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THE RELATIONSHIP OF SPECIFICITY TO AFFINITY OF ANTI-HAPTEN SERA

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SUMMARY

Antisera raised against 17β-oestradiol-6-(*O*-carboxymethyl) oxime-bovine serum albumin was fractionated by partial immunoadsorption on affinity chromatography media. Fractionation yielded low affinity pools which possessed higher specificity towards the homologous hapten than that of higher affinity pools, suggesting that a decrease in affinity leads to higher specificity. The implications of these findings are discussed.

INTRODUCTION

Antisera consist of an heterogeneous collection of antibodies all with different affinity and specificity towards the antigen which elicited its synthesis, and with graded affinities (cross reactivity) towards structurally related compounds. The stereo-complementarity of the antigenic determinants is thought to provide the major contribution towards specificity.

Investigations in this laboratory into the posssibility of obtaining antibodies possessing both parameters of high affinity and high specificity for use in the haptenic radioimmunoassay of steroids and immunisation studies, led us to devise affinity chromatography immunoadsorption techniques for the fractionation of antisera. Goat anti-oestradiol-17β-6-(*O*-carboxymethyl) oxime-BSA sera* was fractionated by partial immunoadsorption into various affinity pools, and the specificity of the respective pools tested. Although numerous studies have characterised fractionated antibody pools, the relationship between the affinity and specificity of these has not previously been critically assessed.

The present reports present data about this relationship and discusses the implications of the findings.

* The trivial names and symbols of the steroids mentioned in the text are given below with their corresponding I.U.P.A.C names.

Symbol	*Trivial name*	*I.U.P.A.C.*
E_1	oestrone	3 hydroxyl-1,3,5 (10) oestratriene-17-one
E_2	oestradiol-17β	1,3,5 (10)-oestratriene-3-17β diol
$E_2$17α	oestradiol-17α	1,3,5 (10)-oestratriene-3-17α diol
E_3	oestriol	1,3,5 (10)-oestratriene-3-16α-17α-oestriol

Glossary of symbols used for steroid derivatives in the text are:

$E_2$6K	6 Keto-oestradiol-17β
$E_2$6CMO	Oestradiol-17β-6-(*O*-carboxymethyl) oxime
$E_1$6CMO	Oestrone-6-(*O*-carboxymethyl) oxime
$E_1$3HS	Oestrone-3-hemisuccinate
$E_1$17CMO	Oestrone-17-(*O*-carboxymethyl) oxime
$E_2$17HS	Oestradiol-17-hemisuccinate

METHODS AND MATERIALS

Materials

Water. All water was deionised and redistilled from glass.

Chemicals. Obtained from British Drug Houses, Poole, Dorset, U.K. were all of Analar grade.

Assay buffer. (pH 7.0) 0.1 M Was prepared as described by Abrahams[1]. Two stock solutions of sodium phosphate buffer were used. Solution A was 0.2 M NaH_2PO_4 and solution B 0.2 M Na_2HPO_4 in water. The assay buffer itself consisted of 195 ml of solution A; 305 ml of solution B, 1 gm of sodium azide and 9 gm of sodium chloride made up to 1 litre with water.

Assay gelatin buffer. 1 gm Of gelatin was added per litre of the assay buffer above for the radioimmunoassay techniques used for assessing the average affinity constant (K_0) and for the specificity tests.

Norit A Charcoal was obtained from Sigma Co., London, U.K. Dextran T40 was obtained from Koch–Light Laboratories, Colnbrook, Bucks, U.K.

Steroids were obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Radioactive [2,4,6,7-^{3}H]-oestradiol-17β (85 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K.

Steroid derivatives were prepared for conjugation to AH-Sepharose-4B as ligands in the affinity chromatographic immunoadsorption. The hapten, oestradiol-17β-6-(*O*-carboxymethyl) oxime ($E_2$6CMO) was synthesised according to the method of Dean, Exley and Johnson[2]. Oestrone-6-(*O*-carboxymethyl) oxime ($E_1$6CMO) was prepared by the method of

Dean, Rowe and Exley[3]. Oestrone 3-hemisuccinate ($E_1$3HS) was prepared according to Exley and Woodhams[4] and oestrone-17-(*O*-carboxymethyl) oxime ($E_1$17CMO) according to the method of Erlanger *et al.*[5].

Immunoadsorbents. AH-Sepharose-4B was obtained from Pharmacia, Uppsala, Sweden. 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Sigma, St. Louis, U.S.A. Immunoadsorbents [^{3}H]$E_2$6CMO-AH-Sepharose-4B etc. were prepared by carbodiimide (EDC) condensation using a modification of the method of Cuatrecasas and Affinsen[6]. The non-covalently bound [^{3}H]$E_2$6CMO was removed from the AH-Sepharose-4B by the washing programme devised by Exley and Avakian[7].

The final products were dried on filter paper and an aliquot combusted and the radioactive [^{3}H]-H_2O counted. This enabled the μM of conjugated hapten per ml of swollen-Sepharose-4B gel to be calculated. The small amount of radioactivity incorporated into the gel was insignificant and did not interfere with any of the subsequent tests.

Immunoadsorbents were stored in assay buffer at 4°C until use.

Antisera. Antisera for this study was raised in a male goat by immunisation with $E_2$6CMO-bovine serum albumin [8]. A 2 mg dose was given for the primary immunisation and this was followed up by monthly 1 mg boosts. Serum collected 13 weeks after immunisation was used because it had a reasonably high titre (1/30,000) (dilution which bound 50% of a 15 pg load of [2,4,6,7-^{3}H]-oestradiol-17β). S.A. 85 Ci/mmol, and high affinity ($K_a = 5 \times 10^9$ L/M). 10 ml Of above serum was partially purified by precipitation of the immuno-globulin fraction with 50% $(NH_4)_2SO_4$. The precipitate was dissolved in 5 ml 0.5% NaCl solution containing 0.1% sodium azide and exhaustively dialysed against 5 l. of this solution. The final product was lyophilised. 120 mg Of the lyophilised material was dissolved in 6 ml of assay buffer ready for the partial immunoadsorption experiments. This was stored at 4°.

Partial immunoadsorption. 0.5 ml Quantities of the above partially purified antisera (10 mg of material) were added to 0.4 ml of the respective Steroid-AH-Sepharose-4B immunoadsorbent, and the small tube kept at 20° for 30 min and then cooled to 4° and the immunoadsorption allowed to carry on for a further 30 min. Slow but constant shaking was maintained during this hour period. A control blank for these immunoadsorption studies was run, in which 0.5 ml quantity of the antisera was shaken with AH-Sepharose-4B only. This helped to check non-specific adsorption on the affinity chromatography media. The filtrate was then removed by a small syringe barrel fitted with porous polythene, the immunoadsorbent washed twice with 2 ml assay gelatin buffer, and the final vol. made to 5 ml with assay gelatin buffer and then stored at 4°.

Affinity measurement

The average equilibrium constant (association or affinity constant) (K_a) to E_2 of the various antibody pools in the respective filtrates was determined by the saturation curve technique [9].

(i) *The saturation curve technique.* The affinity constant (K_a) is defined as the reciprocal of the free hapten concentration (mol/l) at half saturation of the antibody sites, i.e. since $K_a = [HB]/[H]\ [B]$, then when $[HB] = [B]$, $K_a = 1/[H]$, where $[HB]$ is the molar concentration of antibody bound hapten, $[H]$ is the molar concentration of the free hapten.

A modification of the dextran coated charcoal method of Hotchkiss *et al.*[10] was used to obtain the radioimmunoassay data for construction of the respective saturation curves. 0.1 ml Amounts of phosphate gel buffer containing various amounts of [2,4,6,7-^{3}H]-E_2 were added to 0.1 ml of a suitably diluted concentration of antisera. The mixture was equilibrated for 16 h at 4°. 1 ml Of dextran coated charcoal solution was then added, the tubes shaken and then stood in ice for 10 min, then centrifuged after which 1 ml of supernatant was removed for counting. A rapid two-point assay using the above technique was devised for quick assessment of the amount of [^{3}H]-E_2 needed for saturation. This allowed the dilution of each filtrate after immunoadsorption to be adjusted so that each affinity constant determination could be made with approximately 100 pg of [^{3}H]-E_2 needed for saturation. This kept the concentration of antibodies used for each affinity constant determination approximately the same.

(ii) *Scatchard plots.* The filtrates were further diluted for the determination of specificity. Dilution was made so that about 40 pg of E_2 would saturate the antibody sites of 0.1 ml of antisera solution. The affinity constants to E_2 for the antibody pools in the various filtrates were re-assessed to find the effect of dilution and to give a further check on the previous method. These re-assessments were made by Scatchard plots using the actual radioimmunoassay data obtained in the specificity tests. The resultant binding data was analysed employing the following form of the equation.

$B/F = K_a[n] - K_a[B]$.....Scatchard[11] where $[B]$ is the concentration of E_2 in mol/l bound at equilibrium. F is the concentration of free or unbound E_2 at equilibrium and $[n]$ is the concentration of antibody sites in mol/l at equilibrium and K_a is the affinity constant (l/mol). Plots of B/F against $[B]$ enabled K_a and n to be calculated.

Specificity measurement

Specificity was assessed using a modification of the same radioimmunoassay procedure described for the construction of saturation curves. 17 pg Of [^{3}H]-E_2 (85 Ci/mmol) was used as label. 0.1 ml aliquots of anti-sera (suitably diluted to bind approximately 40 pg E_2 at saturation) were used to obtain the radioimmunoassay data of percentage bound label against

log dose. 0.1 ml Of the respective antisera was incubated with 0.1 ml of radioactive label and 0.1 ml of respective standard or cross reactant for 1 h at 37° followed by 1 h at 4° before addition of 1 ml of dextran coated charcoal and counting. These incubation conditions have been shown in our laboratory to provide identical radioimmunoassay data to that obtained by incubating at 4° overnight. The charcoal separation was performed as previously by shaking in ice for 10 min and centrifuging for 10 min.

The specificity of the antibody pools in the respective filtrates was determined relative to the part hapten E_2, and relative to the homologous hapten $E_2$6CMO. These relative specificities were assessed as relative equilibrium constant ratios $R = K_c/K_s$ where K_c is the equilibrium constant for the respective cross reactant and K_s is the equilibrium constant for the standard (E_2 or $E_2$6CMO). The equilibrium constants for the standards i.e. affinity constants K_a were determined for the respective filtrates by assessment from Scatchard plots as discussed above. The ratios $R = K_c/K_s$ were obtained by assessing the potency P at near zero binding of the E_2 radioactive label. As shown by Ekins[12] potency (the reciprocal of specificity) varies with the amount of bound label (B) and the amount of unbound or free label (F) according to the simplified formula (values B and F expressed as ratios of 1.00).

$P = F\ (K_c/K_s) + B$. Thus when $F = 1$ and $B = 0$. $P = K_c/K_s$.

Assessment of P at near zero binding was made by transforming the binding data for each respective radioimmunoassay curve into the logit form below, and plotting these logit transforms against log dose. Using the response variable $Y = B/B_0$ where B is amount bound (c.p.m.) after competition with a known dose, and B_0 is the amount bound (c.p.m.) at zero dose, then

$$\text{logit } Y = \log_e (Y/1 - Y)$$

Plots of logit Y (plotted down to $\log_e = -5$ when $Y = B/B_0 = <0.4\%$ binding, since B_0 is always less than 62% of total) against log dose were constructed. The logit transform/log dose plots linearised the usual sigmoidal radioimmunoassay curves. Extrapolation of the straight line plots to cut the log dose axis at $\log_e = -5$ thus enabled the log dose required to produce near zero binding of the label to be determined. The log dose values for the standards (E_2 or $E_2$6CMO) for each respective filtrate designated (L_s) and the log dose value of the various cross reactants (L_c), provided the ratios L_c/L_s. Since L_c/L_s is inversely proportional to the relative equilibrium constant $R = K_c/K_s$ this enabled this ratio to be obtained. (L_c/L_s values are assessed in mol/l.)

RESULTS

The properties of the fractionated antisera after treatment with the various immunoadsorbents are shown in Table 1. The results show that the most effective immunoadsorbent was the homologous ligand E_2 6CMO which adsorbed 78% of the original antibodies, next was $E_1$6CMO which adsorbed 57%, whilst the 3 and 17 derivatised oestrone ligands were not particularly effective, adsorbing only 17% and 23% respectively. The heterologous ligand $E_1$6CMO appears to have adsorbed considerable quantities of high affinity constant antibodies to E_2, the resultant antibodies in the filtrate (E) have an affinity constant (K_a) relative to the control some 23 times less in value after this immunoadsorption. Table 1 also shows that apart from filtrate C, the saturating amount of E_2 after constructing the saturation curves for the estimation of K_a to this steroid is 100 ± 15 pg. The K_a

Table 1. Properties of antibodies in the filtrates obtained by the various immunoadsorbent treatments

Filtrate label	Immuno-adsorbent	μM Ligand bound/ml Sepharose	Calculated* antibody concentration ($M/L \times 10^{-7}$)	% Original antibody in filtrate	Reciprocal dilution for saturation curve	Saturation level (pg)	Average affinity constant ($K_a \times 10^8$ L/M)
A (Control)	None	—	12.4	100	810	86	44
B	$E_1$3HS	0.85	10.2	83	530	108	38
C	$E_2$6CMO	0.23	2.7	22	260	60	5.7
D	$E_1$17CMO	0.25	9.6	77	470	115	4.0
E	$E_1$6CMO	0.44	5.4	43	300	100	1.9

* Calculated from saturation curve data. MW of antibody = 170,000

Table 2. Properties of antibodies used for the determination of the relative equilibrium constant ratios (R)

Filtrate label	Immuno-adsorbent	Reciprocal dilution original filtrate	Antibody conc. per assay ($M/L \times 10^{-10}$) calc. sat. curve	Antibody conc. per assay ($M/L \times 10^{-10}$) calc. Scatchard plot	$K_a \times 10^8$ L/M (Scatchard plot)	B_0 (Zero load) binding %
A (Control)	None	2000	6.2	5.4	54.0	55
B	$E_1$3HS	1500	6.8	5.8	77.0	62
C	$E_2$6CMO	400	6.8	6.0	6.6	22
D	$E_1$17CMO	1400	6.8	6.4	4.0	19
E	$E_1$6CMO	600	9.0	11.2	2.5	17

Table 3. Relative equilibrium constant ratios ($K_c/K_s \times 10^2$) when $K_s = E_2$ of various cross reactants for the filtrates obtained after immunoadsorption

Filtrate	$K_a = K_s$ (against E_2) $L/M \times 10^8$	E_2	E_26K	E_26CMO	E_16CMO	E_1	E_13HS	E_3	$E_217\alpha$	E_217HS
B	77.0	100	126	84	3.9	2.6	1.11	0.29	0.36	0.075
A	54.0	100	139	94	4.1	2.5	1.21	0.31	0.36	0.079
C	6.6	100	151	101	4.0	2.0	0.98	0.25	0.28	0 068
D	4.0	100	192	164	5.7	2.0	1.00	0.24	0.31	0.066
E	2.5	100	256	213	7.4	1.7	0.85	0.47	0.22	0.061

Table 4. Relative equilibrium constant ratios ($K_c/K_s \times 10^2$) when $K_s = E_26CMO$ of various cross reactants for the filtrates obtained after immunoadsorption

Filtrate	$K_a = K_s$ (against E_26CMO) $\times 10^8$ L/M	E_26CMO	E_26K	E_2	E_16CMO	E_1	E_13HS	E_3	$E_217\alpha$	E_217HS
B	65.0	100	150	119	4.6	3.00	1.33	0.35	0.43	0.09
A	51.0	100	148	106	4.4	2.60	1.29	0.33	0.39	0.08
C	7.7	100	150	99	3.9	2.00	0 97	0.25	0.28	0 07
D	6.4	100	117	61	3.4	1.20	0.61	0.15	0.19	0.03
E	5.3	100	120	47	3.4	0.81	0.41	0.22	0.11	0.03

estimations were therefore made with almost the same concentration of antibodies in the respective filtrates.

Table 2 gives the properties of the antibodies obtained after further dilution for the specificity tests. The dilutions were once again adjusted so that the antibody concentration in each filtrate was approximately the same. The results of the further check on the respective antibody concentrations made by Scatchard plot assessment (constructed from the actual radioimmunoassay data obtained for the specificity tests) showed general agreement between the saturation curve and this method (Table 2). Calculation of affinity constants for E_2 and E_26CMO made by these Scatchard plots reflect the actual affinity values in the specificity tests (ie. the K_s values. Tables 3 and 4). Table 2 shows that filtrate E had a slightly higher concentration of antibodies than the other filtrates whether derived by saturation curve or Scatchard plot assessment. Finally Table 2 gives the binding (B_0) value on zero dose for the specificity tests used for the various filtrates.

Surprisingly the E_13HS immunoadsorbent filtrate B appears to have increased its K_a (K_s for E_2) value on dilution to above that of the control. This higher K_s value is reflected also by the higher R values generally obtained for this filtrate B, i.e. less specificity obtained by this increase in affinity. Tables 3 and 4 are therefore presented with the order of the filtrates given with diminishing affinity and thus the control filtrate (A) data is presented below that of the filtrate (B) data. Comparison of the K_a values presented in Table 1 with the K_a (K_s) values given in Table 3 gives the effect of dilution of the antibodies for the specificity tests. As seen the results are almost the same (except for B) and that they are slightly higher after dilution as expected [13].

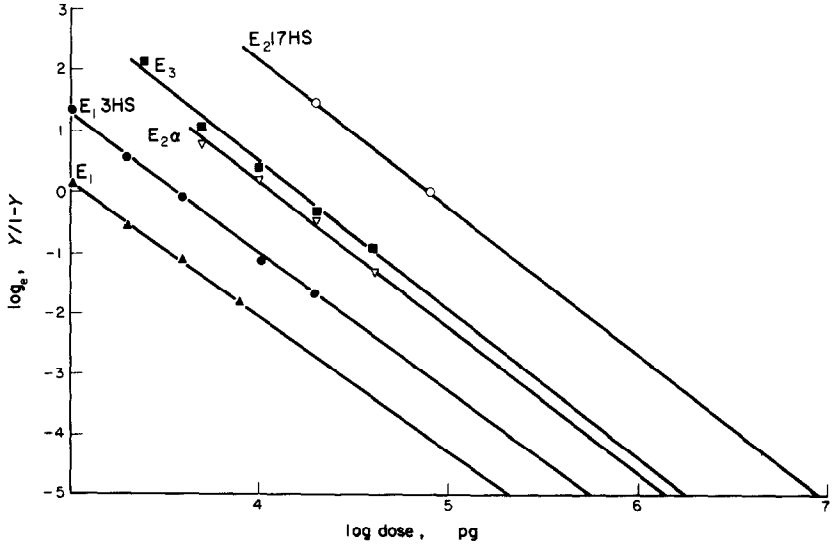

Fig. 1. Typical plots of logit B/B_0 versus log dose, showing extrapolation of data to cut log dose axis at $\log_e (Y/1 - Y)$ values of -5.

Figure 1 shows a typical logit B/B_0 plot where $Y = B/B_0$ and $\log_e (Y/1 - Y)$ is plotted against log dose for the assessment of the relative equilibrium constant ratios ($R = K_c/K_s$). The data (pg) obtained by extrapolation to cut the log dose axis (near zero-binding = 0.4% B) was converted to mol/l to obtain the L_s and L_c values and the K_c/K_s values obtained.

The R values for the various cross reacting steroids and their derivatives which are used as an index of the specificity of the antibody pools in the respective filtrates are shown in Tables 3 (K_s = part hapten E_2) and Table 4 (K_s = homologous hapten $E_2$6CMO). These tables also give the respective K_s values in $L/M \times 10^8$ as assessed by Scatchard plot. Table 3 shows that using K_s assessment of the part hapten E_2, the cross reactants E_1, $E_2 17\alpha$, E_3, $E_1$3HS and $E_2$17HS all tend to have slightly less R values (a slight increase in specificity) with decrease of affinity (K_s). On the other hand, this table shows that the homologous hapten $E_2$6CMO and the part haptens $E_2$6K and $E_1$6CMO (a compound possessing the 6CMO moiety of the original hapten) have increased R values (a decrease in specificity) with decrease in affinity. Table 4 using the K_s assessment of the homologous hapten $E_2$6CMO shows that all the R values decrease (increase in specificity) with decrease in affinity. Thus the specificity of antisera towards its homologous hapten increases with decrease in affinity, i.e. low affinity antibodies are more specific than high affinity antibodies.

DISCUSSION

The affinity constants (K_a) calculated from the Scatchard plot and saturation curve data obtained by the dextran coated charcoal liquid radioimmunoassay technique are not absolute values but they provide information about the affinity constant relative to the original antisera in the control without immunoadsorption ($K_a = 4.4 \times 10^9$ L/M). Charcoal is known to disturb the equilibrium by dissociation of the antibody–hapten complex (Kahn, Andrieu and Dray [13]). This dissociation depends on the magnitude of K_a (the lower the affinity constant the greater the dissociation) on temperature, on the B/F ratio, and on time of contact of the complex with charcoal. The technique of keeping the radioimmunoassay tubes in ice for exactly 10 min and of having in general low B/F ratios helped to keep the dissociation low. Using an identical radioimmunoassay technique, to that used for our specificity tests, experiments conducted in this laboratory indicate that the higher affinity fraction A ($K_a = 5.4 \times 10^9$ L/M) = K_s for specificity test is not seriously affected by this dissociation, but the absolute value for K_s of fraction E will be higher than the 2.5×10^8 L/M determined.

Consideration shows that the present results of showing more specificity in the lower affinity pools cannot be an artefact due to dissociation of the antibody–hapten complex by charcoal. Dissociation of the antibody–hapten complex by charcoal becomes negligible at low B/F ratios (Kahn *et al.* [13]). Assessment of specificity at zero binding is thus not only independent of binding but also independent of dissociation. L_s and L_c values determined by logit B/B_0 plots extrapolated to cut the log dose are not affected by dissociation, but the slope of the logit B/B_0 versus log dose plot increases. The ratios of relative equilibrium constants ($K_c/K_s = R$ values) are independent of dissociation, however the K_s values assessed by Scatchard plot for the various filtrates are lowered by this effect. This effect on K_s has an identical relative effect on all the K_c/K_s values for any particular filtrate, but it is only the absolute value of K_s or K_c which is changed, the ratio remains unchanged. This reasoning also applies when considering any possible effect due to the difference if any of the K_a of radioactive E_2 from the K_a of non-radioactive E_2. Thus despite the difference in affinity constants to E_2 or $E_2$6CMO (i.e. K_s values) in the various filtrates the R values are independent of binding, dissociation, and difference of K_a of radioactive E_2.

The R value for $E_2$6CMO/E_2 is unexpectedly less than the R value for $E_2$6K/E_2 in all the different filtrates. This has been noted in previous specificity tests and it may be due to the fact the particular $E_2$6CMO compound used probably possessed a different isomeric (*syn* or *anti*) composition from that of the original hapten used to raise the antisera used in this study. Despite this lower K_s of the homologous hapten $E_2$6CMO, it is very unlikely it would effect the actual R values for $E_2$6CMO/E_2, in fact the part haptens $E_2$6K and $E_1$6CMO also show the same general trend as shown by this compound.

The determination of specificity of the various filtrates was (despite the difference in affinity) made with approximately the same concentration of antibodies. This eliminated effects on the affinity constants due to dilution. Maintaining this constancy of antibody concentration unfortunately meant it was impossible to arrange that the binding of the radioactive label at zero dose (B_0) be the same for each filtrate. Since cross reactivity is a function of B_0, the commonly used method of determining specificity that based on a 50% reduction of B_0 would give rise to erroneous results. Assessment of specificity was therefore made by determining the ratio of the relative equilibrium constants, which is independent of B_0.

Application of this approach suggests that a substantial amount of previous published data in which this effect on B_0 has not been considered will have supplied erroneous specificity comparisons.

The R values of $E_2$6CMO, $E_2$6K, and $E_1$6CMO all show an increase with decrease of affinity towards E_2. This increase (decrease in specificity) indicates that lower affinity antibodies recognise these substances better than E_2. Calculation shows that the greatest R increase occurs with the homologous hapten $E_2$6CMO (253%), followed by an R increase of 203% for $E_2$6K, and then 189% for $E_1$6CMO. As

expected the homologous hapten is more potent than the other two part haptens in promoting this effect. The antibody sites of the antisera used for these results were clonally processed to recognise $E_2$6CMO, therefore it appears that the lower affinity antibodies are more specific towards its homologous hapten than any of its variants. Thus specificity is best expressed against the homologous hapten and Table 4 clearly shows that it is preferentially bound in the face of all the cross reactants studied. R values of the cross reactants all decrease relative to $E_2$6CMO with decrease in affinity; therefore a decrease in affinity of anti-hapten sera leads to higher specificity.

This finding has been further substantiated by other studies in this laboratory on the affinity/specificity relationship by various antisera collected during the maturation of the immune response to $E_2$6CMO-bovine serum albumin in a goat. Here again as the affinity increased the specificity decreased.

Apart from the E_1-3HS-AH-Sepharose-4B immunoadsorbent, the other heterologous affinity chromatography ligands adsorb more of the high affinity antibodies and produce lower K_a antibody pools in their filtrates than that produced by the homologous ligand $E_2$6CMO. Since high affinity antibodies are less specific than low affinity ones, then the more heterologous the immunoadsorbent the greater the amount of high affinity antibodies should be adsorbed and this is what is found.

The results suggest that the objective of attempting to obtain high affinity pools which possess high specificity is not possible. A compromise has therefore to be made if specificity of radioimmunoassay towards a particular hapten is required since the lower K_a antisera necessary for this specific determination would lead to a more insensitive method. Sensitivity of radioimmunoassay is however not such a strict requirement for those steroid hormones which exist in plasma in reasonable concentration (cortisol, progesterone in pregnancy etc.), thus these hormones could be more specifically determined without chromatographic separation using lower affinity antisera. A decrease in specificity of other haptens and antigens with increase in affinity has been noted previously by a few immunochemists, Eisen *et al.* [14], Little and Eisen [15]. As yet no clear picture of this concept has emerged. The quantitative equilibrated radioimmunoassay cross reaction procedure used in our studies for assessing specificity in contrast to the qualitative radioimmunodiffusion technique used by many immunochemists give stronger credance to this observation.

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