temperature in the 0.2 M sodium acetate buffered solutions. The binding enthalpies and constants at the denaturation temperature can be calculated from the binding constants, the binding enthalpies, and the heat capacity change of the binding reaction determined at room temperature. This appears to be true for the interaction at the lower ionic strength of 0.05, although measurements of the binding constant as a function of pH at 298.2 K and ΔC_{pb} as a function of pH are needed to verify this. Although the binding constants of uridine 3'-monophosphate to ribonuclease *a* are close to those of the cytidine inhibitor at the denaturation temperature and at room temperature, it is difficult to conclude that the interaction of the uridine inhibitor at the denaturation temperature is the same as at room temperature since the binding enthalpies and the heat capacity change accompanying this interaction are unavailable in the literature. Isomerization of the ribonuclease *a*-inhibitor complex following an initial association interaction between the inhibitor and the enzyme has not been observed in the DSC cooperativity determinations. Any enthalpy change resulting from the isomerization may be too small to be detected by the DSC.

Registry No. Cytidine 3'-monophosphate, 84-52-6; uridine 3'-monophosphate, 84-53-7; ribonuclease, 9001-99-4.

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Inhibition of Glucocorticoid Receptor Transformation, Subunit Dissociation, and Temperature-Dependent Inactivation by Various N-Substituted Maleimides†

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ABSTRACT: A series of N-substituted maleimides were synthesized, and their effect on the activation to the DNA binding state of the rat liver glucocorticoid receptor was studied. Unactivated (preincubated at 0 °C) cytosolic [^{3}H]triamcinolone acetonide–receptor complexes were pretreated with various *N*-alkylmaleimides at 0 °C and then heated at 25 °C and assayed for DNA–cellulose binding. No inhibition of the DNA binding activity was observed with either *N*-ethylmaleimide or N-substituted maleimides bearing an ionizable substituent, like *N*-(ω-carboxyalkyl)maleimides and *N*-[2-(trimethylammonio)ethyl]maleimide. On the contrary, treatment with long-chain alkylmaleimides like *N*-heptylmaleimide resulted in significant inhibition. The highest inhibition was obtained with *N*-benzylmaleimide and, to a lesser extent, *N*-(ethylphenyl)-maleimide, whereas *N*-benzylsuccinimide was ineffective. Treatment of cytosol containing unactivated glucocorticoid complexes at 3 °C with *N*-benzylmaleimide also prevents the temperature-mediated conversion of 8S receptor to 4S. Moreover, *N*-benzylmaleimide was able to inhibit the inactivation of the receptor steroid-binding activity caused by heat. *N*-Benzylmaleimide shares with molybdate ions the ability to inhibit glucocorticoid receptor activation, dissociation, and inactivation. However, their respective mechanisms of action are probably distinct, since their effects on receptor inactivation appear additive. It is suggested from the comparison of the various maleimides tested that the sulfhydryl groups essential for receptor activation and dissociation lie in a rather nonpolar environment including aromatic amino acid(s).

Sulhydryl groups are involved in two very important functions of the glucocorticoid receptor, i.e., steroid binding and subsequent acquisition of DNA binding activity by the steroid–receptor complex (Rousseau, 1984). Treatment of unliganded receptors with sulfhydryl-modifying reagents prevents subsequent binding of glucocorticoid (Rees & Bell, 1975; Young et al., 1975). Moreover, the same reagents, when added to preformed steroid–receptor complexes, impede their

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transformation[1] to the DNA binding state and/or their binding to DNA (Young et al., 1975; Tienrungroj et al., 1987). Substantial evidence that the sulfhydryl groups required for steroid binding are different from the groups required for DNA binding was provided by Bodwell et al. (1984b). In a previous paper we reported the results obtained with various *N*-alkylmaleimides used as reporter groups to study the environment of the sulfhydryl residue(s) essential to the steroid binding (Formstecher et al., 1984). These results suggest that these groups lie in a hydrophobic environment, probably in the steroid binding site itself. In the present work, the same approach was applied to the characterization of the sulhydryl group implied in the transformation step. Among the various N-substituted maleimides tested, the ones displaying the most extensive inhibition of transformation were also assayed for their ability to impede the temperature-dependent subunit dissociation of the receptor complexes.

Materials and Methods

Chemicals. [1,2,4(n)-^{3}H]Triamcinolone acetonide,[2] 40 Ci·mmol^{-1}, was obtained from Amersham International (Amersham, U.K.) and unlabeled triamcinolone acetonide from Serva (Heidelberg, FRG). *N*-Ethylmaleimide was purchased from Pierce Chemical Co. (Rockford, IL); maleimide and *N*,*N*-dimethylethylenediamine were purchased from Fluka (Buchs, Switzerland). Utrogel ACA 202 and IBF 10 columns were from Industrie Biologique Française (Villeneuve la-Garenne, France), and DNA–cellulose and *N*-(4-carboxy-3-hydroxyphenyl)maleimide were from Sigma Chemicals (St. Louis, MO). *N*-Ethyl-9α-fluoro-16α-methyl-11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxamide (DXE) was synthesized as described (Formstecher et al., 1980). Other chemicals were of commercial grade.

Synthesis of N-Substituted Maleimides and Succinimides. *N*-Propyl- to *N*-octylmaleimides were prepared from maleic anhydride and the appropriate amine through a two-step procedure according to the method of Heitz et al. (1968), whereas *N*-arylmaleimides, i.e., *N*-phenyl-, *N*-benzyl-, and *N*-(ethylphenyl)maleimides, were obtained by the two-step procedure of Cava et al. (1961). *N*-[2-(Dimethylamino)-ethyl]maleamic acid was prepared by condensation of *N*,*N*-dimethylethylenediamine with maleic anhydride (Cava et al., 1961). Cyclization was then performed by warming in the presence of acetic anhydride and anhydrous sodium acetate and the *N*-[2-(dimethylamino)ethyl]maleimide formed was recrystallized in the presence of methyl iodide as *N*-[2-(trimethylammonio)ethyl]maleimide iodide (El-Merzabani & Sakurai, 1972).

N-(ω-Carboxyalkyl)maleimides were prepared by the two-step procedure of Keller and Rudinger (1975). Briefly, maleimide was derivatized with methyl chloroformate in the presence of *N*-methylmorpholine at 0 °C to yield *N*-(methoxycarbonyl)maleimide, which was recrystallized in ethyl acetate/diisopropyl ether. *N*-(Methoxycarbonyl)maleimide was then coupled to glycine, 4-aminobutyric acid, or 6-aminohexanoic acid to yield *N*-(carboxymethyl)maleimide, *N*-(3-carboxypropyl)maleimide or *N*-(5-carboxypentyl)maleimide, respectively, which were thereafter extracted and purified on a silica column.

Synthesis of *N*-benzylsuccinimide was performed according to the method of Rice et al. (1954). Analytical controls included thin-layer chromatography on plates precoated with silica gel F254 (0.25 mm) (Merck, Darmstadt, FRG). Several solvent systems were used: diisopropyl ether/cyclohexane (50:50 v/v) for *N*-alkyl- and *N*-arylmaleimide; chloroform/acetic acid (95:5 v/v) and chloroform/methanol (50:50 v/v) for *N*-(methoxycarbonyl)maleimide and *N*-(ω-carboxyalkyl)maleimides; methanol/chloroform/glacial acetic acid (60:50:50 v/v) for *N*-[2-(dimethylamino)ethyl]maleimide and *N*-[2-(trimethylammonio)ethyl]maleimide iodide. Maleimide spots were detected on silica plates by using a specific spray reagent (Keller & Rudinger, 1975). The purified compounds were characterized by both ultraviolet and infrared spectrophotometry using a Perkin-Elmer 551S UV spectrophotometer and a Beckman Acculab 1 infrared spectrophotometer.

Cytosol Preparation. Adrenalectomized male Wistar rats (250-g body weight) were killed by cervical dislocation. The livers were removed and perfused with ice-cold TKGE buffer (pH 7.4) (20 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, and 10% glycerol). The tissue was then blotted free of excess buffer, weighed, and homogenized (1.5 mL of buffer/g of tissue) in TKGED buffer (TKGE buffer supplemented with 0.5 mM dithiothreitol and 0.2 mM phenylmethanesulfonyl fluoride) by use of a Teflon–glass Potter homogenizer. The homogenate was centrifuged at 55 000 rpm for 30 min at 2 °C. The supernatant was recovered by aspiration and the pH adjusted to 7.4 with 1 M Tris.

Determination of Binding Sites. The samples were incubated in duplicate at 0 °C with 30 nM [^{3}H]triamcinolone acetonide. After 2 h, the bound radioactivity was determined in duplicate by charcoal adsorption assay (Rousseau et al., 1972). The nonspecific binding was measured by incubating parallel samples in the presence of a 1000-fold excess of nonradioactive triamcinolone acetonide.

Alkylation of the Receptor Complexes by N-Substituted Maleimides. A 100 mM solution of each maleimide was extemporaneously prepared in the appropriate solvent, i.e., ethanol for *N*-alkylmaleimides, *N*-(ω-carboxyalkyl)maleimides, and *N*-phenylmaleimide, 2-methoxyethanol for *N*-benzylmaleimide and *N*-benzylsuccinimide, and dimethyl sulfoxide for *N*-(ethylphenyl)maleimide. Each maleimide derivative was then added to the [^{3}H]triamcinolone acetonide labeled cytosol at the chosen final concentration. Alkylation was performed for various times at 0 °C and ended by addition of β-mercaptoethanol (20 mM final concentration). The alkylated samples were submitted to gel filtration on a Ultrogel ACA 202 column (4 mL) equilibrated in 20 mM Tris-HCl (pH 7.4) containing 50 mM KCl, 1 mM EDTA, and 20 mM β-mercaptoethanol. The excluded fractions containing the receptor complexes were readjusted to a 30 nM [^{3}H]triamcinolone acetonide concentration before the heat activation step (25 °C for 30 min).

DNA–Cellulose Binding Assay. A column procedure was carried out at 4 °C (Idziorek et al., 1985). Columns containing 1.0 mL of packed DNA–cellulose were equilibrated in 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM KCl, 1 mM EDTA, and 20 mM β-mercaptoethanol. Samples (0.1–0.4 mL) of [^{3}H]triamcinolone acetonide labeled cytosolic receptor were applied to these columns, left in contact with the DNA–cellulose for 5 min after penetrating the gel, and then eluted

[1] The term "transformation" is used to describe the process whereby the steroid-bound receptor is converted to a form that binds to nuclei or DNA–cellulose. The transformation step must be distinguished from the DNA binding itself.

[2] Abbreviations: Triamcinolone acetonide, 9α-fluoro-11β, 16α, 17α, 21-tetrahydropregna-1,4-diene-3,20-dione 16,17-acetonide; RU 486, 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-propynylestra-4,9-dien-3-one; DXE, *N*-ethyl-9α-fluoro-16α-methyl-11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

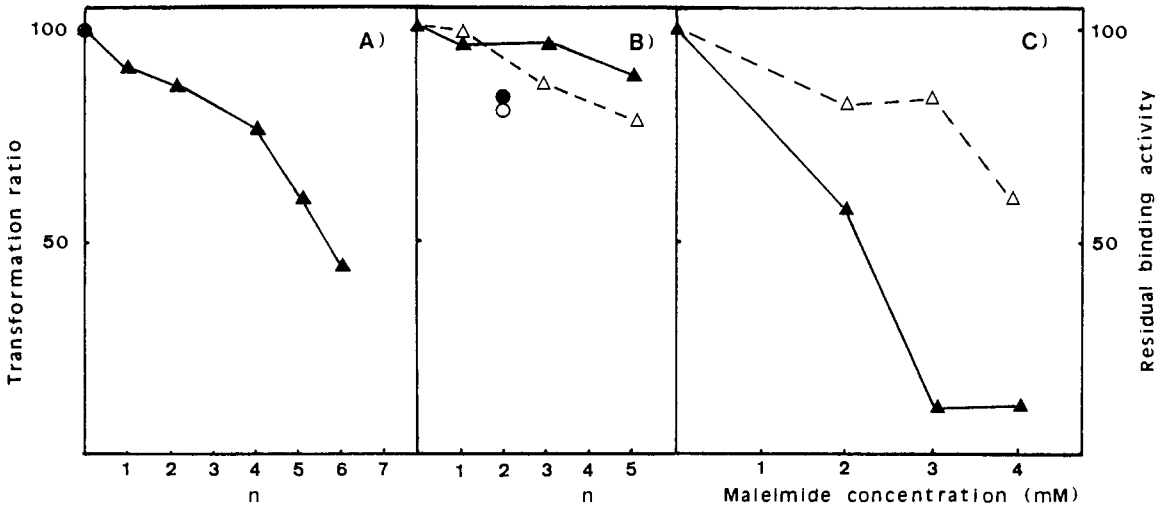

FIGURE 1: Inhibition of the transformation and/or DNA binding of glucocorticoid–receptor complexes by various N-substituted maleimides. Rat liver cytosol prepared in hypotonic TKGE buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide for 2 h at 3 °C. N-Substituted maleimides were added at a final 3 mM concentration; 5.0 min later, the alkylation was stopped by addition of 20 mM β-mercaptoethanol. Each sample was then submitted to gel filtration on a Ultrogel ACA 202 column, and the excluded fractions were submitted to heat activation at 25 °C for 30 min and then assayed by DNA–cellulose chromatography. (A) Effect of *N*-alkylmaleimides of various length [*n* = number of methylene groups in the alkyl side chain R = $(CH_2)_nCH_3$]. Relative transformation ratio (▲) was calculated as explained under Materials and Methods and is expressed in percent. (B) Effect of *N*-alkylmaleimides bearing an ionizable substituent. Relative transformation ratio was computed for (▲) *N*-(ω-carboxyalkyl)maleimides [*n* = number of methylene groups on the carboxyalkyl side chain R = $(CH_2)_nCOO^-$] and (●) *N*-[2-(trimethylammonio)ethyl]maleimide iodide [R = $(CH_2)_2N(CH_3)_3{}^+I^-$]. The residual steroid binding activity (△, ○) was assayed in each sample before the DNA–cellulose step and is expressed in percent of the nonalkylated transformed control. (C) Dose dependence of the *N*-benzylmaleimide effect on the transformation of cytosolic [^{3}H]triamcinolone acetonide–receptor complexes. Relative transformation ratio (▲) and residual steroid binding activity after alkylation and heat treatment (△) are expressed in percent of a nonalkylated transformed control.

stepwise by 10 mL of equilibrating buffer and 10 mL of the same buffer supplemented with 400 mM KCl. Flow rate was 0.75 mL·min^{-1} and 0.3-mL fractions were collected for measurement of radioactivity. Each experiment included two control samples, a heat-transformed and a nontransformed sample, run in parallel to the samples previously submitted to alkylation by the various maleimides and then to heat transformation. The ratio (in percent) of the radioactivity eluted by the high salt buffer to the total receptor-bound radioactivity assayed in the sample just before DNA–cellulose chromatography was calculated. The relative transformation ratio of each sample was computed by using the formula

$$\frac{X - N}{T - N} \times 100$$

where X, T, and N represent the percent of retention on the DNA–cellulose column of the receptor in the alkylated sample, the transformed control, and the untransformed control, respectively.

Sucrose Gradient Centrifugation. Linear (5–20%) sucrose gradients were prepared in 160 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 20 mM sodium molybdate, and 10% glycerol. Samples (200 μL) were layered onto the preformed gradients, and the tubes were centrifuged in a SW 60 rotor (Beckman) at 55 000 rpm and 2 °C for 17 h. Six-drop fractions were collected and counted for radioactivity. Sedimentation coefficients were determined according to the method of Martin and Ames (1962) using aldolase (7.8S) and bovine serum albumin (4.3S) as standards.

Miscellaneous. Protein determination was performed by using the method of Bensadoun and Weinstein (1976). Sulfhydryl groups were assayed with DTNB (Ellman, 1959). Radioactivity was measured in an Intertechnique SL 4000 liquid spectrometer, using Aqualyte (Baker Chemicals, Deventer, The Netherlands) as scintillation cocktail (35% tritium efficiency).

RESULTS

Synthesis of N-Substituted Maleimides. *N*-Alkyl- and *N*-arylmaleimides were easily prepared, whereas *N*-[2-(trimethylammonio)ethyl]maleimide iodide was obtained in a rather low 16% yield, close to the 20% yield reported by El Merzabani and Sakurai (1972). For the preparation of the *N*-(ω-carboxyalkyl)maleimides we first resorted to a procedure including the preparation of the *tert*-butyl ester of the ω-carboxyalkylamine and its coupling to maleic anhydride to give the corresponding maleamic acid (King et al., 1957). This acid could then be cyclized by a carbodiimide method (Trommer & Hendrick, 1973) and finally deprotected by treatment with trifluoroacetic acid. However, the overall yield afforded by this method was poor in our hands, and we turned to the procedure of Keller and Rudinger (1975), which gave excellent results with 60% yield for the preparation of *N*-(methoxycarbonyl)maleimide and 20, 30, and 50% yield for *N*-(carboxymethyl)maleimide, *N*-(3-carboxypropyl)maleimide, and *N*-(5-carboxypentyl)maleimide, respectively. All compounds were homogeneous in thin-layer chromatography and presented a characteristic ultraviolet absorption band near 300 nm, which disappeared after reaction with a thiol derivative like β-mercaptoethanol. Moreover, a characteristic γ-lactam infrared absorption band was always found in the 1700–1750-cm^{-1} region of the spectrum.

Effect of N-Alkylmaleimides on the Transformation and/or DNA Binding of Glucocorticoid–Receptor Complexes. Rat liver cytosol containing untransformed glucocorticoid–receptor complexes was incubated at 0 °C with various *N*-alkylmaleimides. Figure 1A illustrates the effect of lengthening the alkyl chain of the maleimides on their ability to inhibit further heat transformation of the receptor complexes to the DNA binding state. All the maleimides were used at the same 3.0 mM concentration for 5 min at 0 °C. In these conditions, the *N*-alkylmaleimide efficiency as inhibitor of receptor transformation appeared negligible for *N*-ethylmaleimide but increased very significantly with the alkyl chain length to reach

Table I: Effect of *N*-Arylmaleimides on the Transformation to the DNA Binding State of Glucocorticoid–Receptor Complexes[a]

		[^{3}H]TA binding activity (cpm/mg of protein)			
		before transformation	after transformation	% retention on DNA–cellulose	relative transformation ratio %
(A)	nontransformed control	8915		18	0
	transformed control	8915	7195	59	100
	N-phenylmaleimide	4886	4089	52	83
	N-benzylmaleimide	5395	4088	22	10
	N-benzylsuccinimide	8675	8904	59	100
(B)	nontransformed control	12624		7	0
	transformed control	12624	12181	55	100
	N-ethylmaleimide		11310	57	104
	N-(4-carboxy-3-hydroxyphenyl)maleimide	8838	7340	58	106
	N-benzylmaleimide	9261	8389	15	17
	N-(ethylphenyl)maleimide	9271	8660	22.5	32

[a] Rat liver cytosol was labeled with [^{3}H]triamcinolone actonide, treated with various N-substituted maleimides and succinimides for 5 min at a 3 mM concentration, and submitted to heat activation and DNA–cellulose chromatography as described in Figure 1. Receptor binding activity was assayed by using a dextran-coated charcoal assay and corrected for nonspecific binding. Protein concentration was assayed in each sample. Results are reported for two representative experiments A and B.

a 60% inhibition level in the case of *N*-heptylmaleimide. Thus, according to the two-step process postulated for the action of this kind of reporter group (Formstecher et al., 1984; Heitz et al., 1968), the binding of *N*-alkylmaleimide in the vicinity of the sulfhydryl group implied in the receptor transformation could involve a nonpolar region of the receptor protein. The receptor–maleimide complex would be stabilized by hydrophobic interactions, and a higher concentration of the complex would be formed with the longer chain derivatives, resulting in a more extensive alkylation of the receptor. In the present study the steroid ligand appeared unable to protect the receptor steroid binding activity extensively against long-chain maleimides (data not shown) or other N-substituted maleimides (Figure 1A,B dotted lines and Table I). This result contrasts with the complete protective effect previously reported (Formstecher et al., 1984). In the latter case alkylation was performed at a lower temperature (−12 versus 0 °C), and the receptor was previously incubated with [^{3}H]dexamethasone and not with [^{3}H]triamcinolone acetonide. Triamcinolone acetonide–receptor complexes have recently been described as being more easily disrupted by sulfhydryl-modifying reagents than dexamethasone–receptor complexes (Tienrungroj et al., 1987), a result confirmed by the present study. However, whatever the maleimide used there were still enough active steroid–receptor complexes to allow accurate assay of their ability to be transformed to the DNA binding state.

Effect of N-Alkylmaleimides Bearing an Ionizable Substituent. Since acquisition by steroid receptors of DNA binding activity is concomitant with acidophilic transformation and probable exposure at the receptor surface of positively charged groups (Schmidt & Litwack, 1982), a sulfhydryl group involved in the transformation could be expected to be located near or inside the receptor DNA binding site and to lie in a rather polar environment. To test this hypothesis, N-substituted maleimides bearing an ionizable group located at a variable distance from the maleimide nitrogen atom were prepared, and their ability to impede the transformation of glucocorticoid–receptor complexes was measured. Surprisingly, none of the compounds tested inhibited transformation to an extent higher than 16% (Figure 1B). The presence on the receptor surface of a charged residue able to facilitate the binding of the ionized maleimide in the vicinity of the putative cysteinyl group essential for transformation appeared therefore unlikely.

N-Benzyl- and N-(Ethylphenyl)maleimide Inhibit Receptor Transformation and/or DNA Binding. Table I summarizes representative results obtained with four *N*-arylmaleimides. Neither *N*-phenylmaleimide nor its polar derivative *N*-(4-carboxy-3-hydroxyphenyl)maleimide provoked significant inhibition of the transformation of glucocorticoid–receptor complexes. On the contrary, after treatment with *N*-benzylmaleimide only 10–17% of the complexes were still transformable to the DNA binding state. This effect was dose dependent (Figure 1C), and here again a partial inactivation of the [^{3}H]triamcinolone acetonide–receptor complexes, to an extent variable according to the experiments, was observed (Table I). The lower retention on DNA–cellulose of the nontransformed control in experiment B (7%) when compared to experiment A (18%) is explained by the addition of 10 mM sodium molybdate in the DNA–cellulose column buffer in experiment B, where artifactual transformation during the contact with the DNA column was therefore precluded. The mechanism of the *N*-benzylmaleimide action very probably involved an alkylation step, since *N*-benzylsuccinimide, a compound devoid of any alkylating activity and structurally closely related to *N*-benzylmaleimide, did not inhibit transformation. *N*-(Ethylphenyl)maleimide also displayed an antitransformation activity, which was, however, significantly lower than that of *N*-benzylmaleimide. Taken together, these data suggested the presence in the vicinity of the sulfhydryl group necessary for receptor transformation of a residue able to interact rather specifically with *N*-benzylmaleimide. A charge-transfer interaction between the maleimide benzene ring and an aromatic amino acid of the receptor protein could be suggested. For steric reasons this could not be obtained with *N*-phenylmaleimide, in which the phenyl and maleimide rings are directly connected by a single covalent bond, allowing only limited rotation freedom around its axis for both the rigid rings. On the other hand, introduction of a methylene group between the two rings considerably increased the conformational flexibility of the molecule, a property that very probably accounts for the dramatic improvement of its efficiency as transformation inhibitor. However, moving the benzene ring one methylene more distant from the maleimide ring was enough to lead to a significant decrease in the antitransformation activity [32% transformation after *N*-(ethylphenyl)-maleimide alkylation versus 10–17% with *N*-benzylmaleimide].

N-Benzyl- and N-(Ethylphenyl)maleimides Inhibit the Temperature-Mediated Conversion of 8S Receptor to 4S. To test the effect of N-substituted maleimides on the sedimentation pattern of the receptor, samples of cytosol containing untransformed receptors were treated with *N*-ethyl-, *N*-benzyl-, and *N*-(ethylphenyl)maleimide. β-Mercaptoethanol was added to stop the maleimide action, and the samples were heated at

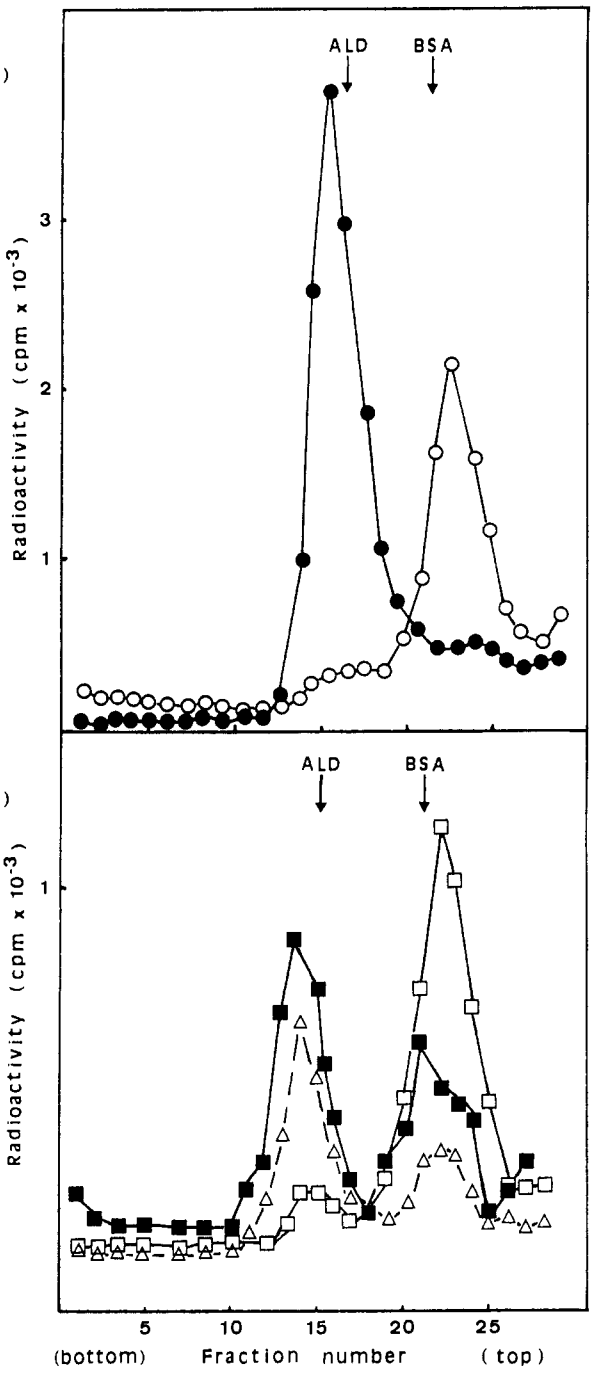

FIGURE 2: Sucrose gradient centrifugation analysis. Aliquots (0.2 mL) of cytosolic [^{3}H]triamcinolone acetonide–receptor complexes submitted or not to alkylation (3 mM for 5 min at 0 °C) by various *N*-alkylmaleimides and to heat treatment (25 °C for 30 min) were treated by dextran-coated charcoal. The resulting supernatants were applied on 5–20% sucrose gradients in high-salt buffer containing 10 mM sodium molybdate and centrifuged at 55 000 rpm for 17 h at 2 °C; 0.14–mL fractions were collected and counted for radioactivity. (A) Nonalkylated unheated (●) and heated (○) control samples. (B) Samples heated after alkylation with *N*-ethylmaleimide (□), *N*-benzylmaleimide (■), and *N*-(ethylphenyl)maleimide (△). Arrows indicate the position of the protein standards aldolase (7.8S) and bovine serum albumin (4.3S).

25 °C. The cytosol was then centrifuged in a sucrose gradient that contained molybdate. As shown in Figure 2, receptor samples that were incubated in the presence of *N*-benzyl- and *N*-(ethylphenyl)maleimide sedimented in a major peak at about 8S and underwent only limited conversion to the 4S form. On the contrary, in the presence of *N*-ethylmaleimide, almost all the receptor was found in the 4S peak. Thus, the N-substituted maleimides able to impede the transformation of the receptor to the DNA binding state were also able to inhibit its temperature-mediated size reduction. When expressed quantitatively, the 4S conversion was about 30%, 42%, and 90% for *N*-benzylmaleimide-, *N*-(ethylphenyl)maleimide-, and *N*-ethylmaleimide-treated samples, respectively, i.e., slightly higher values than the corresponding relative transformation ratio (Table I). Such a discrepancy could be explained by technical reasons: on one hand, the DNA–cellulose assay was reported to yield sometimes underestimated values (Le Fevre et al., 1979), and, on the other hand, some artifactual receptor dissociation could have taken place during the time-consuming centrifugation step.

Inhibition by N-Benzylmaleimide of the Glucocorticoid Receptor Inactivation Caused by Heat. The fact that *N*-benzylmaleimide, like molybdate, inhibits both glucocorticoid receptor transformation and temperature-mediated size reduction prompted us to see if it displayed another well-documented effect of molybdate on the glucocorticoid receptor, i.e., the ability to protect the binding activity of the unbound receptor against inactivation by heat (Dahmer et al., 1984). However, the study of this latter effect was difficult to perform since *N*-benzylmaleimide, like other N-substituted maleimides, was able to rapidly inactivate the unbound receptor by alkylating the sulfhydryl group essential for steroid binding (Formstecher et al., 1985). Therefore, a four-step procedure was used to avoid this inactivation. The first step consisted of the protection of the sulfhydryl group essential for ligand binding and was obtained by incubating the receptor at 0 °C in the presence of a large excess of the low-affinity steroid ligand DXE, a 17β-carboxamide derivative of dexamethasone (Formstecher et al., 1980) displaying a 0.26 μM K_i for the receptor. During the second step, DXE–receptor complexes were alkylated with 3 mM *N*-benzylmaleimide for 5 min at 0 °C. The third step started with gel filtration of the alkylated complexes on an ACA 202 column in order to remove the excess of free DXE and to allow progressive dissociation of DXE–receptor complexes (half-dissociation time at 0 °C was less than 20 min, unpublished results). The resulting unbound complexes were heated at 25 °C in the presence or absence of dithiothreitol and/or molybdate. The fourth and last step consisted of the assay of the residual [^{3}H]triamcinolone acetonide binding activity of the heated samples. Results are summarized in Figure 3. In the absence of molybdate, dithiothreitol alone was unable to protect the receptor binding activity (curve 1). On the contrary, molybdate alone afforded a significant protection (curve 2), which became complete in the presence of both molybdate and dithiothreitol (curve 3). These results were in perfect agreement with earlier work (Dahmer et al., 1984). Previous incubation of the DXE–receptor complexes in the presence of *N*-benzylmaleimide resulted in a significant stabilizing effect, which was independent of the presence or absence of dithiothreitol during the heat treatment (curves 4 and 5). However, some additivity of the effects of *N*-benzylmaleimide alkylation and of molybdate presence during the heating step was observed (curves 6 and 7 versus curve 2).

Several conclusions can be drawn from these results. *N*-Benzylmaleimide apparently displays some molybdate-like stabilizing activity on the glucocorticoid receptor. However, the mechanism of action of the two compounds is clearly different. First, there is no synergy between the effects of alkylation by *N*-benzylmaleimide and further addition of dithiothreitol, contrary to what is observed with molybdate and dithiothreitol. Second, previous treatment with *N*-benzyl-

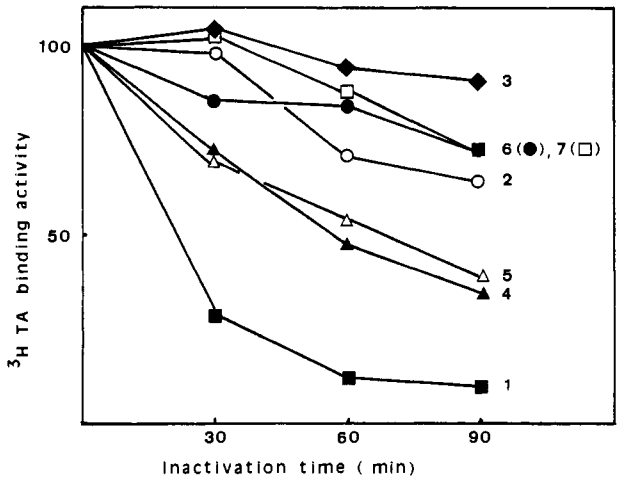

FIGURE 3: Protecting effect of *N*-benzylmaleimide treatment against heat inactivation of the glucocorticoid receptor. Rat liver cytosol was prepared in TKGED buffer and incubated overnight at 0 °C in the presence of 20 μM unlabeled DXE, a 17β-carboxamide derivative of dexamethasone (Formstecher et al., 1980). Aliquots were alkylated or not with 3 mM *N*-benzylmaleimide for 5 min at 0 °C and submitted to gel filtration on an ACA 202 column equilibrated in TKGE buffer supplemented or not with 1 mM dithiothreitol (DTT), 10 mM sodium molybdate, or both. Excluded fractions were heated at 25 °C. After various times, aliquots were brought back to 0 °C and incubated for 2 h in the presence of 30 nM [^{3}H]triamcinolone acetonide. Specific binding activity was assayed by using the dextran-coated charcoal method and is expressed in percent of a nonalkylated unheated control sample and plotted versus duration of the heat treatment. The experimental conditions used for each sample are summarized below.

	curve no.						
	1	2	3	4	5	6	7
N-benzylmaleimide treatment	−	−	−	+	+	+	+
conditions of the heat-inactivation step							
1mM dithiothreitol	+	−	+	−	+	−	+
10 mM molybdate	−	+	+	−	−	+	+

maleimide does not impede the further effect of molybdate on the alkylated receptor. This suggests that *N*-benzylmaleimide and molybdate interact with distinct sites on the receptor molecule.

Discussion

Contradictory results have been reported about the effects of *N*-ethylmaleimide on the transformation and the DNA binding activity of the glucocorticoid receptor. Kalimi and Love (1980) demonstrated that treatment of rat liver cytosol for 30 min at 0 °C with 5 mM *N*-ethylmaleimide, prior to warming at 25 °C, substantially inhibited nuclear binding. However, the alkylating agent was not removed before the warming. On the contrary, when gel filtration was performed before the transformation step, so that *N*-ethylmaleimide was absent during the warming, no decrease in DNA–cellulose binding was observed (Bodwell et al., 1984a), a result confirmed in the present study. Only long-chain *N*-alkylmaleimides, *N*-(ethylphenyl)maleimide, and above all *N*-benzylmaleimide were able to significantly inhibit receptor transformation.

The effects of *N*-benzylmaleimide could be compared with the results of Tienrungroj et al. (1987) for peroxide treatment of the receptor, which led to a similar inhibition of receptor transformation and dissociation. Our data reinforce their assumption that critical sulfur moieties in the receptor complex must be in the reduced form for receptor transformation and dissociation to occur. Transformation of the glucocorticoid receptor is a hormone-dependent phenomenon concomitant with a decrease in molecular weight of the steroid–receptor complexes (Holbrook et al., 1983). Our results provide a new argument sustaining the theory according to which the dissociation step leading to this molecular weight decrease could be the first and critical event of the transformation process. We recently provided another confirmation of this theory by demonstrating that RU 486, a potent antiglucocorticoid, was able to inhibit both glucocorticoid receptor transformation and subunit dissociation (Sablonniere et al., 1986).

The ability of *N*-benzylmaleimide to stabilize the 8S form of the receptor and protect the receptor against inactivation by heat is in good agreement with the higher stability of the high molecular weight form of the receptor reported by others. In intact cells the 8–10S receptor is converted by the steroid ligand to the activated 4S form, which is degraded at in increased rate (McIntyre & Samuels, 1985). Inactivation by heating the receptor in acellular conditions could involve some dissociation or degradation of the 8S complexes, even in their unliganded state, as suggested by recent experiments (unpublished results). In the case of the progesterone receptor it was recently found that, after chemical cross-linking in the absence of molybdate, cytosolic 8S complexes became more resistant to inactivation by heat treatment than non-cross-linked complexes (Aranyi et al., 1988).

No direct characterization of the putative cysteinyl residues alkylated by *N*-benzylmaleimide on the receptor was attempted. The complete sequence of the rat glucocorticoid receptor has recently been deduced from its cDNA (Miesfeld et al., 1986). It is a 795 amino acid polypeptide with 20 cysteines. Cysteine residues 656 and 754, located in the C-terminal part of the protein, have been demonstrated to be directly involved in steroid binding by affinity labeling (Simons et al., 1987; Carlstedt-Duke et al., 1988). The cysteine residues essential for the transformation step and the DNA binding activity are very probably distinct from those involved in steroid binding, as suggested by earlier work (Bodwell et al., 1984b). At the present time it is not known whether the sulfhydryl groups essential for receptor transformation and dissociation are the same as or different from those required for binding to DNA (Tienrungroj et al., 1987). The sulfhydryl groups involved in the latter process could be located in the DNA binding domain of the receptor, which includes 10 cysteins (Miesfeld et al., 1986). On the other hand, the presence of 3 cysteine residues distinct from the 656 and 754 cysteine residues in the so-called steroid binding domain deserves notice. This domain includes a small region highly conserved in all steroid receptors, whose involvement in the ability of these receptors to form 8S complexes (Pratt et al., 1988) and in the repression of their transcriptional activity in the absence of hormone (Danielsen et al., 1987) has recently been postulated.

It is generally considered that molybdate could exert its effects on the receptor by interacting with sulfhydryl groups (Dahmer et al., 1984). However, our data suggest that these groups are distinct from the one(s) alkylated by *N*-benzylmaleimide, since the molybdate effect on the receptor stabilization appears independent of previous treatment by *N*-benzylmaleimide.

Interestingly, several cysteinyl groups borne by the receptor have a neighboring aromatic amino acid that could be involved in some charge-transfer interaction with the benzene ring of *N*-benzylmaleimide. However, a direct approach using radioactive *N*-benzylmaleimide is obviously required to allow a clear identification of the amino acid residue alkylated by *N*-benzylmaleimide. Owing to the low amounts of purified glucocorticoid receptor available, this work will probably be extremely difficult, but the recent data published by Peleg et al. (1988) in the case of the progesterone receptor demon-

strated that such an approach can succeed.

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Registry No. *N*-Ethylmaleimide, 128-53-0; *N*-(4-carboxy-3-hydroxyphenyl)maleimide, 19232-43-0; *N*-propylmaleimide, 21746-40-7; *N*-butylmaleimide, 2973-09-3; *N*-pentylmaleimide, 19775-00-9; *N*-hexylmaleimide, 17450-29-2; *N*-heptylmaleimide, 18559-62-1; *N*-octylmaleimide, 4080-76-6; *N*-phenylmaleimide, 941-69-5; *N*-benzylmaleimide, 1631-26-1; *N*-(ethylphenyl)maleimide, 116503-80-1; *N*-[2-(dimethylamino)ethyl]maleamic acid, 116503-79-8; *N*-[2-(dimethylamino)ethyl]maleimide, 5135-58-0; *N*-[2-(trimethylammonio)ethyl]maleimide iodide, 61638-98-0; *N*-(methoxycarbonyl)maleimide, 55750-48-6; glycine, 56-40-6; 4-aminobutyric acid, 56-12-2; 6-aminohexanoic acid, 60-32-2; *N*-(carboxymethyl)-maleimide, 25021-08-3; *N*-(3-carboxypropyl)maleimide, 57078-98-5; *N*-(5-carboxypentyl)maleimide, 55750-53-3; *N*-benzylsuccinimide, 2142-06-5; cysteine, 52-90-4.

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