The DNA–cellulose-bound receptor was eluted with 75 mM $MgCl_2$, passed through a PD-10 sieving column, and analyzed concurrently with the first sample in another DEAE-Sepharose column (dashed line). The bound hormone is expressed normalized to the fraction with the most radioactivity for each profile. DNA bound, 1164 cpm; unbound supernatant, 1744 cpm.

sults, each normalized to the maximal value obtained with that sample. Peak A was totally absent from the unbound sample but was the major peak in the sample which contained the DNA-bound receptor. In a series of six experiments, peak B has demonstrated a much smaller and varying capacity to bind DNA, while peaks C, D, and E exhibited little ability to be retained on the DNA–cellulose. In the experiment shown in Figure 4, 30% of the receptor was retained on the DNA–cellulose. When the two sets of data were combined, normalized, and replotted, the product (not shown) it a profile similar to that obtained from total cell extracts, demonstrating the additive nature of the component patterns.

Effects of Phosphatase on Conversion to DNA-Binding Forms. The results presented above offer an explanation for the observation that only a portion of the receptor in a cytoplasmic sample binds to DNA. The process of conversion from the large, heteromeric 9S complex results in the generation of a number of receptor species, only one or two of which have a significant affinity for DNA. If the receptor species found in peaks B through E can be converted to that of peak A, it would strongly support the idea that transformation of receptor into a DNA-binding protein is a multistep process. Since the receptor has been shown to contain phosphate, and since peak A could be the result of the removal of negatively charged groups from the more acidic forms, phosphatase was tested for the ability to affect both the column profile and the capacity of receptor to bind to DNA. The results in part B of Table I show that treatment with alkaline phosphatase caused an acceleration of the receptor transformation process. Samples were incubated with hormone at 0 or 27 °C and then at 15 °C in the presence or absence of alkaline phosphatase. After incubation with hormone at 0 °C, incubation at 15 °C resulted in a partial (18.7%) conversion of receptor into the DNA-binding state. The inclusion of alkaline phosphatase resulted in a greater capacity of receptor to bind DNA (33.2%), not quite to the same level as obtained by heating

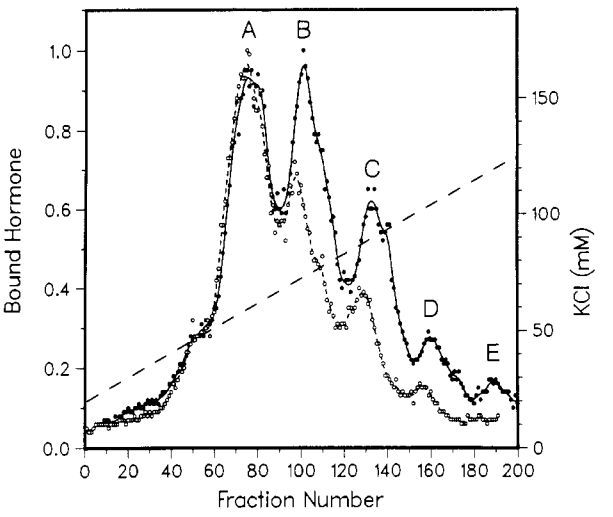

FIGURE 5: Effect of alkaline phosphatase treatment on the DEAE elution profile of cytosol glucocorticoid receptors. A cytosol sample was prepared and incubated with hormone for 30 min at 0 °C. The free hormone was removed by passing the sample through a PD-10 column. The sample was split into two equal parts, and alkaline phosphatase (25 units/mL) was added to each. In one case (solid line), the phosphatase was heated to 100 °C for 5 min prior to its addition to the sample. Both samples were incubated at 15 °C for 1 h and then analyzed using DEAE-Sepharose as in Figure 2. The bound hormone is expressed normalized to the fraction with the most radioactivity for each. Untreated phosphatase (dashed line, 2780 cpm), boiled phosphatase (3564 cpm).

a sample to 27 °C (41%). Samples that had been incubated with hormone at 27 °C and then at 15 °C with alkaline phosphatase had a DNA-binding capacity which was virtually the same (42.0%) as that of heating the samples at 27 °C alone. It would appear, then, that the effect of alkaline phosphatase was to promote or accelerate receptor transformation, but only to a level that would normally be achieved by heating the extract. Since increasing the amount of DNA–cellulose in the assays does not change the percent of receptor bound (not shown), it suggests that there is a property inherent to the samples which determines the maximum capacity of receptor to bind DNA. This could reflect a characteristic of the receptors themselves, or the presence of DNA-binding inhibitors (Simmons, 1977; Atger & Milgrom, 1978; Dahmer et al., 1985) that have been reported by other laboratories.

The result of alkaline phosphatase treatment on the elution profile from a DEAE-Sepharose column is shown in Figure 5. A cytosol sample was split into two equal parts and alkaline phosphatase added to each. In the sample represented by the solid line, the phosphatase had been pretreated by boiling for 5 min to destroy the enzymatic activity. Both samples were heated to 15 °C for an hour. The pattern obtained with the enzyme-inactivated sample was similar to one heated to 27 °C except that the A peak was slightly more prominent. Peak A (fractions 50–90) contained 32% of the total radioactivity recovered from the column. The pattern obtained with the phosphatase-treated sample exhibited a very different profile. In this instance, the peak A fractions contained 43% of the total radioactivity recovered from the column. Concomitant with the increase in peak A there was a sharp decline in the radioactivity observed in the peak C, D, and E positions. There was less than a 6% difference in total radioactivity recovered from the two columns, indicating no net loss of hormone binding.

The results suggest that phosphatase promoted a conversion of the more acidic forms into that of peaks A and B. This possibility was tested more directly by the experiment described

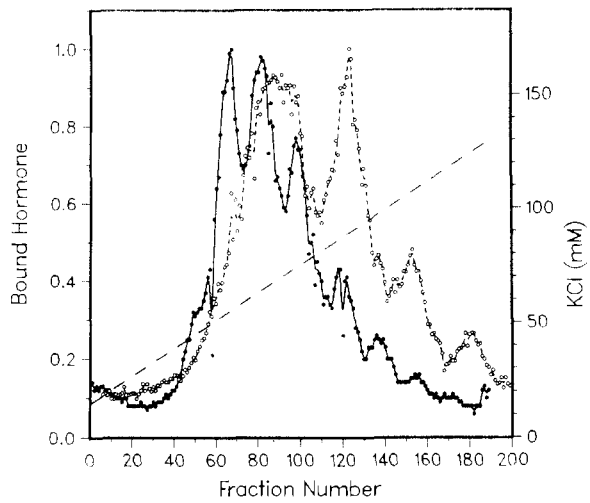

FIGURE 6: Effect of alkaline phosphatase treatment upon glucocorticoid receptors remaining unbound to DNA–cellulose. Cytosol samples were prepared and incubated with hormone at 27 °C for 20 min. The samples were then incubated with DNA–cellulose at 0 °C for 1 h, and the DNA-bound receptor was removed by centrifugation. The supernatant was retained and passed through a PD-10 sieving column. The samples were incubated with (solid line) or without (dashed line) alkaline phosphatase (45 units/mL) at 15 °C for 1 h, loaded onto a DEAE-Sepharose column, and analyzed as in Figure 2. The bound hormone is expressed normalized to the fraction with the greatest radioactivity (914 cpm).

below. An extract was incubated with hormone at 27 °C for 20 min to produce the normal but incomplete transformation of receptor. The extract was chilled and incubated with DNA–cellulose for 1 h to remove the peak A form. The DNA–cellulose was separated by centrifugation, leaving a supernatant containing receptors that had not been bound to DNA (peaks B, C, D, and E). The supernatant was then incubated with alkaline phosphatase for 1 h at 15 °C and analyzed by column chromatography. The results shown (solid line) in Figure 6 demonstrate that the entire pattern had been shifted toward the early eluting forms. It should be noted that the peaks were sharper and the pattern slightly compressed, a behavior which was observed in a number of experiments employing alkaline phosphatase treatment. For comparison, the pattern (dashed line) obtained from a similar sample incubated at 15 °C without phosphatase is also shown. The addition of boiled phosphatase also gave a similar result (not shown). In the phosphatase-treated sample (solid line), 32% of the receptor was found in the first peak. Consistent with the change in elution profile, 40% of the receptor was found capable of binding DNA–cellulose immediately after the phosphatase treatment. Reexposure of untreated samples to DNA–cellulose does not result in significant (<10%) levels of binding. Thus, a significant portion of the more "acidic" forms (C, D, and E) must have been converted into the peak A (and possibly peak B) form during the transformation of nearly half of the receptors into DNA-binding proteins. This change represents conversion since the amount of bound hormone remained constant during the phosphatase treatment.

One potential explanation for the results reported above is that the conversion of the more "acidic" forms to that of peak A could represent the degradation of the receptor protein via proteolysis. To test this possibility, a cytosol extract was incubated with dexamethasone mesylate at 27 °C in order to covalently label the receptor proteins (Eisen et al., 1981). The sample was then split and one portion incubated with alkaline phosphatase as in Figure 6. Both samples were then analyzed using SDS–PAGE. The results (data not shown) detected the specific labeling of a closely spaced protein doublet with molecular weights of 93 000 and 91 000. There was no significant difference between the phosphatase-treated and untreated samples or, in particular, any indication of a specific degradation product with a molecular weight of approximately 40 000 reported by others (Dellweg et al., 1982). Thus, the phosphatase-induced alteration in the column profile was not the result of a measurable change in receptor size.

Discussion

The glucocorticoid receptor exists in a variety of functional states (Housley et al., 1984; Munck & Holbrook, 1984), each one potentially defined by the structure of the protein itself (Carter-Su & Pratt, 1984). The specific mechanisms by which steroid hormones promote conversion of their receptors into DNA-binding proteins are only partially understood. In the case of the glucocorticoid receptor, there is accumulating evidence that this transition involves multiple steps (Grandics et al., 1984; Schmidt et al., 1985; Ben-Or & Chrambach, 1988). Subsequent to binding hormone, the receptor dissociates from a large (9 S), heteromeric complex (Okret et al., 1984; Denis et al., 1987) via a process that is temperature dependent, but which may be promoted in vitro by high ionic strength or sample dilution (Schmidt & Litwack, 1982). Conversely, the complex can be stabilized in vitro by the presence of molybdate (Vedeckis, 1983). Recently, an aminophospholipid has been identified as the endogenous factor that is mimicked by molybdate (Bodine & Litwack, 1988). Our data as well as those of others would appear to indicate that release of the steroid-binding protein from the 9S complex is merely a preliminary step and that there is an additional requirement for receptor modification to produce the active configuration. Our reasons for this conclusion are 3-fold: (1) Manipulations, such as mild heating, result in a quantitative conversion of peak II (the heteromeric complex) into peak I. (2) Peak I is composed of multiple receptor species which contain differing capacities to bind to DNA. (3) The different species comprising peak I appear to be converted from one to another by a process that may include the removal of phosphate groups.

The possibility that a cycle of phosphorylation/dephosphorylation could regulate receptor function was initially addressed relative to the capacity of the glucocorticoid receptor to bind hormone (Munck & Brinck-Johnson, 1973). Treatment with alkaline phosphatase was shown to cause a loss of hormone-binding capacity in receptors that did not contain ligand (Nielsen et al., 1977). The stability of prebound hormone–receptor complexes was unaffected, an observation that we have reaffirmed. In a study of DNA-binding capacity, Barnett et al. (1980) reported a relatively small effect of alkaline phosphatase on the transformation of rat liver receptors (a change from approximately 2% to 10% of receptors bound to DNA–cellulose), but their incubations with the enzyme were carried out at 0–4 °C, rather suboptimal conditions for enzyme activity. Recker et al. (1987) found that phosphatase promoted the conversion of the 9S complex into a smaller 5.2S form. We have extended these observations and shown that the profile of the receptor species which comprise the low-salt (peak I) population shifted as a result of treatment with alkaline phosphatase. The receptor was converted into the forms which eluted from DEAE at lower salt concentrations, the change being consistent with the loss of negative charge. A somewhat similar change can be elicited by heating, which may reflect an endogenous phosphatase activity. In a similar regard, Dalman et al. (1988) recently demonstrated that alkaline phosphatase treatment caused the loss of phosphate from the receptor.

Our results are consistent with the findings of Schmidt et al. (1985), who reported evidence of a heat-stable cytoplasmic activity which promoted conversion of purified rat glucocorticoid receptors into DNA-binding proteins. Their findings are significant in pointing out that the total functional capacity required to affect transformation does not reside entirely within the receptor. Just as thioredoxin may be required to convert receptor into its steroid-binding state (Grippo et al., 1985), another cytoplasmic activity appears necessary to produce the DNA-binding form. Furthermore, Schmidt et al. (1985) demonstrated that receptor transformation is a multistep process. In the model that they have proposed, the first step produces dissociation of the 9S complex and a transition of receptor into the DEAE-peak I form. This is the change that can be inhibited by molybdate. The second step, which is promoted by the putative cytoplasmic activity, results in conversion of receptor within peak I into a DNA-binding form. Our data suggest that the activity observed by Schmidt et al. (1985) may be involved in the conversion of receptors (within peak I) into what we have identified as the peak A form. If this is the case, alkaline phosphatase can either enhance or substitute for the cytoplasmic activity.

While the results presented here strongly support the concept that removal of phosphate promotes receptor transformation, any interpretation of the data must be tempered with the reservation that the location of the putative phosphate group(s) remains to be determined. There would appear to be two likely possibilities: it is located on the receptor itself or on a receptor-associated factor. Since the chromatographic procedure that we have employed did not involve denaturation of the receptor, the individual species could represent the presence of a bound, phosphorylated, regulatory factor. Depending upon the amount of factor and degree of phosphorylation, this alternative would predict a number of different chromatographic species. If removal of the phosphate groups from either the receptor or the associated factor caused a release of the factor, then the DEAE elution profile obtained after phosphatase treatment would represent the receptor minus the factor.

Smith et al. (1986) used 2-D PAGE to resolve two receptor isoforms in IM-9 cells and, somewhat analogous to our observation, found that the more basic form had a selective capacity to bind DNA–cellulose. Neither transformation nor treatment with alkaline phosphatase caused a change in the relative abundance of the two forms, leading the authors to conclude that there was no evidence of receptor dephosphorylation. Mendel et al. (1987), using ^{32}P labeling, estimated the phosphate content of glucocorticoid receptors in WEHI-7 cells to be two to three per receptor. Again, no indication of a change in phosphorylation was seen to result from receptor transformation. Tienrungroj et al. (1987) in a thorough study of receptor phosphorylation in L cells also failed to find evidence of dephosphorylation during the transformation process either in the intact cell or in vitro. Taken together, the weight of the available evidence clearly indicates that the receptor does not undergo an appreciable loss of phosphate as a result of transforming into a DNA-binding protein. It should be noted, however, that in vitro only 30–40% of the receptor gains the capacity to bind DNA and that dephosphorylation could represent the loss of a single (out of several) phosphate group. Thus, the overall degree of phosphorylation may be 15% or less of the total.

The results shown in Figures 5 and 6 also raise another important issue. Extracts treated with alkaline phosphatase contained more than 50% of their receptors in the A and B forms. Somewhat surprisingly, under these conditions there was no net increase in the total capacity to bind DNA (Table IB). This suggests that an additional parameter acts to limit the maximum fraction of receptor which can bind to DNA–cellulose. Thus, generation of the peak A and B forms may be a necessary but not sufficient condition to impart DNA-binding capacity to receptors in crude cytoplasmic extracts.

In conclusion, the data support the concept that transformation of the glucocorticoid receptor into a DNA-binding protein is a multistep process involving a number of intermediate species. Peak A, which elutes from DEAE-Sepharose at the lowest KCl concentration, appears to be a relevant end product form. That is, peak A exhibits the greatest affinity for DNA–cellulose and can be generated from the other receptor species (peaks B, C, D, and E). The nature, location, and role of the labile phosphate in producing receptor transformation remain to be discovered.

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REFERENCES

Atger, M., & Milgrom, E. (1978) *Biochim. Biophys. Acta 496*, 339–348.

Barnett, C. A., Schmidt, T. J., & Litwack, G. (1980) *Biochemistry 19*, 5546–5455.

Bell, P. A., & Munck, A. (1973) *Biochem. J. 136*, 97–107.

Ben-Or, S., & Chrambach, A. (1988) *J. Steroid Biochem. 29*, 47–56.

Bodine, P. V., & Litwack, G. (1988) *J. Biol. Chem. 263*, 3501–3512.

Bodwell, J. E., Holbrook, N. J., & Munck, A. (1984a) *Biochemistry 23*, 4237–4242.

Bodwell, J. E., Holbrook, N. J., & Munck, A. (1984b) *Biochemistry 23*, 1392–1398.

Carter-Su, C., & Pratt, W. B. (1984) *Receptors 1*, 541–585.

Chandler, V. L., Mahler, B. A., & Yamamoto, K. R. (1983) *Cell 33*, 489–499.

Cidlowski, J. A., & Richon, V. (1984) *Endocrinology 115*, 1588–1597.

Dahmer, M. K., Tienrungroj, W., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 7705–7715.

Dalman, F. C., Sanchez, E. R., Lin, A. L.-Y., Perini, F., & Pratt, W. B. (1988) *J. Biol. Chem. 263*, 12259–12267.

Dellweg, H. G., Hotz, A., Mugele, K., & Gehring, U. (1982) *EMBO J. 1*, 285–289.

Denis, M., Wilkstrom, A.-C., & Gustafsson, J.-A. (1987) *J. Biol. Chem. 262*, 11803–11806.

Eisen, H. J., Schleenbaker, R. E., & Simons, S. S., Jr. (1981) *J. Biol. Chem. 256*, 12920–12925.

Granberg, J. P., & Ballard, P. L. (1977) *Endocrinology 100*, 1160–1168.

Grandics, P., Miller, A., Schmidt, T. J., Mittman, D., & Litwack, G. (1984) *J. Biol. Chem. 259*, 3173–3180.

Grippo, J. F., Holmgren, A., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 93–97.

Gruol, D. J., & Dalton, D. K. (1984) *J. Cell. Physiol. 119*, 107–118.

Gruol, D. J., Kempner, E. S., & Bourgeois, S. (1984) *J. Biol. Chem. 259*, 4833–4839.

Gruol, D. J., Campbell, N. F., & Bourgeois, S. (1988) *J. Steroid Biochem. 30*, 287–290.

Harris, A. W., Bankhurst, A. D., Mason, S., & Warner, N. L. (1973) *J. Immunol. 110*, 431-438.
Holbrook, N. J., Bodwell, J. E., Jeffries, M., & Munck, A. (1983) *J. Biol. Chem. 258*, 6477-6485.
Housley, P. R., & Pratt, W. B. (1983) *J. Biol. Chem. 258*, 4630-4635.
Housley, P. R., Grippo, J. F., Dahmer, M. K., & Pratt, W. B. (1984) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) Chapter 12, pp 347-376, Academic Press, New York.
Litman, R. M. (1968) *J. Biol. Chem. 243*, 6222-6233.
Majors, J., & Varmus, H. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5866-5870.
Mendel, D. B., Bodwell, J. E., Gametchu, B., Harrison, R. W., & Munck, A. (1986) *J. Biol. Chem. 261*, 3758-3763.
Mendel, D. B., Bodwell, J. E., & Munck, A. (1987) *J. Biol. Chem. 262*, 5644-5648.
Munck, A., & Brink-Johnson, T. (1968) *J. Biol. Chem. 243*, 5556-5565.
Munck, A., & Holbrook, N. J. (1984) *J. Biol. Chem. 259*, 820-831.
Nielsen, C. J., Sando, J. J., & Pratt, W. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1398-1402.
Okret, S., Wilkstrom, A.-C., & Gustafsson, J.-A. (1985) *Biochemistry 24*, 6581-6586.
Parchman, L. G., & Litwack, G. (1977) *Arch. Biochem. Biophys. 183*, 374-382.
Payvar, F., DeFranco, D., Firesteon, G. L., Edgar, B., Wrange, O., Okret, S., Gustafsson, J.-A., & Yamamoto, K. R. (1983) *Cell 35*, 381-392.
Raaka, B. M., & Samuels, H. H. (1986) *J. Biol. Chem. 262*, 5644-5648.
Reker, C. E., LaPointe, M. C., Kovacic-Milivojevic, B., Chiou, W. J., & Vedeckis, W. V. (1987) *J. Steroid Biochem. 26*, 653-665.
Ringold, G. M. (1985) *Annu. Rev. Pharmacol. Toxicol. 25*, 529-566.
Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533-541.
Sanchez, E. R., Toft, D. O., Schlesinger, M. J., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 12398-12401.
Sanchez, E. R., Meshinchi, S., Tienrungrof, W., Schlesinger, M. J., Toft, D. O., & Pratt, W. B. (1987) *J. Biol. Chem. 262*, 6986-6991.
Sando, J. J., Hammond, N. D., Stratford, C. A., & Pratt, W. B. (1979) *J. Biol. Chem. 254*, 4779-4789.
Schmidt, T. J., & Litwack, G. (1982) *Physiol. Rev. 62*, 1131-1192.
Schmidt, T. J., Miller-Diener, A., Webb, M. L., & Litwack, G. (1985) *J. Biol. Chem. 260*, 16255-16262.
Simmons, S. S., Jr. (1977) *Biochim. Biophys. Acta 496*, 339-348.
Smith, A. C., & Harmon, J. M. (1985) *Endocrinology 115*, 1588-1597.
Smith, A. C., Elsasser, M. S., & Harmon, J. M. (1986) *J. Biol. Chem. 261*, 13285-13292.
Tienrungroj, W., Sanchez, E. R., Housley, P. R., Harrison, R. W., & Pratt, W. B. (1987) *J. Biol. Chem. 262*, 17342-17349.
Vedeckis, W. V. (1983) *Biochemistry 22*, 1983-1989.
Yamamoto, K. R. (1985) *Annu. Rev. Genet. 19*, 209-252.

Dynamics of Proton Diffusion within the Hydration Layer of Phospholipid Membrane†

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ABSTRACT: The diffusion of protons at the immediate vicinity of (less than 10 Å from) a phospholipid membrane is studied by the application of the laser-induced proton pulse. A light-sensitive proton emitter (8-hydroxypyrene-1,3,6-trisulfonate) was trapped exclusively in the hydration layers of multilamellar vesicles made of egg phosphatidylcholine, and the protons were dissociated by a synchronizing laser pulse. The recombination of the proton with pyranin anion was monitored by time-resolved spectroscopy and analyzed by a diffusion-controlled formalism. The measured diffusion coefficient is only slightly smaller than the diffusion coefficient of proton in bulk water. Modulating the width of the hydration layer by external pressure had a direct effect on the diffusibility of the proton: the narrower the hydration layer, the slower is the diffusion of protons.

A precise understanding of proton diffusion on the membrane–water interface is central to experimental observations regarding chemiosmotic proton flux (Chiang & Dilly, 1987). The diffusion of protons between source and sink, located on a membrane, is a sum of two pathways: the lateral proton diffusion through the hydration layer and the bulk diffusion (Nagle & Dilly, 1986). The fast equilibration of protons between surface and bulk (Nachliel & Gutman, 1984) implies that even if the observation time is limited to a few microseconds after protons were released (from a surface source), their reaction (with a surface sink) will be dominated by diffusion through the bulk phase.

In recent years Prats et al. [(1987a,b) and references cited therein] studied the diffusion of protons at a water–monolayer interface. According to their model the proton diffusion at

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