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SPECIFICITY OF THE IMMUNE RESPONSE TO THE OESTRONE-AZO-HAPTEN STRUCTURE

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Summary—Comparison of the cross-reactions between 14 related steroids with eight antibodies showed that each antibody had its individual molecular specificity. During the 183-day period covering eight immunizations, cross-reaction between oestrone and 1-methyloestrone showed only very little change with five antibodies, while the great differences in cross-reactivity values for the individual antibodies were retained. With the same five antibodies, the course of cross-reaction between oestrone and 3-sulphooestrone as well as 6-oxooestrone was also fairly constant, the differences in cross-reactivity in relation to the individual antibodies being considerably large. The blood plasma antibody concentrations measured at the same intervals showed considerable fluctuation, whereas the affinity constants of the respective antibodies, except one of them, showed a moderate upward trend. The suggested molecular parameters of binding sites of the antibodies to the oestrone-4-azo-hapten structure were in surprisingly good agreement with those reported in the literature for mouse myeloma immunoglobulin A proteins possessing dinitrophenyl-binding activity. The individual antibodies exhibited highly sensitive radioimmunoassay curves for both oestrone and 3-sulphooestrone.

INTRODUCTION

Recognition and binding of a steroid ligand by membrane receptors of lymphocytes or target tissue cells are the primary impulses leading to the intracellular signal and subsequent synthesis of specific protein. High-affinity contact of the steroid ligand with the receptor requires a high degree of structural complementarity. Molecular details of the binding interaction of the steroid and its receptor undoubtedly have a direct bearing on receptor affinity and will directly or indirectly influence the triggering of the respective type of biological response [1].

It has been generally accepted that in immunological systems the membrane receptor and the secreted antibody have the same binding properties with respect to a given antigen [2]. According to this concept the structural relations between antigen and antibody reflect with high reliability the details of contact interactions of the antigen and the receptor binding site [3]. The aforementioned specific interactions can be studied remarkably well in complementary steroid–protein systems by means of radiosaturation methods which have been intensively used particularly in endocrinology.

A generalization from the results of cross-reactions in oestrogen-azo-hapten systems in our previous studies prompted us to formulate the molecular nature of the antigenic determinant of these steroid haptens and of the corresponding antibody combining sites [4,5]. The present study extends our previous observations by dealing in more detail with some phenomena of the immune response to oestrone-azo-thyreoglobulin immunogen and comparing them in their dynamic development. Particular attention is given to (a) changes in the average antibody affinity constant in the course of long-term immunization, (b) absence of change in the specificity of antibodies in the course of long-term immunization and (c) definition of the specific areas involved in the response to the oestrone-4-azo-hapten structure.

EXPERIMENTAL

The solution, chemicals, unlabelled steroids and their systematic nomenclature and instruments were the same as those described in previous work [4]. 1-methyloestrone (1-methyl-E_1) and 3-sulpho-E_1 were obtained through the courtesy of the Medical Research Council, London, England.

The immunization of Californian white rabbits and the preparation of immunogen were as previously described [4]. In each group of 5 immunized rabbits the first three animals were males and the remaining two were females (Table 1). For emulsification of the immunogen, Al-Span-Oil Adjuvant (Sevac, Prague, Czechoslovakia) and Freund's complete adjuvant were employed. Al-Span-Oil Adjuvant is a two-component system. Component I is an aqueous suspension of aluminium hydroxide, component II contains paraffin oil of high purity and a detergent of a suitable hydrophil–lipophil equilibrium value. The emulsion with Freund's complete adjuvant was prepared from 7.5 g of heat-killed Mycobacterium tuberculosis organisms, 7.5 ml paraffin oil and 4.5 ml lanolin. To assess the effect of antibody production and

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Table 1. Modes of immunogen treatment before immunization*

Rabbit nos.	1–5	6–10	11–15	16–20	21–25
Immunogen dose (μg)	80	80	320	80	80
Adjuvant stimulation	Al-Span-Oil	$Al(OH)_3$	$Al(OH)_3$	Buffer (controls)	Freund's complete adjuvant

*Rabbits Nos 1–5 and 21–25 were injected with the immunogens after emulsification in the complete adjuvant. Rabbits Nos 6–15 received the immunogens merely adsorbed into a suspension of aluminium hydroxide.

specificity of each of the two Al-Span-Oil Adjuvant components, rabbits were injected with the immunogen either emulsified in the complete adjuvant or merely adsorbed onto component I (omitting the oil component).

Determination of affinity constants

Affinity constants (equilibrium constants) were determined by Scatchard plots using radiosaturation analysis. Antibody dilution was made so that 40–60 pg of [2,4,6,7-^{3}H] E_1 would saturate the antibody sites of 0.5 ml antisera solution. The resultant binding data were analysed by a computer employing the following equation:

$$B/F = K(q) - K(B)$$

where (B) is the concentration of E_1 in mol/l bound at equilibrium, F is the concentration of free E_1 at equilibrium, (q) is the concentration of antibody sites in mol/l at equilibrium and K is the affinity constant (1/mol). The linearity of this relation was defined by correlation coefficients whose values for $n \geqslant 6$ exceeded 0.96 in all cases. In calculating the antibody concentration it was assumed that 2 mol steroid are bound per mol antibody.

The other methods used in this study were described elsewhere [4].

RESULTS

The immune response to oestrone-4-azo-thyreoglobulin (E_1-4-azo-TG) was studied in 25 rabbits (Table 1). Absorption spectrum of E_1-azo-TG in phosphate buffer, pH 7.4, showed a less symmetrical peak in the visible area ($\lambda_{max} = 430$ nm) than that observed with the albumin immunogen used in our previous study [4]. This was caused by low absorption of the carrier TG at about 416 nm. The degree of substitution was determined spectrophotometrically using $\epsilon = 21.7 \times 10^3\,l \cdot mol^{-1}\,cm^{-1}$ (buffer pH 7.0, $\lambda = 430$ nm). The E_1-4-azo-TG used for immunization contained 20.2 mol steroid/mol immunogen.

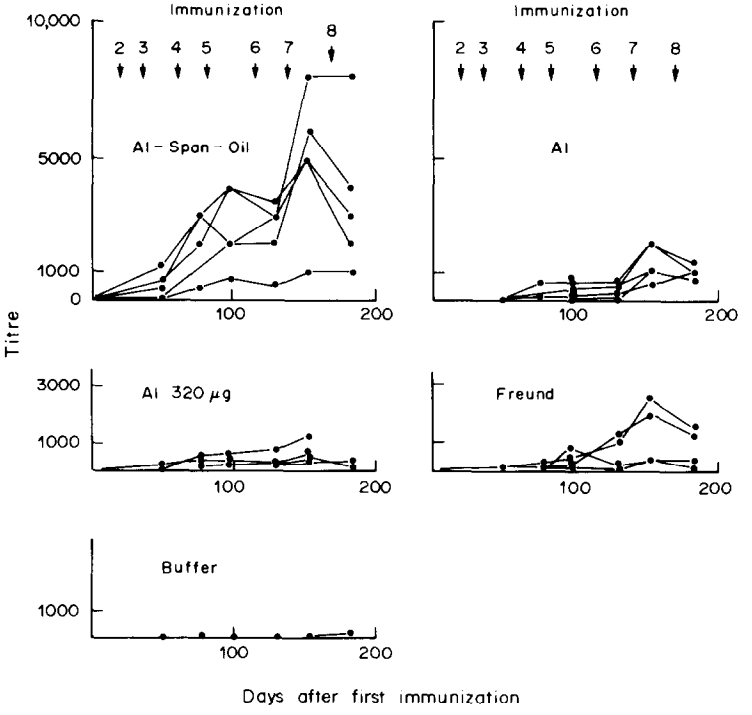


Fig. 1. Effect of adjuvant factors on antibody titres. The antibody titre is defined as the highest antibody dilution at which approx 50% of the radioligand is bound to this antibody under the given conditions of the immunological reaction.

Each of the differently pretreated immunogens was administered to a group of 5 rabbits. The effects of the different adjuvant stimulators on antibody production is shown in Fig. 1. It can be seen that the highest antibody titres were produced in the group of rabbits injected with the immunogen emulsified in complete Al-Span-Oil Adjuvant. Rabbits that were immunized omitting the oil component of Al-Span-Oil Adjuvant, showed a lower antibody production, whether the dose was 80 μg immunogen per animal or 4-fold higher. A similar pattern of results was obtained in rabbits injected with the immunogen emulsified in Freund's complete adjuvant. Control rabbits injected with the immunogen dissolved in phosphate buffer developed a weak antibody response only after the 7th immunization.

Cross-reactions of the homologous E_1 with structurally related ligand were determined according to Abraham [6]. The ligands differed from E_1 by various chemical changes in the A, B and D rings. The positions of these changes are shown in Fig. 2. It can be seen that the modification of the skeleton or functional groups covered areas variously distant from C-4 position, i.e. from the bridge. The large number of changes in the A and B rings permitted a large-scale investigation of the inhibitory properties

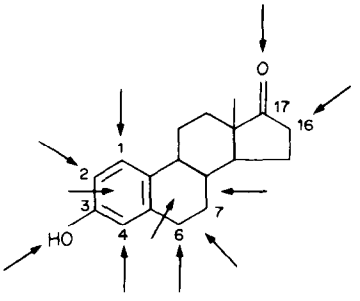
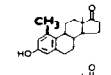
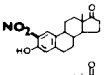
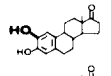
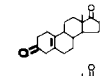
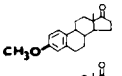
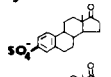
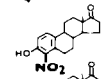
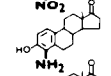
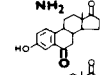
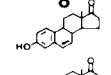
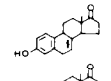
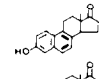
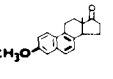


Fig. 2. Sites of chemical modification in the E_1 ligand.

of a variety of structures in the [^{3}H]E_1-antibody system.

The cross-reactivity values for 8 antibodies that were produced in response to the differently pretreated immunogens (see Table 1) are presented in Table 2. The chemical modifications of the E_1 structure are indicated by thickened symbols. All test antibodies were collected on the 183rd day after primary immunization. A wide variety of cross reactions (1.0–79.6%) were exhibited by the antibodies with 1-methyl-E_1. The presence of the nitro group or hydroxy group at C-2 position led to decreased cross-reactivity in four antibodies. On the other hand,

Table 2. Cross-reactions of E_1 and its derivatives with eight antibodies against E_1-4-azo-TG*

Steroid	Ab.1	Ab.3	Ab.4	Ab.5	Ab.7	Ab.12	Ab.22	Ab.23
	1.1	79.6	21.4	68.7	1.0	55.2	16.2	2.3
	1.95	5.35	13.4	5.8	2.8	17.9	29.9	5.9
	4.4	11.6	1.2	3.2	2.7	4.8	5.95	15.6
	4.4	15.8	24.8	26.3	57.5	82.0	48.1	97.9
	31.1	63.7	35.0	74.5	84.3	59.7	54.0	54.9
	97.0	43.4	12.2	45.45	86.9	49.1	48.5	100.0
	60.8	46.1	27.7	69.0	79.7	78.0	57.2	61.5
	32.8	21.4	22.8	11.55	6.0	17.1	35.1	8.2
	95.2	2.9	121.0	5.1	127.1	21.1	110.0	100.0
	80.9	21.3	26.8	18.0	84.3	20.0	59.6	7.1
	2.65	1.4	5.9	0.7	0.9	2.45	3.2	2.5
	28.5	4.6	37.2	2.5	16.8	6.3	7.1	0.8
	2.9	0.7	2.6	0.5	2.0	1.2	1.4	0.2

*Antibody Nos. correspond to rabbit Nos. in the individual groups given in Table 1. The antisera were obtained on the 183rd day after the immunization was started.

these antibodies, with the exception of antibody No. 1, failed to recognize even such a great change as is the A-ring de-aromatization with a concurrent change of 3-OH → 3 = 0. This is in keeping with our previous observations [4, 5]. Approximately an equal range of cross-reaction values were found for 3-methoxy, 3-sulpho and 4-nitro-E_1, whereas the 4-amino-derivative showed, on average, less inhibition. As with 1-methyl-E_1, vast differences in individual cross-reaction values were found for 6-oxo-E_1 where the range was 2.9–127.1%. All the antibodies discriminated between positions of the adjacent double bands in the B ring. Unsaturated 6-en derivative showed much more capacity to inhibit the [^{3}H]E_1 binding to the antibody than did 7-en E_1. Aromatization of the B ring did not produce any marked reduction of cross-reactivity in three antibodies (Nos 1, 4 and 7). A decrease in cross-reaction with this ligand was observed only after the methoxy group was introduced at C-3 position.

In addition to the ligands listed in Table 2, an assessment was made of cross-reactions with ligands modified within the D-ring: in most cases the cross-reaction values for 16α-hydroxy-E_1 and 17β- or 17α-oestradiol did not exceed 1%.

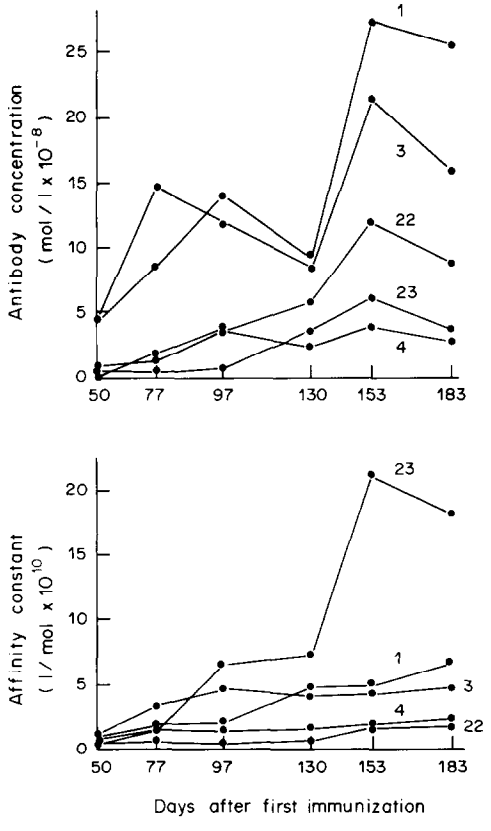


Fig. 3. Upper: Development of the blood plasma antibody concentration in rabbits during long-term immunization. Lower: Development of the affinity constants during long-term immunization of the same animals. The figures with which the individual curves are marked denote the rabbits (see Table 1 or 2) from which the respective antisera were obtained.

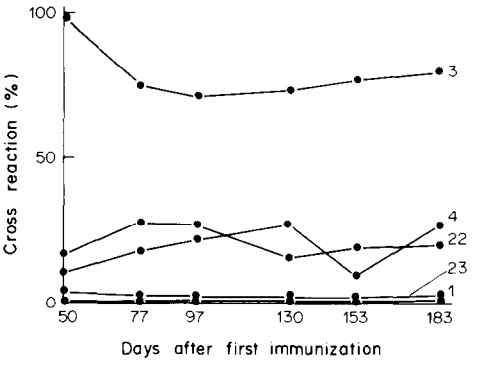


Fig. 4. Development of the cross-reaction with 1-methyl-E_1 during long-term immunization. The figures with which the individual curves are marked denote the rabbits (see Table 1 or 2) from which the respective antisera were obtained.

In 5 rabbits we studied the dynamics of the antibody levels and their affinity constants over a 183-day period covering eight immunizations. Blood plasma samples were obtained at 10–15 days after each immunization. It can be seen from Fig. 3 that the blood plasma concentrations of these antibodies fluctuated within the range of $0.2–27.0 \cdot 10^{-8}$ mol/l and peaked invariably on the 153rd day after the first immunization. In keeping with the theory of antibody maturation during the course of long-term immunization there was a moderate upward trend in the affinity constant values (Fig. 3). The affinity constant of the antibody of rabbit No. 23 showed a marked difference from those of the other antibodies presented in Fig. 3; its peak on the 153rd day after the first immunization reached a value as high as $2.1 \cdot 10^{11}$ l/mol.

At the same point of time we assessed the dynamics of the changes in cross-reaction with the ligands in which chemical modification was made at C-1, C-3 and C-6 positions. During the 183-day period covering eight immunizations very little change was observed in the cross-reactions between E_1 and 1-methyl-E_1 with all the antibodies (Fig. 4) while the great differences in cross-reactivity values between the individual antibodies were retained. Thus antibody No. 3, e.g. showed a cross-reactivity range of 71.0–100%, whereas the cross-reactivity for antibodies Nos 1 and 23 during the same period did not exceed 3%. Similar results were obtained for 3-sulpho-E_1 as well as 6-oxo-E_1. Thus antibody No. 3, e.g. exhibited extremely low cross-reactivity with 6-oxo-E_1 (less than 3%), whereas the cross-reactivity shown by the remaining antibodies ranged from 42 to 155% (Fig. 5). A remarkable rise in cross-reaction was found with 3-sulpho-E_1 in antibody No. 23 (Fig. 6). This antibody was distinguished in addition by the aforementioned marked increase of the affinity constant with time. This trend was not observed with the other antibodies.

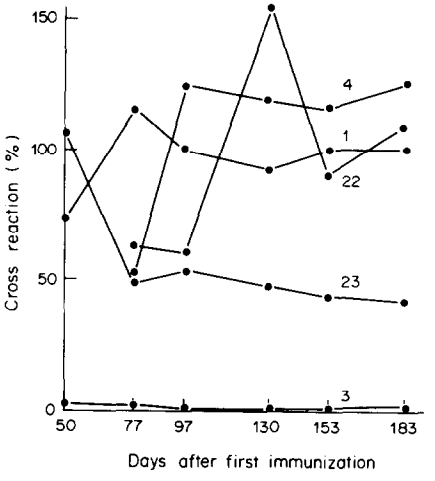


Fig. 5. Development of the cross-reaction with 6-oxo-E_1 during long-term immunization. The figures with which the individual curves are marked denote the rabbits (see Tables 1 or 2) from which the respective antisera were obtained.

The antibodies listed in Table 2 bound, as expected, ^3H labelled 3-sulpho-E_1, E_1-2(4)-iodo- [^{125}I] and E_1-6-o-(CMO)- [^{125}I] iodohistamine. These radioligands were displaced remarkably well by both E_1 and 3-sulpho-E_1. Radioimmunoassay curves representative of the heterologous combination of E_1-6-o-(CMO)- [^{125}I]iodohistamine and anti-E_1-4-azo serum are presented in Fig. 7. The two curves followed a similar pattern, showing a high degree of sensitivity.

DISCUSSION

In previous years we synthetized oestrogen-azo-protein conjugates and prepared antibodies against these immunogens, the characteristics of which were worthy of note both from the practical and theoretical points of view. The cross-reaction results were interpreted by us first with respect to formal chemical aspects (tautomerization of hapten groups) [7] and then in the light of the immunological concept of antigenic determinants and antibody binding sites [4]. The present study provides extended information on the E_1-azo-hapten model particularly in that it deals with the antibody response and characteristics based on a larger number of animals and specifies some details of the E_1-antibody interaction, paying attention to the concurrent development with time of the major parameters of the antibody concentration, affinity and cross-reactivity.

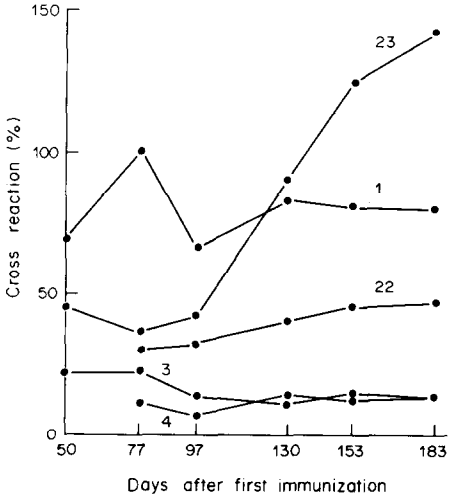


Fig. 6. Development of the cross-reaction with 3-sulpho-E_1 during long-term immunization. The figures with which the individual curves are marked denote the rabbits (see Table 1 or 2) from which the respective antisera were obtained.

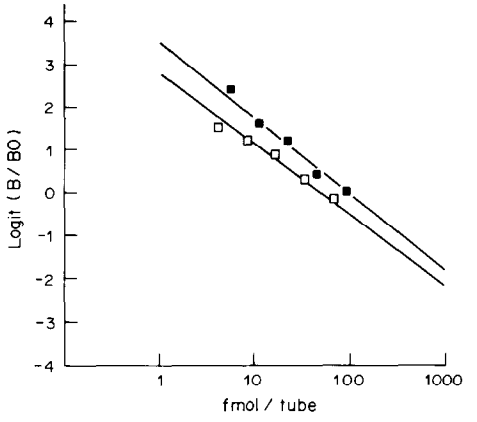


Fig. 7. Radioimmunoassay curves representative of E_1 and 3-sulpho-E_1 using E_1-6-O-(CMO)-[^{125}I] iodohistamine. Antibody against E_1-4-azo-TG bound 50% of iodinated radioligand at the final dilution of 1:6000. The smallest amount of steroid standard that was significantly different from zero at the 95% confidence limit was 0.63 pg (2.34 fmol) for E_1 (■——■) and 0.44 pg (1.20 fmol) for 3-sulpho-E_1 (□——□). Each point is the mean of two determinations.

In the previous experiments with the E_1-azo-protein immunogens we found moderately higher antibody titres when the oil component of Al-Span-Adjuvant was omitted [4]. However, the present evidence shown in Fig. 1, is to the contrary: the antibody titres obtained using complete Al-Span-Oil Adjuvant were markedly higher than those obtained after mere adsorption of the immunogen onto the aluminium hydroxide suspension. In our view, these contradictory results are due mainly to differences in the genetic endowment of the individual animals or whole litters. Therefore any general prognoses or interpretations concerning antibody production would be made with caution and on the basis of sufficiently large numbers of animals.

The data presented in Table 2 show fundamental differences in cross-reaction between 13 structurally related steroids and 8 antibodies. Comparison of the cross-reactions in the individual columns of Table 2 shows that each of the eight antibodies had its individual molecular specificity. In this connexion it should be noted that the distribution of specificity in these antibodies was unaffected by either the immunogen pretreatment or dose (see Table 1).

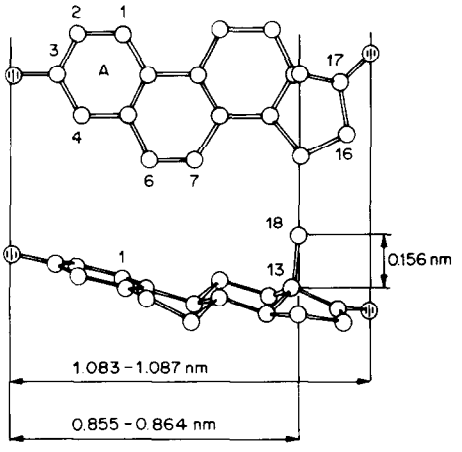


Fig. 8. Crystal structure of E_1 representative of three crystal forms of very similar interatomic distances. The upper drawing shows the skeleton in the plane of the paper viewed from above. The lower drawing is the lateral view in a plane approximately perpendicular to the paper (courtesy of Duax and Norton, 1975). The ranges of the interatomic distances were added to the drawing by the present authors on the basis of the data presented in the book cited above.

The present results show that in assessing the size of the specific binding region of antigen, i.e. antigenic determinant, consideration must be given to the complexity of the respective spatial model. This can be illustrated by the example of different cross-reactions with 1-methyl-E_1 and the 2-nitro- or 2-hydroxy-derivatives, i.e. for the skeleton region around C-1 and C-2. The surprisingly higher cross-reaction values for 1-methyl-E_1 than for the 2-nitro- and 2-hydroxy-derivatives can apparently be interpreted only in the light of stereochemical considerations. In our previous study [4] the results of cross-reactions were generalized to the effect that in the E_1-azo-hapten structure the antigenic determinant area can be ascribed to the size not exceeding 2–3 steroid rings. A point of particular interest in this connexion is the inhibition by 1-methyl-E_1 because its relative strength, expressed in terms of percent cross-reaction, may provide important information on the degree of specificity around C-1. Our testing of cross-reactions with 1-methyl-E_1 in the present study showed that the high-specificity area included also part of the A ring in some antibodies, but not in others. The B-ring region can also be characterized as one with marked differences in specificity. This was documented here particularly for 6-oxo-E_1 and by the case where the double bond situated at the adjacent position resulted in a marked change in cross-reaction (Table 2). The ability of antibody to recognize the position of the double bond, which was demonstrated also in other laboratories [8, 9], can be interpreted in two ways: either the discriminating ability of the antibodies was so high that they recognized even such a small change as is the presence of 2 hydrogen atoms in the B ring or the fit between the unsaturated 6-en ligand and the antibody binding site became tighter in consequence of conformational flexibility which was demonstrated in unsaturated steroids by analysis of X-ray crystallographic data [10, 11]. Although the evidence for the immunodominant role of the D and C rings was obtained only through the ligands modified at C-17 and C-16, general immunological experience allows us to assume that most of the total binding energy is due to this immunodominant part of the skeleton that is most distant from the bridge. More detailed mapping of this region could not be carried out because of the lack of suitable derivatives for cross-reactions.

Our inferences regarding the molecular size of the binding site against the E_1-azo-hapten structure are in remarkably good numerical agreement with the molecular parameters suggested for the binding sites of various mouse myeloma immunoglobulin A proteins that possess dinitrophenyl binding activity [12]. Having compared the dimensions of the combining sites of various myeloma proteins by means of spin-labelled dinitrophenyl haptens, Willan *et al.*[13] found that the depths of the sites were 1.1–1.2 nm, the minimal dimensions of the narrower part were $0.8 \times 0.3 \times 0.8$ nm and that certain differences existed in the lateral dimensions at the entrance to the sites. This entrance is formed by an asymmetrical larger space of minimally $0.8 \times 0.75 \times 0.3$ nm. From our comparison of the interatomic distances in the E_1 ligand (Fig. 8) with the dimensions of the binding sites possessing dinitrophenyl binding activity the following interesting points emerged:

(1) The molecular length of the steroid hapten is approximately equal to the depth of the binding site for the dinitrophenyl ligand.

(2) The narrower part of the dinitrophenyl-binding site corresponds to the size of 2–3 steroid rings which we assume to have a high degree of structural complementarity.

(3) The narrower part of the binding site having 0.3 nm in width is sufficient to accommodate the C and D rings with the C-18-methyl group whose binding axis is approximately perpendicular to the steroid plane and is 0.156 nm long.

The fact that the antibodies prepared in our laboratory showed a remarkable constancy of specificity over the course of 183 days indicates antigenic stimulation of one population of memory cells from which plasma cells, secreting presumably the bulk of antibody, are differentiated. The slight departures from the constant specificity level may have been caused by technical shortcomings such as the fact that we were not able to follow the inhibition reactions invariably under conditions of exactly 50% ligand binding to the antibody. Of particular interest are the properties of antibody No. 23 which showed a marked rise in affinity concurrently with an increase in the cross-reactivity with 3-sulpho-E_1. From this unique case it appears that certain cross-reactions may serve as an

important structural parameter for correlation studies of affinity and immunological specificity.

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