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STRUCTURAL REQUIREMENTS FOR THE BINDING OF DEXAMETHASONE TO NUCLEAR ENVELOPES AND PLASMA MEMBRANES

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Summary—The specificity of dexamethasone binding sites on nuclear envelopes (NE) and plasma membranes (PM) was determined in competition studies with natural and synthetic steroids. The binding affinities for nuclear envelopes and plasma membranes were then correlated with the three-dimensional structures of the ligands. Three major factors are implicated in the ability of the steroid to bind to the membrane sites: (1) the separation between the terminal oxygen atoms substituted at atoms C3 and C17, or attached to the substituent at C17, is found to be longer than 10 Å for the medium and high affinity steroids; (2) the β-orientation of the oxygen atom in the C17-substituent to the D-ring is favored over α-orientation; and (3) bulky substituents and nontypical configurations are not accepted by the binding sites. A nearly linear correlation between the O3···O (substituted at C17) distance and the binding affinity of the tested steroids is observed; explanations for the lack of linear correlation of some steroids are given. A preliminary model for the interaction of steroids with these membrane sites is proposed which requires two hydrogen bonding regions that interact with the 2 oxygen atoms and some steric restriction sites that prevent the binding of steroids with large substituents. The hydrophobicities of the steroids do not correlate with binding affinities to the dexamethasone binding sites; hydrophobicity seems to play a minor role in these steroid–membrane interactions. Comparisons of the specificity of the dexamethasone binding sites on membranes to the specificity of various steroid receptors are also presented.

INTRODUCTION

Steroid hormones must traverse the plasma membrane and the nuclear envelope of the target cell before exerting their action at the chromatin. However, relatively few studies have been done to elucidate the mechanisms by which steroids traverse membrane barriers. As steps towards investigating the transport mechanisms, we have characterized the binding sites for dexamethasone on plasma membranes [1] and nuclear envelopes [2] prepared from male rat liver. In the course of these investigations, competition studies done to determine the specificity of the dexamethasone binding sites in both membrane systems were of interest for two reasons: (i) while properties of dexamethasone binding to the two membranes differed in some respects, the orders of potency of the competing steroids were similar in the plasma membrane and in the nuclear envelope, and (ii) while the membrane binding sites showed marked specificity, they displayed a broad range of specificity in contrast to the strict steroid specificity of the cytosolic glucocorticoid receptor (GR) [3–6]. Some of the steroids that show strong binding to the membrane binding sites do not bind to GR while, a potent glucocorticoid, triamcinolone acetonide, binds poorly to the membranes. As these membrane sites may be important in the modulation of steroid hormone action in some pathophysiological states, we investigated the structural requirements for the membrane binding. To establish the exact structural requirements, the known crystal structures of the steroids used in the competition were examined via molecular graphics comparisons and least squares superposition of relevant structural features, and the correspondences of binding features were determined. In this paper, we

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report that three factors are implicated in the ability of dexamethasone to bind to the membrane sites: (1) the separation between the oxygen atom at C3 and the oxygen atom at C17 or attached to a substituent at C17; (2) the relative orientation of the C17 oxygen atom to the D-ring; and (3) the overall shape of the molecule: extra rings, bulky substituents or an atypical configuration lower the ability of the steroid to bind.

EXPERIMENTAL

Materials

[^{3}H]Dexamethasone (38–48.9 Ci/mmol) was purchased from New England Nuclear (Lachine, Canada). Unlabeled steroids were from Steraloids Inc. (Wilton, N.H.) except for cholesterol, triamcinolone, triamcinolone acetonide and triamcinolone diacetate which were from Sigma Chemical Co. (St Louis, Mo.). Sucrose, ultra-pure, was from Schwartz–Mann (Cambridge, Mass). Desoxyribonuclease 1 (DNAse 1), buffers and dithiothreitol (DTT) were obtained from Sigma Chemical Co.

Animals

Male Sprague–Dawley rats weighing 200–250 g were obtained from Charles River Canada Inc. (Montreal, Canada) and maintained on a diet of Wayne Lab Blox (Allied Mills, Chicago, Ill.) and tapwater *ad libitum*. The rats were killed by decapitation and the livers were quickly removed, stripped of connective tissue, weighed and placed on ice.

Preparation of membranes

Plasma membranes [1] and nuclear envelopes [2] were isolated as previously described.

Competition studies

Optimum conditions for dexamethasone binding to plasma membranes [1] and nuclear envelopes [2] had been determined previously.

(a) Plasma membranes. Rat liver plasma membranes (100 μg protein) were incubated with 50 nM [^{3}H]dexamethasone for 16 h at 0–4°C in the absence or presence of 50 μM (1000-fold excess) of the unlabeled steroid in a total volume of 59 μl of 25 mM TAPS, pH 8.6, containing 1% ethanol to ensure solubility of steroids. The incubations were stopped by centrifugation for 1 min in a Microfuge B and aspiration of the supernatant following the addition of 1 ml of excess buffer. The pellet was then washed twice with vortexing in 1 ml of buffer as described above. The tip of the tube containing the pellet was cut and incubated overnight at 50°C in 5 ml Ready Solv (Beckman) containing 500 μl of NCS tissue solubilizer (Amersham Canada Ltd, Oakville, Canada). The radioactivity was determined by scintillation counting.

(b) Nuclear envelopes. Rat liver nuclear envelopes (100 μg protein) were incubated with 30 nM [^{3}H]dexamethasone for 16 h at 0–4°C in 25 mM TAPS, pH 8.6, containing 1 mM DTT in the presence or absence of 30 μM (1000-fold excess) of the unlabeled steroid. The incubations were stopped and the radioactivity bound to the membranes was determined as described above for the plasma membranes.

Molecular comparisons

Atomic coordinates of the molecular structures of all but 3 of the steroids used in the competition studies and of diethylstilbestrol were obtained from the crystallographic literature [7–40] or from the Cambridge Crystallographic Database [41]. The crystal structure of 5β-dihydrotestosterone (5β-DHT) was determined especially for this project; crystallographic data on 5β-DHT are presented elsewhere [42]. The three-dimensional structures of triamcinolone and triamcinolone diacetate are not available; discussion of their structures is based on the observed structure of triamcinolone acetonide [15] and on the chemical similarity of triamcinolone to dexamethasone. All the comparisons of molecular structures were performed with the least-squares superposition program PROFIT [43] and the graphics program MMS [44].

RESULTS AND DISCUSSION

Figure 1 contains the structures of all of the compounds tested. The first two columns of Table 1 show the membrane binding affinities of these compounds.

Our studies of the characterization of dexamethasone binding sites on the plasma membranes [1] and nuclear envelopes [2] prepared from rat liver showed that both membrane systems possessed two dexamethasone binding sites; a low capacity, high affinity site and a high capacity, low affinity site. The high affinity site on the nuclear envelopes resembled the nuclear glucocorticoid receptor in its binding affinity and in hormone responsiveness. In further

studies, we have now shown that a population of glucocorticoid receptors is resident at the periphery of the nucleus and in nuclear envelope preparations [45]. The higher affinity plasma membrane site has a molecular weight of 45,000 Da and does bind anti-glucocorticoid receptor antibody [1]. In the studies described in this paper, we were interested in further characterization of the lower affinity dexamethasone binding sites on both membrane systems as they may function at pharmacological doses of steroids to modulate steroid hormone action. The specificities determined represent principally those of the low affinity site as the high affinity

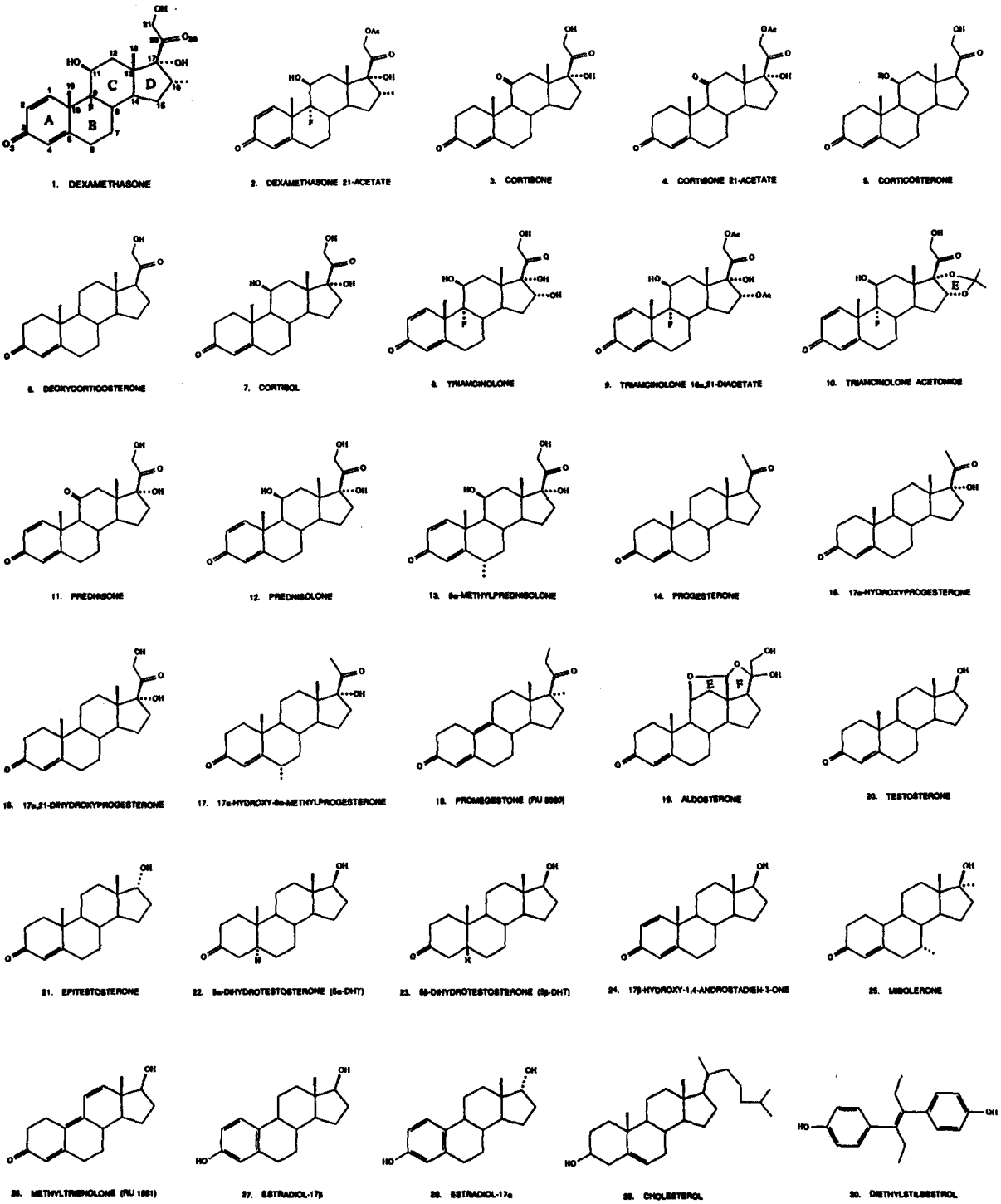

Fig. 1. Structures of compounds used in the competition studies. Labeling scheme of the steroid nucleus is shown for dexamethasone.

Table 1. Specificity of [^{3}H]dexamethasone binding to male rat liver nuclear envelopes (NE) and plasma membranes (PM) and the separations between the terminal oxygen atoms, O3 and Ox, for the compounds used in the competition studies. Total binding of [^{3}H]dexamethasone to NE in these studies was 4.19 pmol/mg of protein and to PM 1.03 pmol/mg of protein. The % inhibition by dexamethasone of [^{3}H]dexamethasone binding to NE was 92.6 ± 2.8% and to PM 84.9 ± 2.8%. For ease of comparing data from the two membrane systems, the data are expressed as % inhibition of total binding, with the inhibition of [^{3}H]dexamethasone binding by a 1000-fold excess of unlabeled dexamethasone having been set at 100%. For each hormone and both membrane systems values of a mean ± SEM and a number of experiments (n) are given. O3···Ox distances were calculated from crystallographic atomic coordinates taken from the literature—the references are listed in the last column

Unlabeled hormone	% Inhibition of total binding for: Nuclear envelope		Plasma membrane		O3···Ox distance [Å]	x	Ref.
1. Damethasone[1]	100.0 ± 3.0	n = 13	100.0 ± 3.7	n = 8	11.57	20	[7]
2. Dexamethasone 21-acetate	94.1 ± 2.0	n = 4	96.0 ± 4.7	n = 4	11.47	20	[8]
3. Cortisone	101.3 ± 3.8	n = 3	94.3 ± 3.3	n = 4	11.43	20	[9]
4. Cortisone 21-acetate	87.9 ± 2.2	n = 2	93.5 ± 2.8	n = 3	11.66	20	[10]
5. Corticosterone	93.4 ± 7.5	n = 2	87.9 ± 6.7	n = 2	11.83	20	[11]
6. Deoxycorticosterone	98.8 ± 0.2	n = 2	97.2 ± 2.0	n = 2	11.89	20	[12]
7. Cortisol[1]	100.0 ± 1.4	n = 2	87.6 ± 4.3	n = 2	11.72	20	[13, 14]
8. Triamcinolone[6]	96.4 ± 0.3	n = 2	92.1 ± 4.2	n = 2	—	—	—
9. Triamcinolone 16α,21-diacetate[6]	92.3 ± 0.5	n = 2	81.9 ± 3.3	n = 2	—	—	—
10. Triamcinolone acetonide	30.0 ± 7.7	n = 2	36.5 ± 26.6	n = 2	11.48	20	[15]
11. Prednisone	104.5	n = 1	100.2 ± 1.3	n = 2	11.28	20	[16]
12. Prednisolone	102.6 ± 1.0	n = 3	101.4 ± 3.0	n = 3	11.62	20	[17]
13. 6α-Methylprednisolone	98.9 ± 1.2	n = 2	98.4 ± 1.2	n = 4	11.42	20	[18]
14. Progesterone[1]	100.7 ± 0.1	n = 2	100.9 ± 0.5	n = 5	11.92	20	[19, 20]
15. 17α-Hydroxyprogesterone	101.8 ± 3.1	n = 3	95.5 ± 1.7	n = 4	11.69	20	[21]
16. 17α,21-Dihydroxyprogesterone	101.8 ± 4.9	n = 3	99.9 ± 2.9	n = 3	11.69	20	[22]
17. 17α-Hydroxy-6α-methylprogesterone	96.2 ± 1.2	n = 2	96.8 ± 1.7	n = 4	11.90	20	[23]
18. Promegestone (RU 5020)	99.3 ± 1.4	n = 2	85.5 ± 3.1	n = 2	11.84	20	[24]
19. Aldosterone	55.5 ± 2.0	n = 2	70.5 ± 8.3	n = 2	11.27	20	[25]
20. Testosterone[2]	71.0 ± 1.7	n = 2	68.2 ± 3.0	n = 2	10.89	17β	[26, 27]
21. Epitestosterone	30.5 ± 3.7	n = 2	51.8 ± 14.4	n = 4	9.64	17α	[28]
22. 5α-Dihydrotestosterone (5α-DHT)[3]	74.0 ± 5.5	n = 2	58.8 ± 11.3	n = 2	10.75	17β	[29, 30]
23. 5β-Dihydrotestosterone (5β-DHT)	21.6 ± 3.4	n = 2	23.9 ± 7.4	n = 2	9.83	17β	[42]
24. 17β-Hydroxy-1,4-androstadien-3-one	88.2 ± 6.1	n = 3	88.0 ± 7.4	n = 3	10.24	17β	[31]
25. Mibolerone	94.6 ± 0.1	n = 2	97.3 ± 2.8	n = 2	10.92	17β	[32]
26. Methyltrienolone (RU 1881)	65.7 ± 0.8	n = 2	77.9 ± 11.6	n = 2	10.86[4]	17β	[33]
					10.65[5]	17β	[33]
27. Estradiol-17β[3]	84.1 ± 6.5	n = 2	87.8 ± 2.2	n = 3	10.98	17β	[34–36]
28. Estradiol-17α[1]	50.2 ± 6.9	n = 2	59.4 ± 0.7	n = 2	10.43	17α	[37]
29. Cholesterol	14.5 ± 1.5	n = 2	16.8 ± 0.7	n = 2	—	—	[38]
30. Diethylstilbestrol	86.0	n = 1	80.1	n = 1	12.13[7]	—	[39, 40]

[1]O3···Ox distance is an average for the two conformationally similar molecules; [2]an average for four molecules; [3]an average for three molecules; [4]flat conformation; [5]bent conformation; [6]crystal structure not determined; [7]distance between the terminal hydroxyl oxygen atoms—an average for four independent determinations.

site represents only approximately 1% of the total number of [^{3}H]dexamethasone binding sites [1, 2].

Plasma membrane dexamethasone binding sites have been characterized which show similar steroid specificity to the site described here [46]. Further, the broad specificity of membrane associated dexamethasone binding proteins is not unique to glucocorticoid systems as such sites have been reported for estrogen on anterior pituitary microsomes [47] and triiodothyronine binding sites on rat liver nuclear envelopes [48].

Competition studies were carried out at pH 8.6 as we intended to perform affinity labeling studies with dexamethasone mesylate which gives optimal labeling of cytosolic glucocorticoid receptor at pH 8.6. We have verified that binding assays at pH 8.6 give identical results to those obtained at pH 7.4 (results not shown).

A survey of the chemical structures and the affinity data provides several generalizations. First, the sidechain at C17 is important to binding affinity. All but two of the pregnanes (**1–19**) show high affinity (over 81% of [^{3}H]-dexamethasone binding) while the androstanes (**20–24**) and the estranes (**25–28**) show competition with [^{3}H]dexamethasone over a wide range of values (between 21 and 98%). Cholesterol (**29**) does not compete with dexamethasone. The major difference between the pregnanes and the other steroids used in the binding studies is in the substitution pattern at atom C17; all pregnanes have a common 17β-sidechain with a C20 keto function, androstanes and estranes have instead a 17β- or 17α-hydroxyl substituent, while the 17β-sidechain on cholesterol is aliphatic and contains no oxygen atoms. The lower affinities of the androstanes and estranes may be due to their 17-hydroxy function. The oxygen atom of the 17-hydroxy group is located in a different position than the 20-carbonyl oxygen of the pregnane 17-sidechain; and, the character of the oxygen atom differs in these two cases

because the hydroxyl oxygen has weaker hydrogen accepting properties than does the carbonyl oxygen atom. Supporting this hypothesis, we find that diethylstilbestrol (**30**), which has a hydroxyl substituent, behaves similarly to the endogenous estrogen, 17β-estradiol (**27**).

Second, either a carbonyl or a hydroxyl substituent at C3 can be tolerated by the binding site. All of the steroids have a carbonyl group attached to C3 except the estradiols and cholesterol which are 3-hydroxylated; yet, 17β-estradiol displaces 84–87% of [^{3}H]dexamethasone and thereby confirms that a hydroxyl group is accepted.

Third, in contrast to the specificity of the glucocorticoid receptor, a number of substituents can be tolerated between positions C3 and C17, with the exception of substitutions at C21. Comparison of the binding affinities of related compounds shows that 11β-, 16α-, 17α- and 21-hydroxy or 11-keto substitutions in pregnanes are not important for binding at the dexamethasone site on membranes. This finding, that the membrane binding site is insensitive to substituents at C11, as different from the specificity of the glucocorticoid receptor where it is suggested that the pregn-4-en-11-ol-3,20-one substitution pattern contains the minimal structural requirements for binding and hormonal expression [5, 49 and references therein]. Similarly, there is no significant effect found for 6α-, 16α-, 17α- and 21-methyl or 9α-fluoro substitution although these substituents influence the molecular conformation. Again there is a difference to the pattern of binding to GR, because a 9α-fluoro substituent enhances binding to GR [5]. Comparison of the binding of the 21-acetylated pregnanes: dexamethasone acetate, cortisone acetate and triamcinolone diacetate, with the binding of their parent compounds shows that the 21-acetyl group slightly decreases affinity. The same effect was observed for the glucocorticoid receptor [5]. The data set does not include sufficient examples to test the effect of substitutions at C10 and C7.

Fourth, the binding site appears to be sensitive to the configuration at C17 and C5. In the androstanes and estranes, a β-orientation of the 17-hydroxy group rather than the α-orientation appears to be favored at the membrane binding site. This effect is seen by comparing pairs of 17β- and 17α-hydroxy substituted epimers, testosterone with epitestosterone, and estradiol-17β with estradiol-17α. In each case, the natural 17β-hydroxy-steroid shows significantly higher affinity than its 17α-epimer. The 17β-hydroxy group in steroid androgens is known to be essential for high affinity binding to the androgen receptor [50]. The 5β-configuration results in a strong bending of the 5β-DHT molecule [42]. Comparison of the binding of the endogenous hormone, 5α-dihydrotestosterone (5α-DHT), to that of its 5β-epimer, suggests that 5β-configuration of the ligand is not well accepted by this site.

Finally, attempts to find a correlation between the calculated or experimental hydrophobicities of the steroids and their binding affinities for the membrane binding sites were unsuccessful.

All of these features, together with an understanding that the oxygen atom substituents on a steroid nucleus contribute to the biological properties of the steroid, led to a suggestion that the O3 atom on the A-ring and the O20 on the 17β-pregnane sidechain, or O17 of the 17-hydroxy group of androstanes and estranes, are probably important for steroid-to-membrane interaction in the dexamethasone binding sites. Moreover, the separation between atom O3 and the appropriate oxygen atom at the 17-substituent could be an important factor. To quantify the structure–activity relationships, we hypothesized that the binding of dexamethasone to membranes depends on the distance between the two terminal oxygen atoms: O3 in all cases and O20 in pregnanes, or O17 in androstanes and estranes. These distances, O3$\cdots$Ox, were calculated from the atomic coordinates determined by crystal structure analysis and are listed in Table 1. For several compounds, **1**, **7**, **14**, **20**, **22**, **27** and **28**, the structures of more than one molecule has been reported, either from multiple observations in one crystal or by crystallization from different solvents. In all of these cases, the conformations of molecules of the same compound are almost identical; the value of the O3$\cdots$Ox distance listed in Table 1 is an average of the individual measurements. However, in the case of methyltrienolone (RU 1881, **26**), two significantly different conformations were observed [33]; thus, two values of the O3$\cdots$Ox distance are listed. The O3$\cdots$Ox distance is not provided for cholesterol as this steroid is lacking an oxygen atom at the D-ring terminus, and for triamcinolone and triamcinolone diacetate because the crystal structures of these compounds have not been determined.

A plot of the binding affinities to nuclear envelopes vs O3···Ox distance for all steroids except cholesterol is shown in Fig. 2. Triamcinolone (**8**) and triamcinolone diacetate (**9**) were plotted by assuming a value for the O3···Ox distance which is equal to that of triamcinolone acetonide (**10**). Diethylstilbestrol (**30**) was also included; however, compounds **8**, **9** and **30** were not included in the regression analysis.

All of the 20-keto steroids, except **10** and **19**, are located in one group around the average values of 11.7(2)Å for the O3···Ox distance and 98(4)% for the binding affinity. The 17β-hydroxy steroids, with the exception of **23**, are scattered in the center of the plot. 5β-DHT (**23**) has a 17β-hydroxy group but a different configuration (5β) and thus a unique O3···Ox separation; compound **23** lies in the lower left corner of the plot. When steroids **10**, **19** and **24** are excluded, O3···Ox distances for the other steroids exhibit an approximately linear correlation with the binding affinity (the regression line is shown as a dashed line in Fig. 2). The correlation coefficient is 91.9%. Excluding only triamcinolone acetonide (**10**), a high correlation is still obtained, $R = 83.3\%$ (chain-dashed line), while for all steroids the correlation drops to 72.0% (solid line). If the linear correlation is correct, then the positions of steroids **10**, **19** and **24** in this plot need some explanation.

Before explanations are provided for the outliers, the validity of a distance obtained from a single molecular conformation must be considered. Since the 17β-pregnane sidechain may rotate around the C17—C20 single bond, a number of orientations of the C20=O20 carbonyl group are possible for the binding conformer. Duax *et al.* [51, 52] found that all of the pregnanes with a 20-one and a 16β-hydrogen substituent have values for the torsion angle C16—C17—C20=O20 (φ) in the range of 0° to −46°. They also found that these pregnane conformations are normally distributed about the average value, −21°, which they assumed is a minimum energy conformation. The conformations with torsion angles outside the observed range are not favored because of steric hindrance between the C17 sidechain and the methyl group on C13. The torsion angles for the pregnanes included in this study, with the exception of aldosterone which does not have a comparable C17 sidechain, are between 0° and −34° with an average of −22(±2)°. To check the influence of the C17—C20 rotation on the O3···O20 distance, the 17β-sidechain of steroids **1–7** and **10–18** was rotated [44] around the C17—C20 bond to φ-values of 0° and −46°. For $\phi = 0°$ the O3···O20 distance was slightly longer, 11.48–12.00 Å, than the observed crystallographic distance and for $\phi = -46°$ the distance was slightly shorter, 11.18–11.69 Å, but always longer than 11 Å. Thus, the scatter of the pregnanes along the O3···Ox axis in Fig. 2 may be due, to some extent, to the orientation of the C17 sidechain. The scatter may also be due to

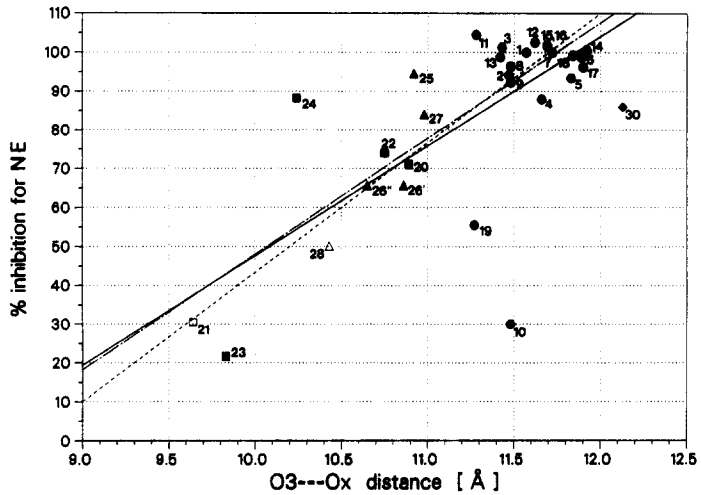

Fig. 2. A plot of the percentage inhibition of [^{3}H]dexamethasone binding at the site on nuclear envelopes versus the O3···Ox distance for all tested steroids except cholesterol. All compounds are numbered as in Fig. 1 and Table 1; circles represent pregnanes; squares, androstanes; and triangles, estranes; the open figures are for steroids with only a O17α substituent on the D-ring; diethylstilbestrol (**30**) is marked with a diamond and is excluded from all regression calculations. Regression lines are: solid—for all steroids shown except **8** and **9**, chain-dashed—excluding **8**, **9** and **10**, and dashed—excluding **8**, **9**, **10**, **19** and **24**.

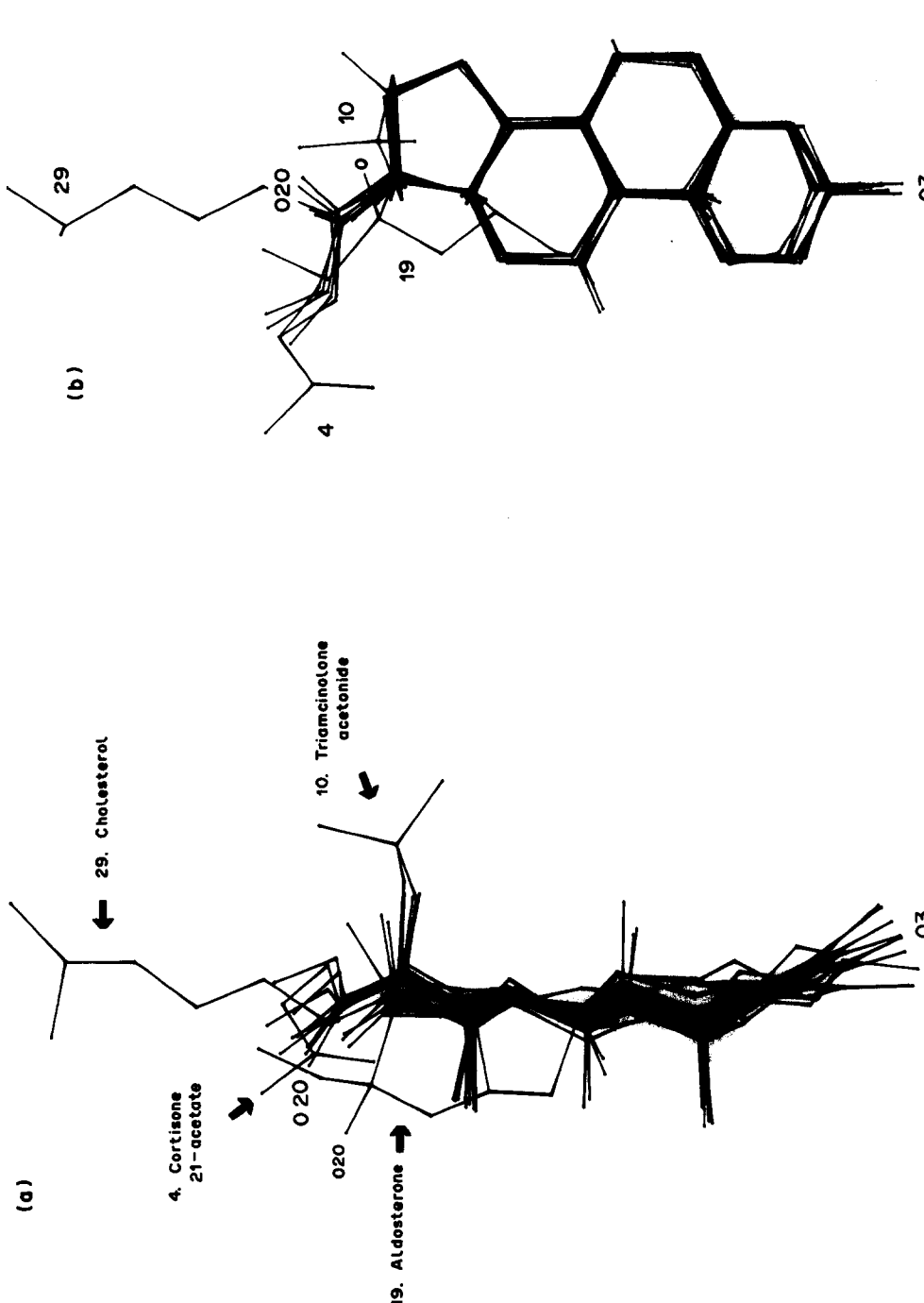

Fig. 3. (a) Superposition of steroids **4**, **10**, **11**, **18**, **19**, **25**, **26**, **27** and **29** with dexamethasone (**1**) viewed from a direction parallel to the average molecular plane. The four compounds which occupy additional space in comparison to the six others are indicated by their numbers. Large lettering indicates the position of atom O20 in dexamethasone and small lettering indicates position of atom O20 in aldosterone. (b) A view perpendicular to that of (a).

differences in the overall bending of the steroid nucleus due to differences in substitution and unsaturation. It can be assumed, however, that the best fit of the steroid to the binding site on the membrane may involve a rotation of the 17β-pregnane sidechain which could lead to a similar O3···O20 distance for all pregnanes.

To explain the steroids whose O3···Ox distances do not lie on the regression line shown in Fig. 2, molecular comparisons of all of the steroids with the structure of dexamethasone were performed. A superposition was calculated with the program PROFIT [43] for all of the atoms of the steroid backbone (C1–C17, C18 and O3) that are common in the structures listed in Table 1 and the results were displayed with MMS [44]. Figure 3 shows two perpendicular projections of the superposition of 10 steroids: **1, 4, 10, 11, 18, 19, 25, 26, 27** and **29**. Four of the steroids shown: cholesterol, aldosterone, triamcinolone acetonide and cortisone acetate are labeled so that the differences between these unique steroids and the common space occupied by the other six molecules are evident. The structural differences between each of these four steroids and the others are discussed below.

The position of the potent glucocorticoid, triamcinolone acetonide (**10**), in the correlation plot (Fig. 2) indicates that it has low affinity for NE, in contrast to the affinity of triamcinolone (**8**); this lower affinity may be due to the bulky acetonide group on the α-side of triamcinolone acetonide as shown in Fig. 3. The NE dexamethasone binding site clearly accommodates

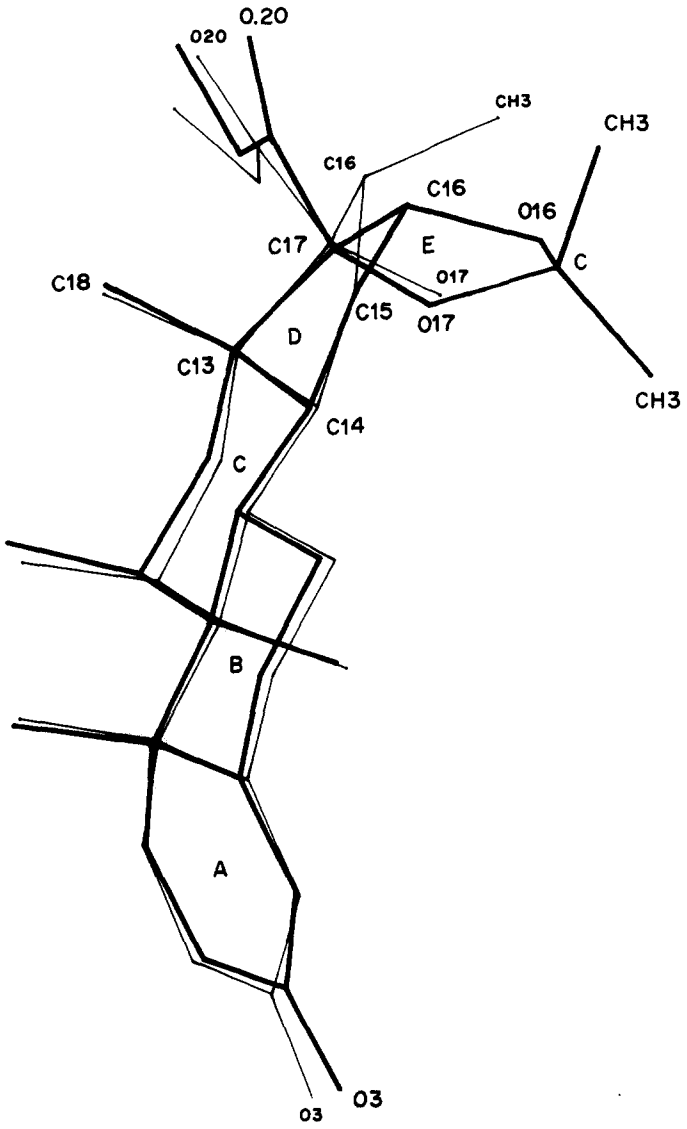

Fig. 4. Superposition of triamcinolone acetonide (thick line) with dexamethasone (thin line).

small substituents in this region: both 16α- and 17α-hydroxy and methyl substituents have little effect on binding affinity; however, the much larger acetonide ring may not be accepted in the site. Another molecule with a large side chain attached to C16 is triamcinolone diacetate (**9**) which has only slightly lower affinity than triamcinolone itself; a reduction in affinity that may be due to 21-acetylation rather than 16α substitution. The structure of **9** has not been determined so the effect of the acetonide group on the molecular conformation can only be postulated from a comparison of the conformations of triamcinolone acetonide [15] and dexamethasone [7]. This comparison, shown in Fig. 4, indicates that the addition of the acetonide ring changes the conformation of the D-ring in the triamcinalone molecule. The conformation of the D-ring in dexamethasone is 13β-envelope and the pseudorotation parameter [53], Δ, is 36.2 and 40.3 in two independent molecules, respectively. In contrast, the additional acetonide ring attached at 16α and 17α in trimanciolone acetonide causes a conformation change of the D-ring to 14α-envelope with a $\Delta = -25.8$. The 14α-envelope conformation is not usually observed in a D-ring consisting of five sp^3 carbon atoms [54]. Thus, if the 16α-methyl group of dexamethasone is replaced with an acetoxy group, to form triamcinolone 16α-acetate, the D-ring should not change conformation and the acetoxy group may not overlap with acetonide group of triamcinolone acetonide. Indeed, the activity of **9** suggests that the 16α-acetoxy group, which is smaller than acetonide and is free to rotate around the C16—O16α bond, occupies less space, or alternatively, different space than does the acetonide group.

Figure 3 shows also a molecule of cortisone acetate (**4**) superimposed with the dexamethasone: the 21-acetyl group of **4** occupies a

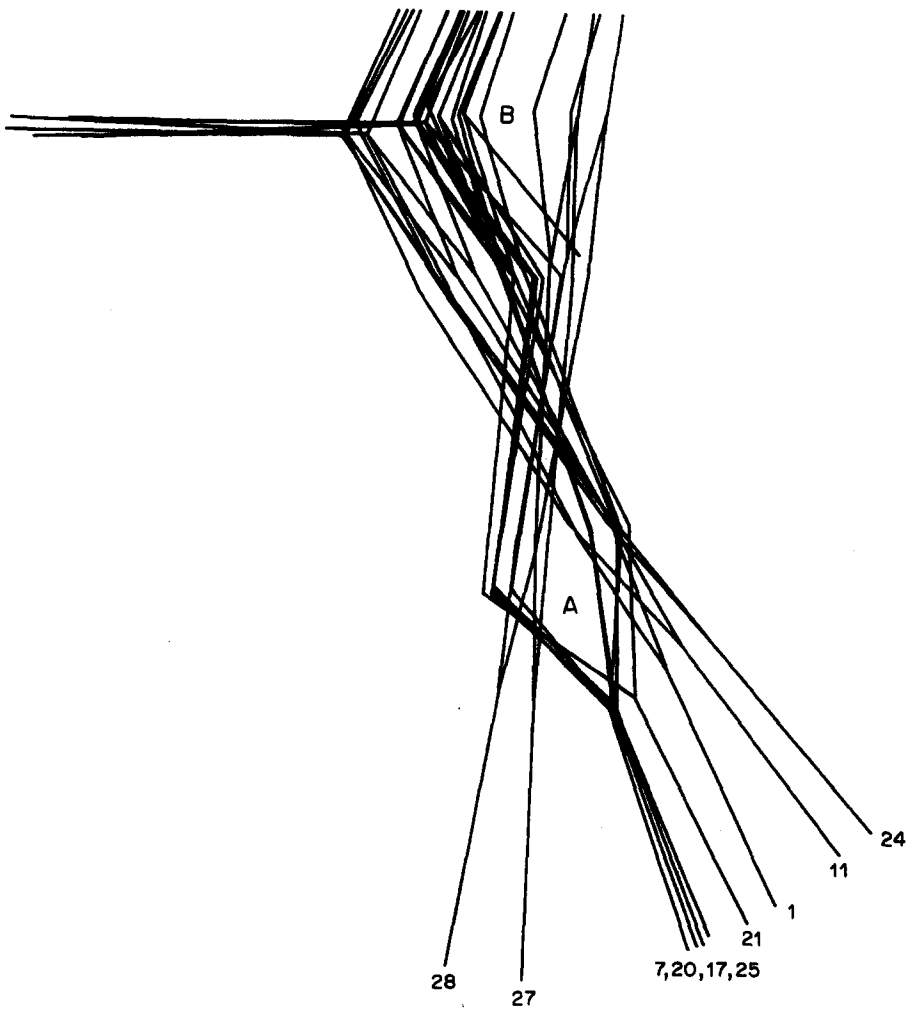

Fig. 5. Superposition of steroids **7**, **11**, **17**, **20**, **21**, **24**, **25**, **27** and **28** with dexamethasone (**1**)—only the A-ring is shown. The O3 atoms are numbered to identify the appropriate steroid.

different space from the common volume of the other 17β-pregnane sidechains. As mentioned above, 21-acetylation produces a decrease in binding affinity, which suggests that the dexamethasone binding site on membranes can not accept large 21-substituents on pregnanes.

Figure 3 also presents the structure of cholesterol with one of the observed conformations of its 17β-aliphatic sidechain [38]. The large space occupied by this sidechain, together with a lack of the oxygen atom at the D-ring terminus of the steroid, may be the reason that cholesterol has low affinity for the NE binding site. Still, some interaction between the steroid and membrane occurs; this could be due to hydrogen bonding through the 3-hydroxy group or due to purely hydrophobic interactions between this steroid and the site.

The binding affinity of 17β-hydroxy-1,4-androstadien-3-one (**24**) is higher than would be predicted from the linear correlation of O3···O*x* distance with affinity. To examine the structural basis for the affinity, the structure of **24** was compared to those of 8 other steroids (**7**, **11**, **17**, **20**, **21**, **25**, **27** and **28**) and to dexamethasone. Examination of the A-rings in this superposition, shown in Fig. 5, shows that androstane has a ring conformation similar to that of prednisone (**11**) and that in both molecules the O3 atom is on one side of the O3 position in dexamethasone while in the majority of steroids, even those not shown in Fig. 5, the O3 position is either close to the dexamethasone position or on the other side. Since prednisone (**11**) has high affinity despite a relatively short O3···O20 distance for a pregnane and androstane (**24**) is also of high affinity compared to the linear correlation, this difference in the orientation of O3 for the two molecules suggests that the preferred orientation of the hydrogen bond from a donor on the membrane to O3 is directed toward the side that favors these steroids (see Fig. 7). Another explanation for the high affinities of steroids **11** and **24** could be the bowing of the A-ring relative to the plane through rings B, C and D which results in an orientation of their semiquinone A-ring that favors a π-stacking interaction with the membrane. The possibility that such an interaction takes place can be supported by the fact that 5β-dihydrotestosterone which also shows the bowing [42], but has a saturated A-ring, has low affinity.

The natural and potent mineralocorticoid aldosterone (**19**) is one of the outliers from the regression line. The results for aldosterone are difficult to interpret because there are more than 5 structural isomers [55] and it is not known which of the isomers is responsible for mineralocorticoid receptor binding and hormone action. Two isomers of aldosterone, the 18-acetal-20-hemiketal and the 11β,18-oxide, were confirmed in solution [56] while the 18R-acetal-20S-hemiketal of aldosterone was stable in the crystal [25]. This crystal structure was used in our comparisons; the structure has the shortest O3···O20 distance, 11.27 Å, of the pregnanes tested. The affinity found for **19** is about 30% lower than the value predicted from the regression line and from the affinity of cortisone acetate (**4**) which has the lowest affinity of the other, similarly substituted pregnanes. The short O3···O20 distance is a result of the incorporation of the aldosterone 17β-sidechain into an additional F-ring; thus, the configuration at atom C20 is sp^3 and O20 is not a carbonyl but a hydroxyl oxygen atom and is located far from the typical position of O20 in other pregnanes. A superposition of the 18R-acetal-20-hemiketal isomer (**19**) with dexamethasone is shown in Fig. 3; the distance between the positions of the O20 atoms is about 1.9 Å. The different location could affect the postulated hydrogen bond interaction with the membrane binding site; additionally, the extra E- and F-rings of this aldosterone isomer could cause steric hindrance on the β-side of the steroid. These differences may explain the lower affinity of aldosterone compared to the other pregnanes; however, the presence of the other isomer must be considered. Another aldosterone isomer, the 11β,18-oxide, was crystallized as a 18R-α-D-glucosiduronic acid tetraacetate methyl ester [57]; it has one extra ring (E), a free 17β-sidechain, and a O3···O20 distance is 12.00 Å. Comparison of the aldosterone 11β,18-oxide derivative with aldosterone 18R-acetal-20S-hemiketal and dexamethasone shows that unique substituents on the 11β,18-oxide derivative do not distort the basic steroid and sidechain conformations; therefore, this crystal structure should be representative of the conformation of this isomer. The O20 atom of this isomer is only 0.3 Å from the O20 atom of dexamethasone when these two molecules are superimposed which suggests that this isomer of aldosterone would have high affinity for the binding site, assuming that the free aldosterone 11β,18-oxide in solution has a similar molecular structure to that found in the crystal. Therefore, the lower

affinity of aldosterone at the dexamethasone binding site on membranes could arise from a mixture of isomers as observed in spectroscopic studies [56]: the 11β,18-oxide which would be as competitive as the other pregnanes and the more stable 18-acetal-20-hemiketal which does not bind well.

The nonsteroidal, synthetic estrogen, diethylstilbestrol (**30**) has the same binding affinity as estradiol-17β even though the O···O distance in **30** is about 1.1 Å longer than in estradiol. Thus, compound **30** exhibits a lower affinity than would be predicted from the linear correlation plotted in Fig. 2. The lower affinity could be due to the nonsteroidal structure of diethylstilbestrol, to steric hindrance from the ethyl sidechains, or to a long O···O distance that exceeds the tolerance of the membrane binding site.

Figure 6 shows a correlation plot for the affinities of steroids for the binding site on plasma membrane (PM); as in Fig. 2 steroids **8** and **9** were included with the O3···Ox distance equal to that of steroid **10**, while they, similarly as the diethylstilbestrol (**30**), were not included in the regression analysis. The correlation coefficient for all steroids is $R = 66.4\%$ (solid line), for all except **10** is 78.5% (chain-dashed line) and for all except **10**, **23** and **24** is 79.2%. The difference between Fig. 6 and Fig. 2 is clear: binding affinities to PM are less dependent on the O3···Ox distance, i.e. the specificity of binding to plasma membranes is lower than to nuclear envelopes. Normalization of the binding data for PM was achieved by multiplying the data so that the inhibition for dexamethasone was equal to 100%. After normalization, all compounds are similarly distributed around the regression line as they are for NE. Interestingly, steroid **23**, which has a different configuration at C5, and steroid **10**, which has a large extra ring perpendicular to the D-ring, show the same low level of affinity to both membranes. This suggests that the residual binding of these two steroids may consist of nonspecific interactions.

Figure 7 summarizes the features of the dexamethasone binding site on membranes which can be proposed on the basis of the structure–activity relationships discussed above. The binding sites consist of two regions which contain possibly hydrogen donors: one close to the O3 atom and the other close to atoms O20 and O17β of steroids. The optimal distance between these two donors, measured between the acceptor oxygen atoms on the ligands, is between 11 and 12 Å; the binding site may also change its conformation slightly to accept ligands with an O···O distance of 10.2 to 11 Å and, possibly, those slightly longer than 12 Å. There are at least two regions in the binding site which will accept only small substituents: at atoms O16α and O17α, and at atom O21. Additionally, we found some evidence for a hydrophobic region which could interact through the π-stacking with the A-ring of

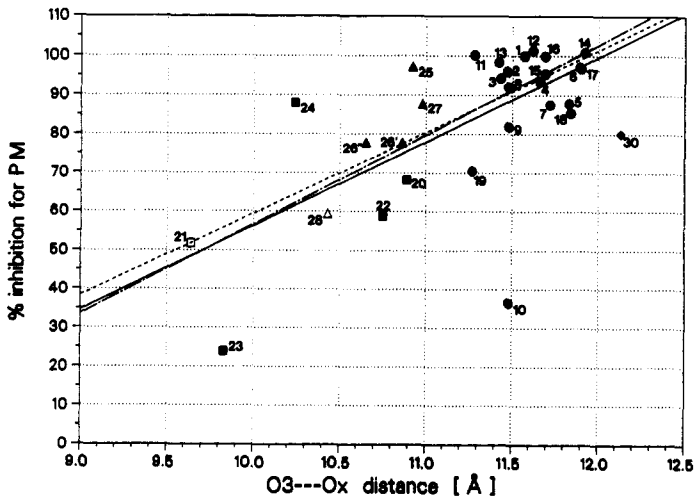

Fig. 6. A plot of the percentage inhibition of [^{3}H]dexamethasone binding at the site on plasma membranes versus the O3···Ox distance for all tested steroids except cholesterol. All compounds are numbered as in Fig. 1 and Table 1; circles represent pregnanes; squares, androstanes; and triangles, estranes; the open figures are for steroids with only a O17α substituent on the D-ring; diethylstilbestrol (**30**) is marked with a diamond and is excluded from the regression calculations. Regression lines are: solid—for all steroids except **8** and **9**, chain-dashed—excluding **8**, **9** and **10**, and dashed—excluding **8**, **9**, **10**, **23** and **24**.

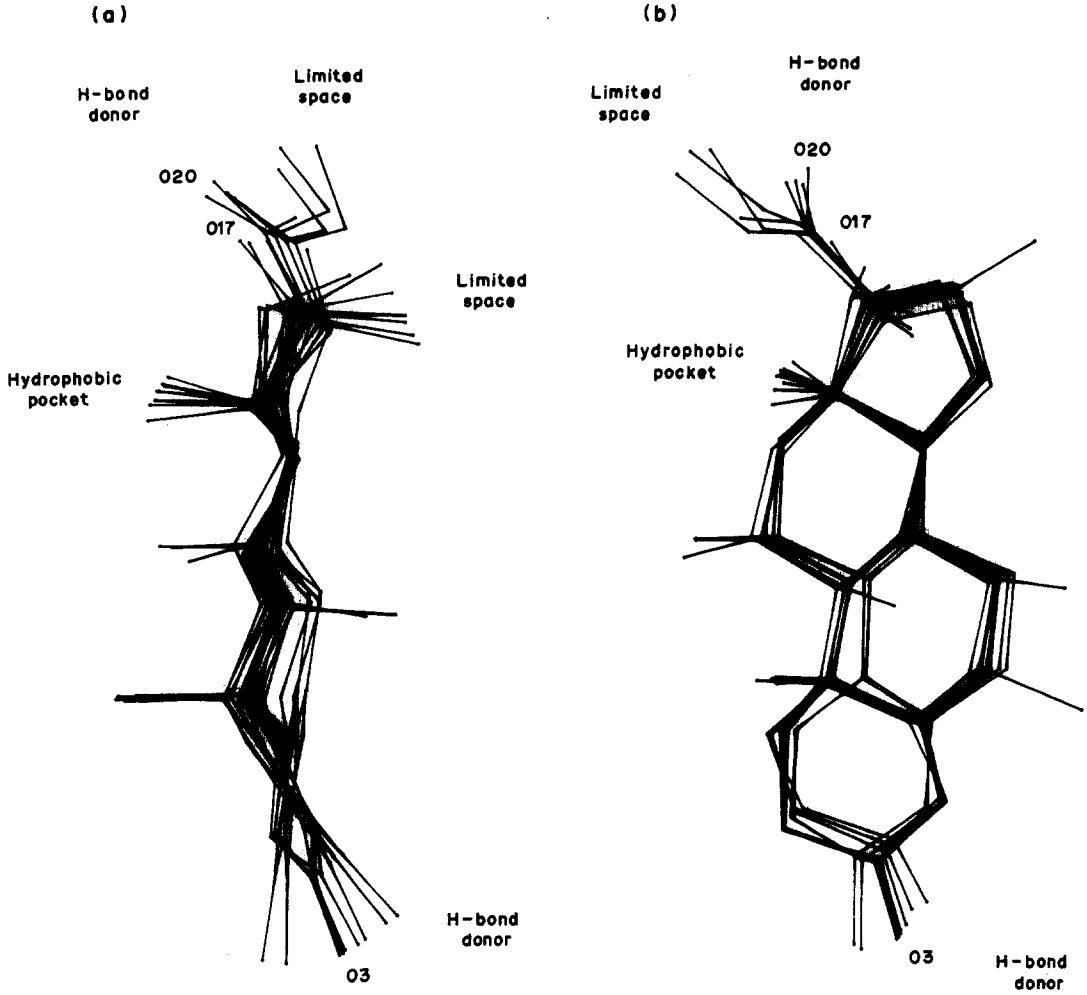

Fig. 7. Features of the dexamethasone binding sites on the nuclear envelopes and plasma membranes shown for steroids in the same superposition as in Fig. 5. The two projections shown: view (a) and view (b), are approximately perpendicular to each other.

steroid. This study shows that the binding site accepts substituents of limited size, like methyl and hydroxy groups or fluorine atoms, at various other positions of the steroid backbone. Binding of 5β-steroids seems unfavorable.

To confirm these features and to further develop this model, it will be necessary to expand the study to many related or significantly altered steroids and to nonsteroidal hormones which interact with steroid receptors and steroid binding sites.

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