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Interaction of the Activated Cytoplasmic Glucocorticoid Hormone Receptor Complex with the Nuclear Envelope†

Peter Smith and Claus von Holt*

ABSTRACT: Highly purified activated cytoplasmic glucocorticoid hormone receptor binds with high affinity to sites in the nuclear envelope. Nuclear envelope fragments can be isolated from purified chromatin. They bind activated cytoplasmic glucocorticoid receptor with the same equilibrium constant as nuclear envelopes. The presence of envelope components in chromatin is confirmed by the virtual identity of the gel electrophoretic glycoprotein pattern of nuclear envelope, chromatin nonhistones, and nuclear envelope fragments from chromatin.

It is well established that under "in vitro" and "in vivo" conditions steroid hormones including the glucocorticoids initiate increased transcription (Young et al., 1974; Jensen & de Sombre, 1972; O'Malley et al., 1977). There are good indications that such inductions of messenger ribonucleic acid (mRNA) synthesis in general are accompanied or preceded by enzymatic differential modifications of nuclear proteins (Stein et al., 1974). The initiation of the enzymatic nuclear protein modification which in turn may render the DNA transcribable must be preceded by the interaction of the inducing hormone with a suitable macromolecule in the nucleus. It is generally accepted that prior to its localization in the nucleus steroid hormones are bound by cytoplasmic receptors which in turn interact with a nuclear acceptor site (Spelsberg, 1974). A considerable amount of literature has been generated over the years, purporting that the nonhistone chromosomal proteins contain these acceptor sites, thus exerting a controlling function on the transcriptional activity of the genome (O'Malley et al., 1977). However, a number of authors have leveled criticism at the criteria of purity of nonhistone prep-

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arations, particularly with regard to contamination by nuclear envelope components (Tata et al., 1972; Harlow et al., 1972; Jackson, 1976).

In view of these results, we have investigated whether the nuclear envelope under in vitro conditions will bind the charged cytoplasmic steroid hormone–receptor complex as a first step in relaying a hormonal cytoplasmic signal to the nucleus. As a model steroid hormone, we chose the analogue triamcinolone acetonide (9α-fluoro-16α-hydroxyprednisolone 16,17-acetonide).

Two types of cytoplasmic glucocorticoid hormone receptors have been described. A large molecular weight form of 7 S (Beato & Feigelson, 1972) with a Stokes radius of 6.1 nm (Wrange & Gustafsson, 1978) binds initially the hormone or a fluorinated analogue at low ionic strength and +4 °C (Koblinsky et al., 1972). On exposure to +20 °C or an ionic strength of $I = 0.3$, this 7S receptor is converted (activated) into the 3–4S form (Koblinsky et al. 1972) with a Stokes radius of 3.6 nm (Wrange & Gustafsson, 1978).

The activation can apparently also be effected in vitro by proteolytic enzymes (Wrange & Gustafsson, 1978). It has been suggested that lysosomal enzymes may be involved in that process (Carlstedt-Duke et al., 1979). It is this low molecular weight form which preferentially binds to nuclei (Wrange & Gustafsson, 1978). The activated cytoplasmic glucocorticoid receptor used in the present investigation is a several thousand times purified preparation (Climent et al., 1977).

In binding studies of cytoplasmic glucocorticoid receptor to nuclear acceptor sites, the resolubilization of the radioactively charged cytoplasmic steroid hormone–receptor complex from the insoluble cytoplasmic steroid hormone receptor–nuclear acceptor complex in the presence of 0.3–0.4 M KCl has been widely accepted as being indicative of the presence of specific binding sites and forms the basis of methods to determine such sites (Schrader et al., 1977). The interpretation of results from such tests may be difficult because of the aggregation of unbound cytoplasmic steroid hormone receptor (Climent et al., 1977). We have therefore investigated the binding of charged cytoplasmic steroid hormone receptor to potential acceptor sites under conditions of sucrose gradient centrifugation at sucrose concentration ranges which keep the essential components of the test system well separated according to their buoyant densities.

Materials and Methods

Isolation of Nuclei. Nuclei were isolated according to the method of Blobel & Potter (1966).

White female rats were starved overnight and killed by cervical dislocation. The livers were excised, trimmed, and homogenized in 3 volumes of 250 mM sucrose in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 25 mM KCl, and 1.5 mM $MgCl_2$ (TKM) in a Teflon–glass Potter homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 600g for 20 min. The supernatant was removed and the pellet resuspended in 9 volumes of 2.3 M sucrose in TKM and centrifuged at 45000g for 70 min. The resulting milky white pellet, when examined by phase contrast microscopy, showed nuclei free of cells and debris. If not used immediately, the nuclei were resuspended in 2.3 M sucrose in TKM and stored in liquid nitrogen (−170 °C).

Isolation of Chromatin. Chromatin was prepared according to the mehod of Bonner et al. (1968). Nuclei from two rat livers were suspended in 50 mL of 0.05 Tris-HCl, pH 8.0, and homogenized at +4 °C in a glass–Teflon Potter homogenizer. The homogenate was centrifuged at 10000g for 10 min, and the pellet was resuspended in Tris buffer and centrifuged again. The final pellet was resuspended in a small volume of 50 mM Tris buffer layered over 1.7 M sucrose in 10 mM Tris-HCl, pH 8.0, and centrifuged for 2 h at 50000g. The pellet, designated chromatin, was resuspended in 250 mM sucrose and 2 mM phosphate, pH 7.5, and stored at −20 °C.

Nuclear Envelope Isolation. Nuclear envelopes were prepared according to the method of Bornens (1977). Nuclei or chromatin were suspended in 250 mM sucrose and 2 mM sodium phosphate, pH 7.85, to a DNA concentration of 250 μg/mL at +4 °C. Heparin (lithium salt, Sigma) was added slowly with stirring to give a DNA/heparin ratio of 1. The solution was stirred gently for 1 h and then centrifuged for 1 h at 50000g. The pellet, designated crude nuclear envelope, was taken up in 250 mM sucrose and 2 mM sodium phosphate, pH 7.5, layered over a 25–50% (w/v) sucrose gradient, and centifuged for 3.5 h at 170000g. The gradient was analyzed on a ISCO density gradient fractionator, Model 640, using a 280-nm filter. Material banding at 37% sucrose (d 1.18–1.20 g/mL) was diluted 5-fold with 2 mM sodium phosphate and pelleted by centrifuging at 50000g for 1 h. This preparation was taken as pure nuclear envelope. The mass of the nuclear envelope in the various experiments is given in micrograms of protein.

Isolation of Nonhistone Proteins. Purified chromatin was suspended in 10 mM Tris, pH 8.0, to a concentration of 0.5 mg of DNA per mL. H_2SO_4 (2 N) was added dropwise to a final concentration of 0.4 N. The precipitate was pelleted by centrifugation and washed with 10 mM Tris, pH 8. The supernatants containing the histones were discarded. The pellet was resuspended in 1% sodium dodecyl sulfate ($NaDodSO_4$) and 50 mM Tris-HCl, pH 8, by homogenization and stirred overnight at room temperature. The DNA was removed by centrifugation at 170000g for 24 h, and the supernatant containing the nonhistones was dialyzed against sample application buffer prior to $NaDodSO_4$ gel electrophoresis.

Isolation of the Activated Cytoplasmic Triamcinolone–Receptor Complex. The method of Climent et al. (1977) was followed. All operations were carried out at +4 °C. Principle buffers used were TSS [50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA), and 1 mM β-mercaptoethanol] and TGA [10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 1 mM β-mercaptoethanol, 100 mM NaCl, 10% glycerol, and 0.1% bovine serum albumin (BSA)]. Four male white rats were killed, and their livers were perfused with 50 mL of 0.25 M sucrose in TSS. Livers were homogenized in 3 volumes of 250 mM sucrose in TSS in a glass–Teflon Potter homogenizer. The homogenate was centrifuged at 300000g for 60 min in a Beckman 60 Ti-rotor. The supernatant was incubated with [1,2,4-^{3}H]triamcinolone acetonide (TA) at 5×10^{-8} M with a specific activity of 7.0 μCi/nmol for 30 min and passed over two phosphocellulose columns. Unbound radioactivity was collected and incubated at +20 °C for 30 min, cooled to 0 °C, and applied to a third phosphocellulose column. This column was washed with TGA buffer until the eluant was free of radioactivity. The NaCl concentration in the eluant was then raised to 500 mM and the receptor eluted from the column. Radioactive fractions were pooled and precipitated overnight with 0.5 volume of saturated ammonium sulfate adjusted to pH 7.0 with NH_4OH. After centrifugation at 50000g for 1 h, the precipitate was resuspended in about 300 μL of TGA buffer without NaCl, and an aliquot was checked for radioactivity. The receptor was dispensed in 10- or 20-μL quantities

Table I: Purification of Cytoplasmic Triamcinolone Acetonide-Receptor (8)

fraction	volume (mL)	total protein (mg)	total radioactivity (dpm)	dpm/mg of protein	purification	% yield (radioactivity)
cytosol	31	796.7	4.005×10^7	5.026×10^4	1	100
column 3 eluant (0.5 M NaCl)	16.5	0.0825	8.968×10^5	1.0870×10^7	216.25	2.23
ammonium sulfate precipitate	0.5	0.005	6.169×10^5	1.240×10^8	2466	1.53

into small plastic vials and stored in liquid nitrogen until used.

In several preparations, the triamcinolone–receptor complex had been purified between 2400-fold and 3000-fold (see Table I), similar to the results of Climent et al. (1977). The yield per isolation varied from 25 to 50 pmol of triamcinolone–receptor complex, assuming a 1:1 stoichiometry between hormone and receptor. Between 40% and 50% of this preparation was inactive—i.e., unable to bind to chromatin. Less than 3% of radioactivity was present as free steroid as determined by both dialysis against TSS buffer and gel exclusion chromatography on a Bio-Gel P2 column using TSS as eluant. After binding to nuclear components, 80% of the complex was extractable with 0.3 M KCl. The receptor sedimented in a sucrose gradient at 3 S with myoglobin, BSA, and ovalbumin as standards. This *s* value has been found typical for the activated complex prepared by the above method (Climent et al., 1977). The receptor was stable for at least 3 months when stored in liquid nitrogen. Repeated freezing and thawing, or heating to 25 °C for 1 h, inactivated the complex. On average, between 0.5 and 1.0 pmol of steroid receptor complex was used per incubation with either nuclear envelope or chromatin. For competition experiments, "unlabeled" receptor complex was prepared by the same methods by using triamcinolone acetonide with a specific activity of one-tenth, sufficient to monitor the purification steps.

Incubation of Nuclear Envelope, Nuclei, and Chromatin with Activated Cytoplasmic Hormone–Receptor Complex. All incubations were carried out in TGA buffer. Identical binding occurs when BSA is omitted from the buffer, indicating that the BSA neither promotes nor inhibits binding. Nuclear envelope and nuclear envelope fragments (120–150 μg of protein) or nuclei and chromatin (1.5–2.0 mg of DNA) were incubated with 0.5–1.0 pmol of triamcinolone–receptor complex (3–6 nCi) in a total volume of 300 μL of TGA for 1 h at +4 °C. The incubates were then layered over a 25–50% (w/v) sucrose gradient in TGA and centrifuged at +4 °C for 3.5 at 170000*g*. The gradients were analyzed on an ISCO density gradient fractionator with a 280-nm filter. Pellets were dissolved in 0.4 mL of 2% (w/v) $NaDodSO_4$ and checked for radioactivity. Where incubations took place in the presence of 0.3 M KCl, the gradient was also made 0.3 M KCl. Fractions of 0.4 mL were collected and assayed for radioactivity in a Beckman LS 250 liquid scintillation counter, using the following scintillator solution: 300 g of Triton X-100, 50 mL of 20% $NaDodSO_4$, and 5 g of 2,5 diphenyl-1,3-oxazole made up to 1 L with toluene.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$ (1% w/v) (sodium dodecyl sulfate) and 10% (w/v) acrylamide slab gels were prepared essentially according to the method of Panyim & Chalkley (1971). Gels were buffered with 50 mM glycine–NaOH, pH 10. The spacer gel was buffered with 50 mM Tris-HCl, pH 6.7. Envelope and chromatin samples were prepared for electrophoresis by incubation at +100 °C for 5 min in sample application buffer (10 mM Tris-HCl, pH 6.7, 2% $NaDodSO_4$, 4 M urea, and 1% β-mercaptoethanol). Gels were stained for protein with Coomassie Brilliant Blue R250 and destained by diffusion. The fractionation range of the gels was determined with molecular weight (M_r) markers in the range from 53000 to 265000 (BDH Biochemicals No. 44230).

Gel Staining with Fluorescein-Labeled Con-A. Electrophoresis gels were stained for carbohydrate by using fluorescein-labeled concanavalin A (Con-A). Con-A was isolated according to Agrawal & Goldstein (1967) and covalently coupled to fluorescein isothiocyanate (Mallucci, 1976). The electrophoresis gels were fixed overnight in 25% ethanol and 7% acetic acid, subsequently equilibrated in 0.1 NaCl and 0.01 M sodium phosphate, pH 7.5, and stained with a solution of fluorescein–Con-A (1 mg/mL) in the same buffer containing 5 mM Ca^{2+} and Mn^{2+} each for 3 h. Nonspecific binding was monitored by staining in the presence of 0.1 M of methyl α-D-glucopyranoside, a ligand specific for Con-A. Gels were destained by diffusion until the background was free of fluorescence. Gels were scanned for fluorescence on a Vitatron TLD 100 densitometer with Hanau ST 41 mercury lamp as source. In our hands, this procedure detects approximately 0.5 μg of standard glycoprotein (horseradish peroxidase). Fluorescein–Con-A stained gels were photographed over a short-wave (mainly 254 nm) transilluminator (UV Products) through a Wratten type 61 filter.

Incubations with DNase. DNase I, EC 3.1.4.5 (Merck, 200 U/mg), was incubated with nuclear envelope for various lengths of time at +4 and +25 °C in 10 mM sodium phosphate, pH 7.5. The nuclear envelope was then pelleted by centrifugation and the supernatant assayed for DNA. Protein was determined according to Lowry (Lowry et al., 1951) and DNA according to Burton (Burton, 1956).

Preparation of Plasma Membrane. Plasma membrane was purified essentially as described by Aronson & Touster (1974). Briefly, white female rats were starved overnight and killed by cervical dislocation. Livers were perfused with 50 mL of 0.25 M sucrose and 5 mM Tris, pH 8.0, excised, trimmed, and homogenized in 3 volumes of perfusion buffer in a Teflon–glass Potter homogenizer. The homogenate was passed through 4 layers of cheesecloth and then spun at 1000*g* for 10 min. The pellet was rehomogenized twice in 0.25 M sucrose and 5 mM Tris, pH 8.0, and repelleted. Supernatants were pooled and pellets saved.

The supernatant was spun at 33000*g* for 7.5 min. The resultant pellet was rehomogenized twice and the step repeated. Supernatants were pooled, and the pellet was discarded. The supernatant was spun at 78000*g* for 100 min. The pellet from this spin was homogenized with 2.5 volumes of 57% sucrose and 5 mM Tris, pH 8.0. Twelve milliliters of the homogenate was placed in a SW25.1 rotor tube and overlayed with 15 mL of 34% sucrose and 5 mM Tris, pH 8.0. The rest of the volume was made up with 0.25 M sucrose and 5 mM Tris, pH 8.0. Tubes were centrifuged at 100000*g* for 3 h. The plasma membrane was obtained at the 0.25 M/34% sucrose interface.

The nuclear pellets obtained after the first 1000*g* spin were processed exactly as the supernatants except that the 78000*g* spin was omitted and, in the final centrifugation, 37.2% sucrose rather than 34% was used. The plasma membrane was collected at the 0.25 M/37.2% sucrose interface.

Preparation of Rough and Smooth Microsomes. Rough and smooth microsomal fractions were produced essentially as described by Adelman et al. (1974). Briefly, rats were

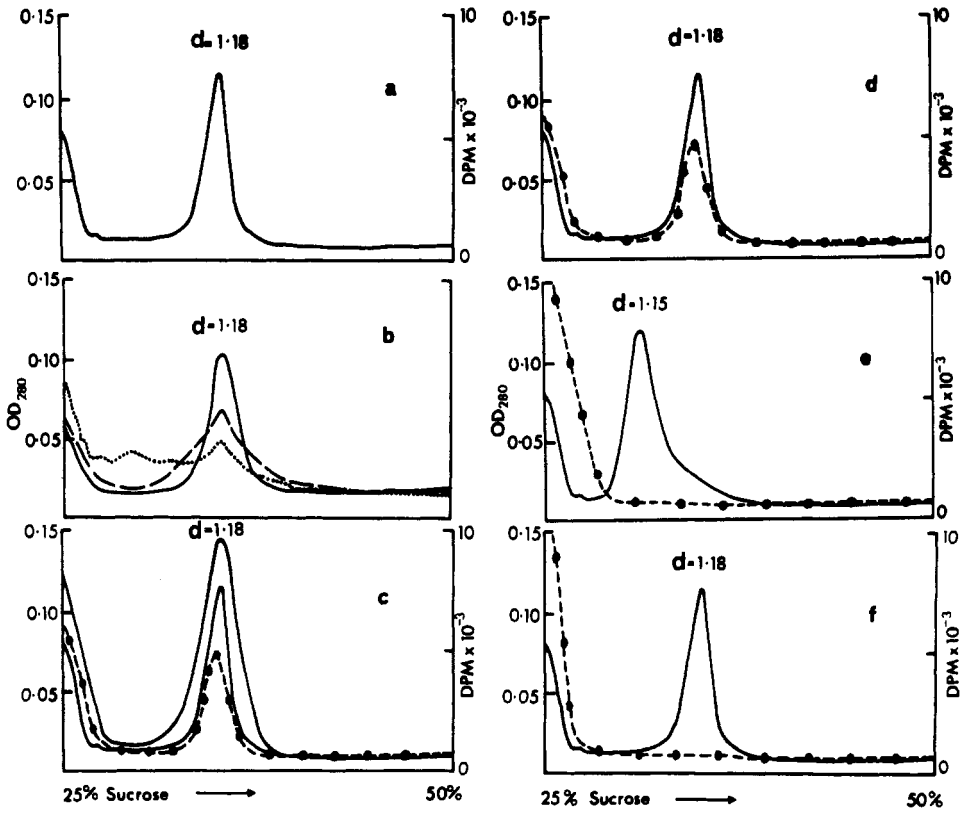

FIGURE 1: Sedimentation diagrams of nuclear envelope under a variety of conditions, in a 25%–50% sucrose gradient in TGA buffer. Centrifugation was for 3.5 h at 170000*g*. (a) Purified nuclear envelope (—) (OD_{280}). (b) Nuclear envelope incubated with DNase. (—) OD_{280} incubated with DNase for 30 min at +4 °C; (---) OD_{280} incubated with DNase for 1 h at +4 °C; (···) OD_{280} incubated with DNase for 30 min at +25 °C. (c) Nuclear envelope incubated with hormone–receptor complex. Increasing amounts of nuclear envelope were incubated with a constant amount of cytoplasmic hormone–receptor complex for 1 h at +4 °C prior to centfiguation in the absence of KCl. (—) OD_{280}; (---) dpm. (d) Incubation of nuclear envelope and hormone–receptor complex in TGA containing 0.15 M KCl. Conditions as in (c) prior to centrifugation. The gradient contains 0.15 M KCl. (—) OD_{280}; (---) dpm. (e) Incubation of nuclear envelope and hormone–receptor complex in TGA containing 0.3 M KCl. Conditions as in (c) prior to centrifugation. The gradient contains 0.3 M KCl. (—) OD_{280}; (---) dpm. (f) Incubation of nuclear envelope with hormone–receptor complex. Repeatedly frozen and thawed cytoplasmic hormone–receptor complex has been incubated as in (c) prior to centrifugation. (—) OD_{280}; (---) dpm.

killed, and livers were perfused and homogenized as for plasma membrane preparation except that perfusion was with unbuffered 0.25 M sucrose and homogenization was in 2 volumes of 1.0 M sucrose. The homogenate was strained through 1 layer of cheesecloth, and an equal volume of 2.5 M sucrose was added and mixed well. The homogenate was transferred to 60 Ti-rotor tubes, overlayed with 1 M sucrose, and spun at 100000*g* for 45 min. The supernatant was removed, and 0.5 volume of water was added during homogenization. The homogenate was then spun at 22000*g* for 15 min. The supernatant was decanted and saved, and the pellets were suspended in 25 mL of 0.5 M sucrose and centrifuged for 15 min at 20000*g*. The supernatant was pooled with the previously saved supernatants and spun for 15 min at 20000*g*. Supernatants were decanted and stored and the pellets discarded. The supernatant was transferred to centrifuge tubes, underlaid with 4 mL of 1.5 sucrose and 1 mL of 2.0 M sucrose, both in 50 mM Tris, pH 7.5, 25 mM KCl, and 5 mM $MgCl_2$, and spun for 20 h at 200000*g*. The smooth microsomal fraction was obtained at the homogenate supernatant/1.5 M interface and rough microsomal fraction at the 1.5 M/2.0 M sucrose interface. Each fraction was finally purified by centrifugation on a 25–50% linear sucrose gradient. Peaks were pooled and pelleted.

Results

Treatment of nuclei with heparin results in almost immediate solubilization of chromatin and release of nuclear envelopes. The latter band has a single peak at its characteristic buoyant density of 1.18 (Kashnig & Kasper, 1969) in a 25–50% sucrose gradient (Figure 1a). We found between 0.5% and 1% of the total nuclear protein in this fraction. The composition of the protein component of the nuclear envelope as revealed by $NaDodSO_4$ gel electrophoresis is very similar to that reported by other authors (Jackson, 1976; Bornens & Kasper, 1973; Aaronson & Blobel, 1975). In particular, the characteristic high molecular weight band (M_r ~170000) and the triplet at M_r ~55000were both present. The preparation is slightly contaminated by low molecular weight material moving with the mobility of histones (Figure 2Aa). A large number of the electrophoretically separated polypeptide chains do bind fluorescein-labeled Con-A. This interaction between the lectin and the protein fractions can be inhibited by methyl α-D-glucopyranoside, one of the ligands of the lectin, thus characterizing the proteins as glycoproteins. This is true in particular for the M_r 170000 band and the triplet at M_r 55000. The pattern of both Coomassie and Con-A binding proved to be constant from batch to batch of nuclear envelope preparations (Figure 2Aa). We find the protein/DNA ratio in the envelope to be 12:1; a protein content for the envelope has been reported between 60% and 65% (Harris, 1978). On the basis of this, our nuclear envelope preparations contains 5% DNA by weight. This is in good agreement with values obtained by other authors (Harris, 1978; Franke, 1974). It is well established that the morphological integrity of the nuclear envelope depends on the presence of a small amount of bound DNA (Agutter, 1972; Peterson & Berns, 1978). This is borne out by the result presented in Figure 1b. Incubation of nuclear

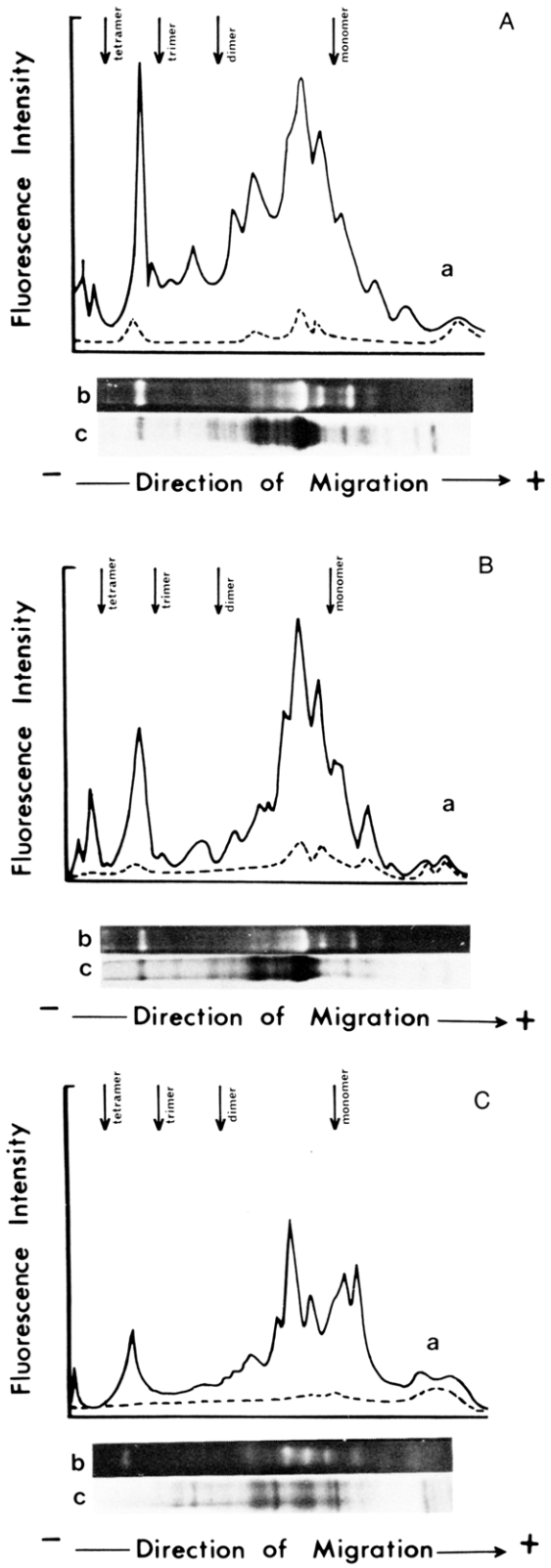

FIGURE 2: $NaDodSO_4$ gel electrophoresis of nuclear envelope (A), nuclear envelope fragments (B), and nonhistone proteins (C). Gels were stained for protein with coomassie brillant blue, and glycoproteins were visualized by using fluorescein-labeled Con-A. Nonspecific binding of Con-A was monitored by staining in the presence of 0.1 M methyl α-D-mannoside. The monomer of the molecular weight marker protein has a molecular weight of 53000 daltons (BDH product No. 44230). (a) Scans of the fluorescent gels; (—) binding in the absence of methyl α-D-mannoside; (---) binding in the presence of 0.1 M methyl α-D-mannoside. (b) Photograph of fluorescent gels. (c) Protein stain of gels.

Table II: Competition of Radioactive and Nonradioactive Cytoplasmic TA-Receptor Complex for Nuclear Envelope Sites

input radioactive cytoplasmic TA-receptor (dpm)	TA-receptor bound to envelope (dpm)		
	no pretreatment with competitor	pretreatment with competitor[a]	
		6.6 nM	9 nM
2950	710	352	150
5670	1420	948	740
9212	3015	1503	1260

[a] Nuclear envelopes were preincubated at 6.6 and 9 nM concentration of unlabeled TA-receptor for 60 min, pelleted, washed, and reincubated with radioactive TA-receptor (see Materials and Methods). Input radioactivity represents true cytoplasmic TA-receptor activity (see also legend to Figure 3).

envelope with DNase I releases only a fraction of that DNA after 30-min incubation at +4 °C. Longer incubation with DNase I at +4 °C or incubation at +25 °C for 30 min results in significant disintegration of the envelope and release of protein and DNA into solution. Such DNase-treated envelope no longer sediments at *d* 1.18 on sucrose gradient centrifugation but tends to form a broad zone near the top of the gradient.

On incubation of a given amount of nuclear envelope with TA-receptor complex, up to 50% of the radioactivity is found associated with the nuclear envelope (Figure 1c). Forty percent of the radioactivity remains at the top of the gradient. It is not possible to mop up this unbound radioactivity by increasing the amount of nuclear envelope (Figure 1c). A small pellet representing about 10% of the radioactivity is usually obtained, representing probably aggregated cytoplasmic receptor. These results show that 50% of the radioactivity present in the TA-receptor preparation represent active cytoplasmic steroid hormone receptor complex. Exposure of the envelope-bound cytoplasmic receptor to 0.3 M KCl in a sucrose gradient in order to remove the bound radioactive cytoplasmic hormone receptor from the envelope results in a complete dissociation of the radioactivity from the envelope and a structural change of the latter witnessed by the sedimentation at the lower density of *d* 1.14–1.15 (Figure 1d,e). Neither the TA-receptor complex, which has been inactivated by repeated freezing and thawing (Figure 1f), nor the triamcinolone acetonide (results not shown) binds to nuclear envelope. The binding of radioactivity is thus a protein-mediated process.

Binding sites for the TA receptor on the nuclear envelope are saturable. Increasing the amount of TA receptor for a fixed amount of nuclear envelope leads to increased binding until saturation is reached at 0.015 pmol of TA receptor per μg of membrane protein (Figure 3a). The shape of the saturation curve indicated more than one binding site. The Scatchard plot (Figure 3b) reveals the presence of two high affinity sites in the nuclear envelope for cytoplasmic steroid hormone–receptor complex with an equilibrium constant in the order of 10^{-9} or 10^{-10} M. More than one nuclear acceptor site for cytoplasmic steroid hormone–receptor complex has been revealed by others for progesterone in oviduct cells (Spelsberg et al., 1976). The retention of radioactivity by the nuclear envelope after exposure to highly labeled cytoplasmic TA-receptor complex is cytoplasmic receptor mediated as shown by the experiment recorded in Table II. Unlabeled cytoplasmic TA-receptor complex purified by means identical with those used for the highly labeled TA-receptor complex competes with the latter for binding sites on the nuclear envelope. The binding of the cytoplasmic TA receptor by other membranes of the liver cell is considerably less. The plasma

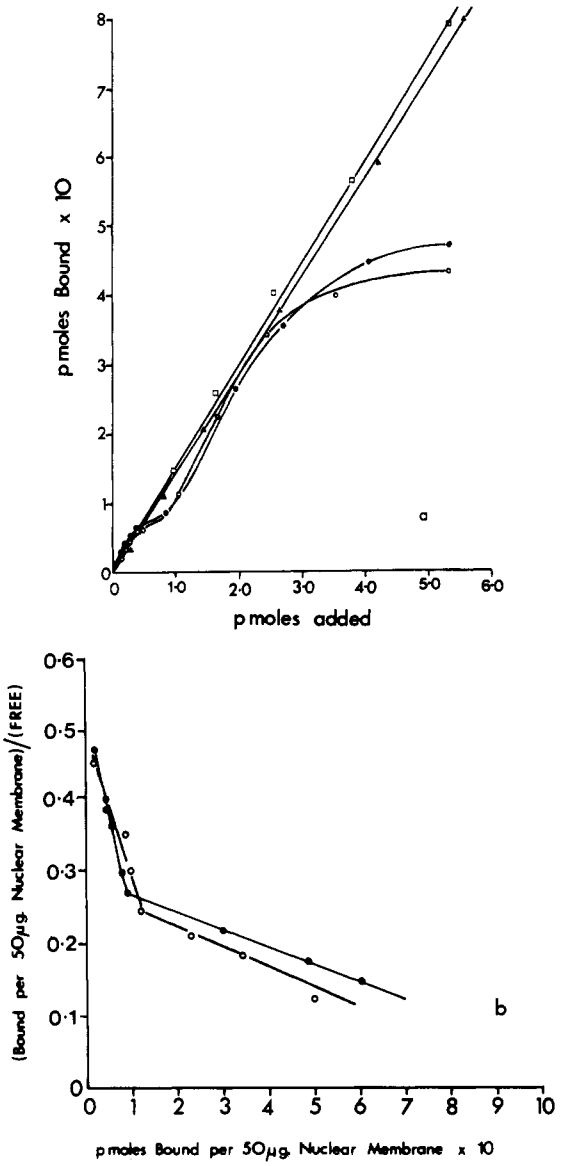

FIGURE 3: (a) Saturation plot of hormone–receptor binding to a constant amount of nuclear envelope. Prior to incubation with nuclear envelope, aggregated material was removed from the hormone–receptor complex by centrifugation (100000*g* for 1 h); in this supernatant, the concentration of the cytoplasmic TA–receptor complex was determined by titrating against an excess of nuclear envelope (see also legend to Figure 1c). The input of cytoplasmic receptor complex recorded in the abscissa represents true receptor concentration. Incubation took place in 300 μL of TGA buffer for 1 h at +4 °C after which the nuclear envelope hormone–receptor complex was pelleted by centrifugation. The supernatant was carefully removed and the pellet washed with 100 μL of 0.3 M KCl, which was assayed for radioactivity. Aggregation of the hormone–receptor complex during the experiment was monitored by using blanks with appropriate cytoplasmic receptor concentrations which did not contain nuclear envelope. Nonspecific hormone receptor aggregation was found to be between 10% and 20%. (□) Chromatin; (△) nuclei; (●) nuclear envelope; (○) nuclear envelope fragments isolated from chromatin. (b) Scatchard analysis of the data in Figure 2a. (●) Nuclear envelope; (○) nuclear envelope fragments isolated from chromatin.

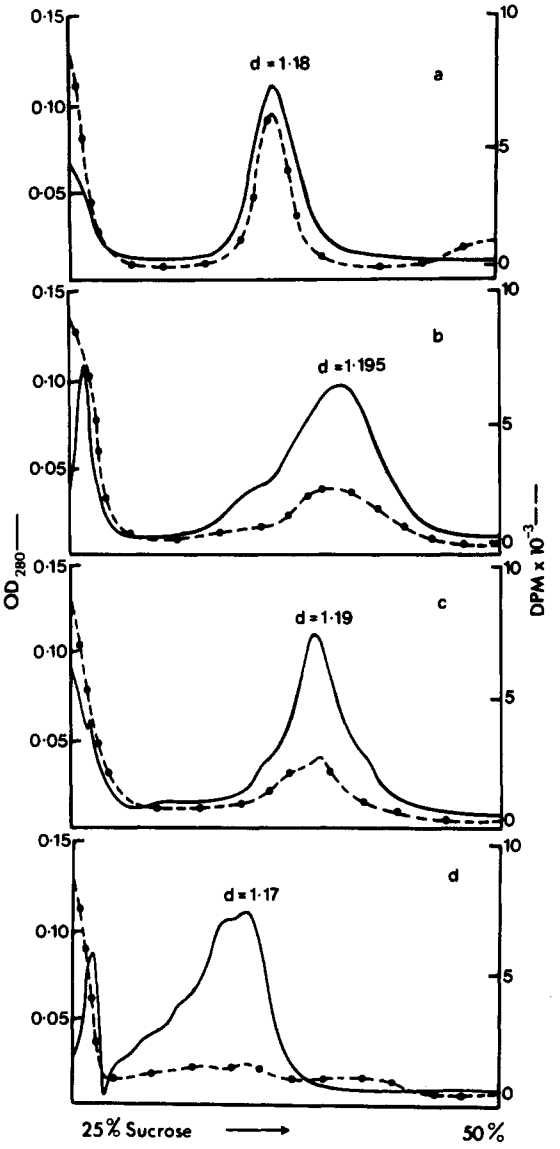

FIGURE 4: Sedimentation diagrams of nuclear, microsomal, and plasma membranes in a 25%–50% sucrose gradient in TGA buffer after incubation with hormone–receptor complex for 1 h at 4 °C. The amount of membrane in each incubation was identical with respect to protein. Centrifugation was for 3.5 h at 170000*g*. (a) Nuclear envelope. (b) Rough endoplasmic reticulum. (c) Smooth endoplasmic reticulum. (d) Plasma membrane.

Table III: Binding of Cytoplasmic [$^3H_3$]Triamcinolone–Receptor Complex[a] to Nuclear, Endoplasmic, and Plasma Membranes

	dpm/100 μg of protein	μg of DNA/100 μg of protein
nuclear envelope	6430	8.5
rough endoplasmic reticulum	2140	0.53
smooth endoplasmic reticulum	2064	1.25
plasma membrane	542	1.15

[a] Radioactivity bound by membranes was assayed after density centrifugation as in Figure 1.

membrane exhibits less than 10% and the two endoplasmic membrane types less than 30% binding if compared to the nuclear envelope under identical conditions (Figure 4 and Table III). The three nonnuclear membrane preparations are slightly contaminated with nuclear material (Table III). The two endoplasmic membranes and the plasma membrane sediment at sucrose densities distinctly different from that of the nuclear envelope. The nuclear envelope preparation on density gradient centrifugation appears to be free from any significant contamination by the other membranes or radioactivity bound by the latter.

The activated glucocorticoid receptor binds to intact nuclei as well as to chromatin (Figure 3) as reported previously by others (Milgrom et al., 1976). However, within the concentration range tested, it appears that these binding sites are entirely different from those in the isolated nuclear envelope.

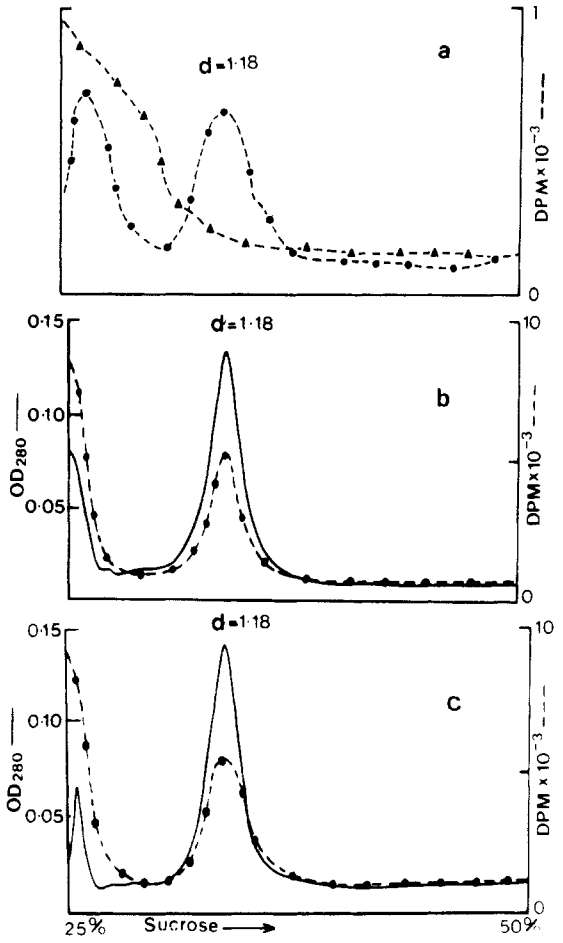

FIGURE 5: Sedimentation diagrams: (a) Nuclear envelopes isolated from nuclei preincubated with TA receptor. TA–receptor input 25 000 dpm; nuclear pellet 7700 dpm; envelope pellet after heparin treatment 2500 dpm; gradient purified nuclear envelopes 1500 dpm (●) sucrose gradient in 100 mM KCl and (▲) sucrose gradient in 300 mM KCl. (b) Nuclear envelope fragments isolated from chromatin. The envelope fragments were incubated with cytoplasmic steroid–receptor complex prior to gradient centrifugation. (c) Nuclear envelopes treated as in (b). All gradient centrifugations were done in 25–50% sucrose gradient in TGA buffer at 170000*g* for 3.5 h. The amounts of envelope fragments or envelopes were identical with respect to protein in each experiment. The incubation with TA receptor was for 1 h at +4 °C.

Whereas the latter are saturable sites, the former belong to the nonsaturable type (Figure 3a). This is in accordance with the well-established affinity of the activated glucocorticoid receptor to DNA or other polyanionic matrices which forms the basis of a number of purification methods for the receptor (Climent et al., 1977; Wrange et al., 1979). Nevertheless, the binding of the glucocorticoid receptor to the specific nuclear envelope sites does take place in the intact nuclei in spite of the presence of the large excess of polyanionic competitor in the form of DNA. Nuclear envelopes prepared from nuclei which had been pretreated with labeled cytoplasmic glucocorticoid receptor become labeled with the receptor (Figure 5a). Approximately 20% of the glucocorticoid receptor pelleted with the nuclei can be recovered in the nuclear envelope isolated from these nuclei. The labeled receptor dissociates typically from the envelopes at elevated ionic strength (Figure 5a). Chromatin contains in addition to the nonsaturable sites saturable acceptor sites for cytoplasmic receptor. Their presence is apparently masked by the large excess of polyanionic nonspecific binding sites. This becomes evident when chromatin is used as starting material for the isolation of nuclear envelopes. On gradient centrifugation, these membrane fragments from chromatin have a density identical with that of nuclear envelopes (Figure 5b,c), and the radioactive hormone–receptor complex cosediments with it. The protein components in the envelope fragments as assessed by $NaDodSO_4$ gel electrophoresis appear to consist of polypeptide chains with molecular weights identical with those of the nuclear envelope. In particular, the fluorescein–Con-A binding appears to be virtually identical (Figure 2Aa,Bb). The DNA/protein ratio of 12:1 is also identical with that found before for the nuclear envelope. These nuclear envelope fragments isolated from chromatin bind activated cytoplasmic glucocorticoid receptor in a fashion identical with that of nuclear envelope with virtually the same equilibrium constant (Figure 3a,b). The presence of nuclear envelope fragments in chromatin explains the similarity of the polypeptide pattern between nuclear envelope and the nonhistone fractions as assessed by $NaDodSO_4$ gel electrophoresis and concanavalin A binding of the polypeptide chains (Figure 2Ca).

Discussion

The experiments reported show that the nuclear envelope binds activated cytoplasmic steroid hormone receptor with a specific activity 10 times higher than the plasma membrane and more than 3 times higher than the two endoplasmic type of membranes (Table II).

The nuclear envelope prepared by the heparin method comprises a two-leaflet structure (Bornens, 1977) representing the outer and inner membrane of the nucleus. Our results provide evidence that this nuclear envelope contains saturable high affinity acceptor sites for a cytoplasmic steroid hormone–receptor complex which become labeled when intact nuclei interact in vitro with the activated cytoplasmic glucocorticoid–receptor complex. Furthermore, they show that such high affinity acceptor sites for cytoplasmic steroid–receptor complexes are also present in sucrose gradient purified chromatin as a contaminant of the latter with nuclear envelope fragments (Figures 2 and 5) and that, in confirmation of the report by Jackson (Jackson, 1976), a large number of the polypeptide chains present in the chromatin nonhistones result from this contamination of chromatin (Figure 2).

The property of the nuclear envelope to bind cytoplasmic steroid hormone receptor may explain why the nuclear matrix has been reported to retain labeled steroid hormone after injection into rats (Barrack et al., 1977). The nuclear matrix consists of a large number not only of intranuclear chromosomal and nucleolosomal proteins but also to a considerable extent of residual nuclear envelope proteins insoluble under the conditions of matrix isolation (Berezney & Coffey, 1976). Similarly, the binding of cytoplasmic steroid hormone receptor by nonhistone (O'Malley et al., 1977) is probably partly due to the high degree of contamination of chromatin with nuclear envelope proteins found in our experiments and also reported earlier (Jackson, 1976).

The equilibrium constants in the order of 10^{-10} and 10^{-9} M for the interaction between the cytoplasmic steroid receptor and the envelope acceptor show that, given the low intracellular hormone concentration, the nuclear envelope sites have those high affinity properties necessary for playing a biologically significant role in cytoplasmic–nuclear interaction. Calculated on the basis of 8.3 pg of DNA per rat cell nucleus (Altman & Dittmer, 1964), our experiments reveal an average of 18 000 binding sites in the envelope per nucleus. The figure is of a similar order as those determined by Webster (Webster et al., 1976), who found 6000–10 000 sites per nucleus for progesterone receptor in avian oviduct cells, Beato (Beato et al., 1974), who determined 15 000 sites per nucleus for dexamethasone in rat liver, and Bugany (Bugany & Beato, 1977),

who found 5000–10000 sites per haploid genome in rat liver. The possibility that binding of the receptor complex to the nuclear envelope may have been caused by the 5% DNA in the membrane can be discarded on two grounds. The binding characteristics of DNA as revealed by the binding of glucocorticoid receptor to unfractionated nuclei and chromatin are entirely different from those of the nuclear envelope (Figure 3a). Furthermore, the binding of steroid hormone receptor complexes by DNA is considerably diminished already at 0.1 M salt (Rousseau et al., 1975; Milgrom et al., 1976), the concentration of sodium chloride in the binding test (see Materials and Methods). That binding in our experiments does not decrease at 0.15 M NaCl (Figure 1d) but is only abolished at higher salt concentration indicates that the nuclear envelope represents a nuclear subfraction enriched in specific, high affinity acceptor sites other than DNA for cytoplasmic steroid hormone–receptor complex.

The nonhistone protein fraction of chromatin is most frequently cited as containing the nuclear acceptor sites for steroid hormone receptors [for review, see O'Malley et al. (1977)]. However, in contrast to the nuclear envelope, which is a reasonably well-defined subfraction of the nucleus (Harris, 1978; Franke et al., 1976), the nonhistone fraction of chromatin may be expected to vary considerably, depending on the method chosen for either the chromatin or the subsequent nonhistone isolation. Most authors include a density gradient centrifugation step in the preparation procedure for chromatin which serves as starting material for the isolation of nonhistones. This step is designed to remove the nuclear envelope. Our results show, however, that centrifugation through 1.7 M sucrose used to isolate the chromatin preparation leaves a considerable amount of nuclear envelope fragments in the chromatin. Also, a nonionic detergent such as Triton X-100 has been used by others to remove the outer nuclear membrane. As the outer nuclear membrane is no longer visible by electron microscopy after such a Triton treatment, and over 80% of phospholipid has been removed (Frederiks et al., 1978), the outer membrane is generally assumed to be solubilized in toto. However, it has been demonstrated that the nuclear pore complex remains associated with the nucleus after Triton X-100 treatment (Aaronson & Blobel, 1975) and that 1% Triton X-100, though removing virtually all of the phospholipids, removes only a small amount of nuclear envelope protein (Jackson, 1976). The single layer residual nuclear envelope with readily visible nuclear pores seen by electron microscopy (Berezny & Coffey, 1976) after Triton treatment of nuclei thus represents a collapsed bileaflet membrane, and the assumption that Triton X-100 treatment removes the outer leaflet in toto is not justified.

Part of the concanavalin A binding sites shown to be present in the nuclear membrane (Monneron & Segretain, 1974) are due to glycoproteins which are well-established components of the nuclear envelope [for review, see Franke et al. (1976)]. A number of authors (Stein et al., 1975; Rizzo & Bustin, 1977; Goldberg et al., 1978) have described the occurrence of glycoproteins in chromatin even after rigorous treatment with Triton. Though identity of mobility in $NaDodSO_4$ gels only indicates similar molecular weights and the binding of concanavalin A only proves the presence of a ligand within the limits of the specificity of the lectin, the striking similarity of the glycoprotein pattern revealed by the uptake of fluorescein–concanavalin A after $NaDodSO_4$ gel electrophoresis (Figure 2) for nuclear envelopes, nuclear envelope fragments isolated from purified chromatin, and nonhistones argues strongly that most of the glycoproteins in chromatin derive from envelope contamination.

The binding of the cytoplasmic steroid hormone–receptor complex to the nuclear envelope established in the experiments together with findings from other laboratories on the specific binding of insulin (Vigneri et al., 1978; Horvat, 1978) and also of neural growth factor (Yanker & Shooter, 1979) to the nuclear envelope reveals that the nuclear envelope may be the target for specific cytoplasmic hormonal signals which are known to lead to increased nuclear activity. The nuclear envelope may thus be considered as the first relay station in such cytoplasmic nuclear communications.

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Phase Behavior of Ether Lipids from *Clostridium butyricum*†

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ABSTRACT: Ether lipids have been isolated from the phospholipid fraction of *Clostridium butyricum* IFO 3852 cells which had been grown in media devoid of biotin with added elaidic acid or oleic acid. The plasmalogen form of phosphatidylethanolamine (plasmenylethanolamine) from elaidate-grown cells was highly enriched with 18:1 in both the alk-1-enyl and acyl chains. A transition from the gel to liquid-crystalline state, with a peak maximum (T_m) at 33 °C and enthaply ΔH = 5.7 kcal/mol, was observed by differential scanning calorimetry. With the fluorescent probes *cis*- and *trans*-parinaric acids, transitions were observed at 33 °C on heating and at 29 °C on cooling. These transition temperatures are 5-6 °C lower than those reported for the corresponding diacyl lipid, dielaidoylphosphatidylethanolamine. A similar study of the phase behavior of both the elaidate-enriched and oleate-enriched glycerol acetal derivative of plasmenylethanolamine from *C. butyricum* revealed a large hysteresis of 12.5-16 °C. Hysteresis in the polar head group motion was also observed by ^{31}P nuclear magnetic resonance. The elaidate-enriched lipid, which melted between 28 and 33 °C, appears to undergo supercooling prior to the transition to the gel state at about 18-13 °C, depending on the scanning rate. The formation of a more ordered gel state relative to plasmenylethanolamine was indicated by a 2-fold increase in ΔH. Electron microscopy revealed a marked reorganization from typical multilamellar liposomes above T_m to large needle-like structures below T_m. The oleate-enriched glycerol acetal lipid formed the gel phase at −4 °C, which is 10 °C above the transition temperature reported for dioleoylphosphatidylethanolamine. Stabilization of oleate-enriched glycerol acetal lipid bilayers may result from hydrogen bonding between polar head groups. The relationship of the phase behavior of the ether lipids to the lipid composition and phase behavior of *C. butyricum* membranes is discussed.

Plasmalogens, 1-(*O*-alk-1′-enyl)-2-acylglycerophospholipids, are major lipids of the membranes of many anaerobic bacteria including Gram-positive and Gram-negative organisms belonging to a variety of genera (Kamio et al., 1969; Goldfine & Hagen, 1972; Hagen, 1974; Clarke et al., 1976; vanGolde et al., 1973, 1975). The presence of these unsaturated ether lipids in animal tissues has been recognized for many years (Debuch & Seng, 1972), and in certain tissues, such as brain white matter and heart, they represent >30% of the total phosphoglycerides (Horrocks, 1972).

Although some work has been reported on the physical behavior of plasmalogen monolayers (Shah & Schulman, 1965), there have been no detailed studies on the phase behavior of these lipids. Presumably the difficulties encountered in the chemical synthesis of plasmalogens with the natural

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