ESTUDIO DE LA REACCION DE FIJACION DE UN LIGANDO
A SU RECEPTOR: EFECTO DE LA VALENCIA DEL LIGANDO,
COMPETENCIA CLONAL Y COOPERATIVIDAD

TESIS

PARA OBTENER EL GRADO DE:

MAESTRO EN CIENCIAS

(Investigación Biomédica Básica)

QUE PRESENTA:

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Dear Dr. Celis:

I am pleased to inform you that your manu-
script entitled "Effects of the Extent of DNP
Substitution on the Apparent Affinity Constant
and Cooperation ...." has been accepted
for publication in Immunochemistry and dispatched
to Pergamon Press.

Sincerely yours,

Fred Karush

FK:sk
encl.

RESUMEN

Con el fin de estudiar los aspectos físico-químicos de
la reacción de un receptor celular con un antígeno se elaboró un
modelo químico de los receptores acoplando anticuerpos purifica-
dos a agarosa, para simular la parcial inmovilización que su po-
sición membranal les confiere, mientras que el antígeno se mantu-
vo en solución. En este trabajo se describe el efecto de la va-
lencia del antígeno, expresada como el número de moles del radi-
cal dinitrofenil (DNP) por mol de seroalbúmina humana (HSA), so-
bre la reacción con anticuerpos anti-DNP y anti-HSA inmovilizados.
La constante de afinidad aparente de la reacción (K) de los anti-
cuerpos anti-DNP aumentó con incrementos en la valencia de grupos
DNP del antígeno mientras que disminuyó la de la reacción con los
anticuerpos anti-HSA. También se detectó la presencia de un trazo
ascendente en las gráficas de Scatchard a concentraciones peque-
ñas de antígeno pegado, que es compatible con la existencia de un
fenómeno de cooperatividad entre los sitios activos de los anti-
cuerpos, según lo demuestra el análisis computacional de un pro-
ceso de fijación teórico donde se modifica positivamente el núme-
ro total de sitios activos o la constante de afinidad de la reac-
ción al ocuparse los primeros sitios activos.

Los cambios en K con la valencia del antígeno se ofre-
cen como explicación de la mayor habilidad que tienen los antí-
genos polivalentes sobre los oligovalentes en alcanzar niveles

inmunogénicos y toleragénicos. Por primera vez se estudia detalladamente la existencia de una porción ascendente en el trazo de Scatchard y se demuestra que es compatible con cooperatividad entre sitios activos del anticuerpo, lo que puede corresponder a la primera magnificación de la señal de haberse encontrado un antígeno en el interior de un organismo y, de ser suficientemente grande e intermolecular, podría representar un mecanismo de incorporación a la reacción de moléculas de anticuerpos que solas no podrían iniciar la reacción.

ABSTRACT

Increments in the extent of dinitrophenyl (DNP)[*] substitution of human serum albumin (HSA) increases the apparent affinity constant of its reaction with anti-DNP antibodies coupled to agarose and decreases that of its reaction with anti-HSA antibodies also fixed to the same solid phase. An ascending limb of the Scatchard plot in the low levels of bound ligand is described and is shown to be also dependant of the degree of DNP substitution of the antigen. Results are discussed as indicative of a probabilistic affect of the antigens' valence on the apparent affinity constant for antibodies coupled to agarose while the ascending limb of the Scatchard plots as compatible with cooperation between antibody active sites.

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[*] Abbreviations used: DNP, dinitrophenyl; HSA, human serum albumin; PBS, phosphate buffered saline.

INTRODUCTION

Many cellular activities are initiated by the reaction of cell membrane bound receptors with specific ligands. The immune response is no exception: the production of antibody, the proliferation of immunocompetent cells, the activation of T lymphocytes to reject tumors and transplants of foreign tissue and some states of hypersensitivity are perhaps triggered by the reaction of antigen with cell surface receptors (Feldman and Globerston, 1976; Warner, 1974). Although some attempts have been made to closely examine the reaction of cell bound receptors with antigen (Davie et al, 1973; Karush and Hornick, 1969), the low concentrations of specific cells and the restrictions imposed on the instrumentation by whole live cells limit the depth of such studies. In an effort to identify the most important factors in the binding of antigen by cell receptors, we have covalently coupled purified antibodies onto agarose beads and began a systematic exploration of the receptor-antigen reaction presumably occuring on the cells by way of this model system. In this paper special emphasis is made in describing the effects of the antigens' valence or number of identical binding sites per molecule on the binding process.

Our results certainly point to the importance of the number of dinitrophenyl (DNP) determinants carried by human serum albumin (HSA) on the apparent affinity (avidity) of its reaction

with anti-DNP and anti-HSA antibodies fixed to a solid phase, and on the height of an ascending limb found in the Scatchard plots at low concentrations of bound ligand, compatible with cooperation between antibody sites.

MATERIALS AND METHODS

The overall experimental design consists in studying the reaction of anti-DNP or anti-HSA purified antibodies coupled onto agarose with tritium labeled HSA dinitrophenylated to different extents (α) ($DNP_\alpha HSA-^3H$).

Immunogens and Immunization Procedures

To obtain anti-DNP antibodies, $DNP_{23}BSA$ was prepared by reacting 2 g of bovine serum albumin (BSA, Sigma Chem. Co.) with 350 mg of 1-chloro-2,4-dinitrobenzene (DNClB, Sigma) in a 2 % solution of K_2CO_3 at a pH of 9 and room temperature for 4 hours. Free DNClB was eliminated by extensive dialysis against a phosphate-saline buffer, PBS (0.15 M NaCl, 0.01 M PO_4, pH 7.4). Protein concentration was determined by Nesslerisation (Campbell et al, 1970) after total digestion of the conjugates with H_2SO_4 in a micro-Kjeldahl digestor. The DNP:protein molar ratio was calculated from the conjugates' optical density at 363 nm using 17530 as the molar extinction coefficient for DNP-Lysine (Eisen, 1964). Anti-HSA antibodies were obtained using 99 % pure human serum albu-

min (Sigma) as immunogen. Two adult male sheep were immunized, one
with HSA and the other with $DNP_{23}BSA$; both animals recieved 5 mg
of antigen every 15 days intramusculary using Freund's Complete
Adjuvant with the antigen in only the first occasion. Sheep were
bled one week after each immunization by puncture of the jugular
vein obtaining 500 ml of blood each time. Sera were evaluated for
antibody content by conventional agar double-diffussion precipita-
tion methods (Campbell et al, 1970). Precipitating sera were used
as sources of purified specific antibodies.

Antibody Purification

Anti-DNP and anti-HSA gammaglobulin fractions were puri-
fied by amonium sulphate precipitation (Campbell et al, 1970) and
later incubated with their respective immunoadsorbents, HSA-Sepha-
rose and DNP-Lysine-Sepharose, prepared by the standard techniques
(Cuatrecasas, 1970), for 12 hours at 25 C° under continous gentle
shaking. After extensively washing the immunoadsorbents with PBS,
specific antibody fractions were eluted with the hapten (10^{-3} M
2,4-dinitrophenol in PBS) to obtain the anti-DNP antibodies and
with 3 M sodium thiocyanate for the anti-HSA antibodies. Fractions
containing the antibodies were concentrated by ultrafiltration
and dialysed with PBS to eliminate the eluent solutions. No hapten
was detected in the anti-DNP antibodies as measured by their opti-
cal density at 365 nm.

Antibody Coupling to Agarose Beads

Antibodies to DNP and to HSA were coupled onto Sepharose (Pharmacia) 6B and 4B, respectively, by cyanogen bromide, following the method described by March et al (1974). Approximately 500 mg of purified anti-HSA or anti-DNP antibodies were added to 30 ml of cyanogen bromide activated agarose. The reaction was left for 12 hours at a pH of 7.4 in PBS and at 4 C°. Afterwards, the agaroses were washed with 1 M Glycine in PBS and then with PBS to inactivate the remaining cyanogen bromide bridges and to remove the excessive aminoacid before storing at 4 C° until usage.

Radioactive Antigens for Binding Studies

Following the method of Cuatrecasas (1972), 250 mg of HSA in 20 ml of 0.1 M $NaHCO_3$, pH 8.8 were labeled with tritium adding 25.5 mg (100 mCi) of acetic-^{3}H anhydride (New England Nuclear). A specific activity of 1.38×10^7 cpm/mg of protein was obtained. $DNP_\alpha HSA-^3H$ conjugates were then prepared by Eisen's method (1964) reacting the appropiate concentrations of $HSA-^3H$ and 2,4-dinitrobenzensulfonic acid (Eastman) to obtain the desired $DNP:HSA-^3H$ molar ratio (α), which was determined as described above. The hapten ε-2,4-dinitrophenyl, $3,5-^3H$ (N)-L-Lysine (5.2 Ci/mmole) was purchased from New Englan Nuclear and diluted with ε-2,4-dinitrophenyl-L-Lysine (Sigma) at different ratios. Fifteen dilutions of the three ligands were prepared in PBS to span the interval from 10^{-5} to 10^{-8} M of each ligand.

Binding Reactions

100 µl of a 10 % suspension of agarose coupled with anti-
bodies in PBS and 100 µl of each concentration of radioactive ligand
also in PBS were mixed in a small siliconized glass test tube,
incubated at 30 C° and shaked for one hour. After studying the kine-
tics of the reactions and establishing that equilibrium had been
reached after such interval, a 100 µl sample of each reaction mix-
ture was withdrawn and placed in conical centrifuge plastic tubes
(Beckman) at the top of 0.3 ml of 2.5 M saccharose. After centri-
fuging for 15 minutes at 6,100 g the tubes were buried in pulveri-
zed dry ice for a couple of minutes to solidify their contents,
and cut with a scalpel blade in two halves. Each half tube was
placed in a scintillation vial containing 1 ml of NCS solubili-
zer (Amsterdam-Searle) and left incubating overnight at 37 C°.
Next day, 10 ml of scintillation mixture (4 g PPO and 0.4 g POPOP
per liter of toluene) were added to each vial and mixed vigourosly
in a vortex. Samples were allowed to cool and the estimates of the
BOUND and FREE ligand concentrations were calculated from the
bottom and top halves of the tubes, respectively, from the radio-
activity present as counted by a Nuclear Chicago Mark III scin-
tillation counter with 50 % efficiency.

Estimation of $[S]_o$ and K

The antigen binding capacity in moles per liter of each

agarose-antibody conjugate, $[S]_0$, was estimated as the X intercept of the linear regression of the molar concentration of bound ($[B]$) to free ligand ($[F]$) on bound ligand, in the Scatchard (1949) fashion:

$$\frac{[B]}{[F]} = K[S]_0 - K[B] \qquad (1).$$

Due to some curvature in the Scatchard plots, the apparent affinity constant (K) or avidity constant, was estimated from the Sips (1948) linear regression:

$$\log \frac{R}{1 - R} = \underline{a} \log K + \underline{a} \log[F] \qquad (2),$$

where R is the proportion of the total antigen binding capacity occupied by antigen at each concentration of free ligand ($[B]_i/[S]_0$ at $[F]_i$), and $\underline{a}$ is an heterogeneity index.

RESULTS

Binding Capacity with Monospecific Ligands

Figures 1 and 2 show the Scatchard plots of the reactions of anti-DNP agarose with DNP-Lysine-^{3}H and of anti-HSA agarose with HSA-^{3}H; the estimates of $[S]_0$ and K for each curve are shown in Table I. It is clear that both antibody coupled agaroses have active sites that are capable of binding their specific ligands with an apparent affinity within expected limits.

Effect of the Antigens' Valence on the Apparent Affinity of the Reaction of DNP$_{\alpha}$HSA-^{3}H with the Anti-DNP Agarose

Four dinitrophenylated antigens with different DNP:HSA-^{3}H molar ratios (α = 6.5, 10.7, 21.4, 32.5) were reacted with the anti-DNP agarose under the same experimental conditions. Representative Scatchard plots of these reactions are shown in Figure 3 and the estimates of $[S]_0$ and K are in Table II. Two features of these plots should be noted. Firstly, the positive correlation between K and the degree of dinitrophenylation (Figure 4) and, secondly, the ascending limb of the plots at very low concentrations of bound antigen that reach maximal values of bound/free ($(B/F)_{max}$) that are also positively correlated with the DNP:HSA-^{3}H molar ratio of the ligand (Table II). In contrast, all values of $[S]_0$ for these plots appear to be similar.

Effect of the Antigens' Valence on the Apparent Affinity of the Reaction of DNP$_{\alpha}$HSA-^{3}H with the Anti-HSA Agarose

Figure 5 shows the Scatchard plots of the binding reactions of the anti-HSA agarose with four antigens of different DNP:HSA-^{3}H molar ratios (α = 0, 10.1, 26.4, and 33.9). The estimates of $[S]_0$ and K from these reactions are summarized in Table III. The results are strikingly consistent with those of anti-DNP agarose: all $[S]_0$ being roughly similar, the K for each antigen diminished with increasing degrees of dinitrophenylation (Figure 6). However, no significant difference in the ascending limbs were

appreciable in the Scatchard plots, they all tended to similar

$(B/F)_{max}$ values (Table III).

DISCUSSION

The binding of tritiated and dinitrophenylated human serum
albumin by purified sheep anti-DNP and anti-HSA antibodies fixed to
agarose was studied in search of evaluating the effects of the de-
gree of dinitrophenylation upon the apparent affinity, or avidity,
constant (K) of the reactions. And, indeed, the number of DNP groups
carried by the protein was proved an important variable in the an-
tigens' binding to both the anti-DNP and anti-HSA agaroses. Incre-
ments in the DNP to HSA molar ratio resulted in higher values of
K in the reaction with the anti-DNP agarose and in lower values of
K in the reaction with the anti-HSA. If the number of DNP determi-
nants per HSA molecule effectively increases with the extent of
dinitrophenylation, while the number of native determinants decrease,
the effects of the DNP:HSA molar ratio upon K may be easily
attributable to the relation between the number of binding sites
and affinity postulated by Klotz (1953):

$$K_i = \frac{a - i + 1}{i} k \qquad (3),$$

on the basis of valence influencing the number of possible reaction
products and the likelihood of a stable union for any product,
where a is the number of binding sites on the ligand, i is the

number of sites bound by the ith reaction and k is the intrinsic association constant. Naturally, some corrections of the above equation would be necessary to account for the multivalency of the reactants used in our particular case, but no serious departure from Klotz's expected trend of apparent affinity on valence are brought about from such condition. The actual values of K estimated in these experiments are, of course, of no great use in the study of thermodynamic quantities. However, their positive correlation with the degree of DNP substitution serves as an indicator of the importance of the antigen valence in its reaction with immovilized antibody, an issue of interest in the explanation of the _in vivo_ increased ability of polysubstituted antigen to reach immunogenic, toleragenic and memory priming dose levels over that of oligosubstituted antigen (Larralde and Janof, 1972; Larralde and Lagunoff, 1974, 1975). Presumably, such _in vivo_ effects of the antigens' valence are caused by an increase in the affinity for cellular receptors, while the different immune responses relate to the extent of receptoroccupation per unit cell and/or receptor polymerization on the cell membrane (Feldman, 1971, 1972). Our results show how, if native antigen ever encounters a cellular receptor, the number of combining sites could certainly influence the ensuing immunological events.

The ostensible ascending limb of the Scatchard plots at very low concentrations of bound antigen are somewhat puzzling on the surface, since Equation 1 is that of a straight line and hetero-

geneity of binding sites is well known to curve the plot in the
opposite direction (Day, 1972). The chances of an artifactual
source for such points, due perhaps to different magnitudes of
error along the plot, are practically nullified by the finding
that there is a significant positive slope in that part of the
curves (Tables II and III). Also, the absence of ascending
limbs in the binding plots of non-immune Ig speak against other
sources of error such as the equilibria of labile labelled groups
on the protein and in solution. The possibility of such equi-
libria being altered by the reaction of antigen with antibody
is considered of minimal significance since no labile O-acetyl-
tyrosine was found in our tritiated proteins by spectral analysis
(Riordan and Vallee, 1972) and antigen free label was found to be
slightly less than 1% of the total radioactivity by thin layer
chromatography, and remained constant at different antigen-antibody
reactions performed at different concentrations of antigen. It
occured to us that increments in the product $K[S]_o$ with the ini-
tial binding of antigen could account for the positive slopes in
the Scatchard plots. And positive changes in $K[S]_o$ do indeed curve
the plot upwards as is shown in the theoretical curves in Figures
7 and 8, where K is increased exponentially with respect to bound
ligand, while heterogeneity and nonspecific binding do not have
such effect. So, since systematic error is ruled out and the other
recognized causes of curvilinearity affect Scatchard plots in
a manner opposite to that described here, we propose that the

ascending limbs of the plots are consistent with the hypothesis
that upon the binding of the first antibody sites some change
is occuring in the reacting system that increases the number of
binding sites, the apparent affinity of the remaining sites, or
both. Under our experimental conditions, where antibodies are
covalently bound to a practically solid phase and probably not
proximal to each other, we feel very little of the cooperation
must come from the formation of geometrical antigen-antibody
arrangements optimal for further binding but, rather, from re-
arrangements or "contortions" (Metzger, 1974) of the antibody
molecules that affect only the other site in the same molecule
and not other antibody molecules. The influence of the antigens'
valence on the extent of the cooperative effect in the reaction
with anti-DNP antibodies could reflect the different increments
in the concentration of DNP determinants about the microenviro-
ment of the second site after the first becomes bound. It seems,
however, that either there is a maximal effective valence or a
maximal cooperative effect since $DNP_{21.4}HSA$ reached similar
$(B/F)_{max}$ than $DNP_{32.5}HSA$ (Table II). The apparently ineffectual
role of the DNP:HSA molar ratio on the cooperative effect in the
reaction with anti-HSA antibodies could be explained on the basis
of there still being native determinants of the $DNP_\alpha HSA-{}^3H$ mo-
lecule in a number that is maximal with respect to cooperation
effects or, they being probably of different specificities, the
valence of any particular one is not significantly altered by

dinitrophenylation although the total valence of the antigen is lowered.

We are aware that cooperation in the antigen-antibody reaction is not usually recognized in the literature, although Metzger (1974) briefly points that some reported ascending limbs in radioimmunoassays of hormones could be interpreted as such. Cooperation has been mentioned to account for increments in the free energy of the reaction of antibody with polyvalent antigen (Davie and Paul, 1972; Paul, Siskind and Benacerraf, 1966), but we would like to distinguish between those effects of polyvalence on the overall affinity constant of the system due to probabilistic considerations (Klotz, 1953) and due to increased complexity of the reaction products, from our proposition of a change in the apparent affinity of the reacting system as the reaction progresses. Our impression is that equilibrium binding studies, not usually including such very low levels of bound ligand, and velocity measurements being still technically deficient for so fast a reaction, must account for the practically universal oversight of such a notable accident in the Scatchard plots of antigen and antibody reactions. Clearly, the study of cooperation in the antigen-antibody reaction needs of diversified experimental designs to obtain finer information but is particularly lacking of a mathematical expression that includes such important variables as the multivalency of both reactants, besides that of heterogeneity, if we are

Fig. 1

[BOUND] / [FREE]

.4
.3
.2
.1

5   10   15   20   25   30   35

[BOUND] × 10⁸

Fig. 2

[BOUND] / [FREE]

5
4
3
2
1

50   100   150   200   250   300   350   400

[BOUND] × 10⁵
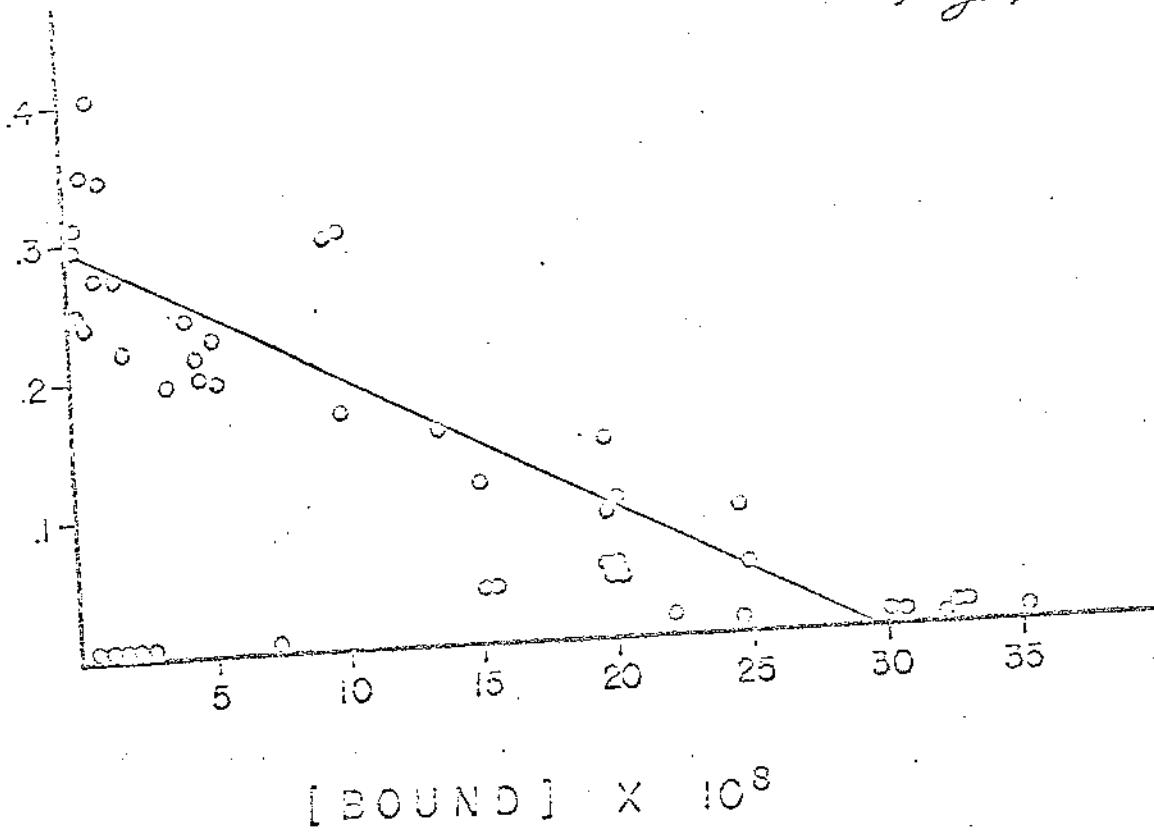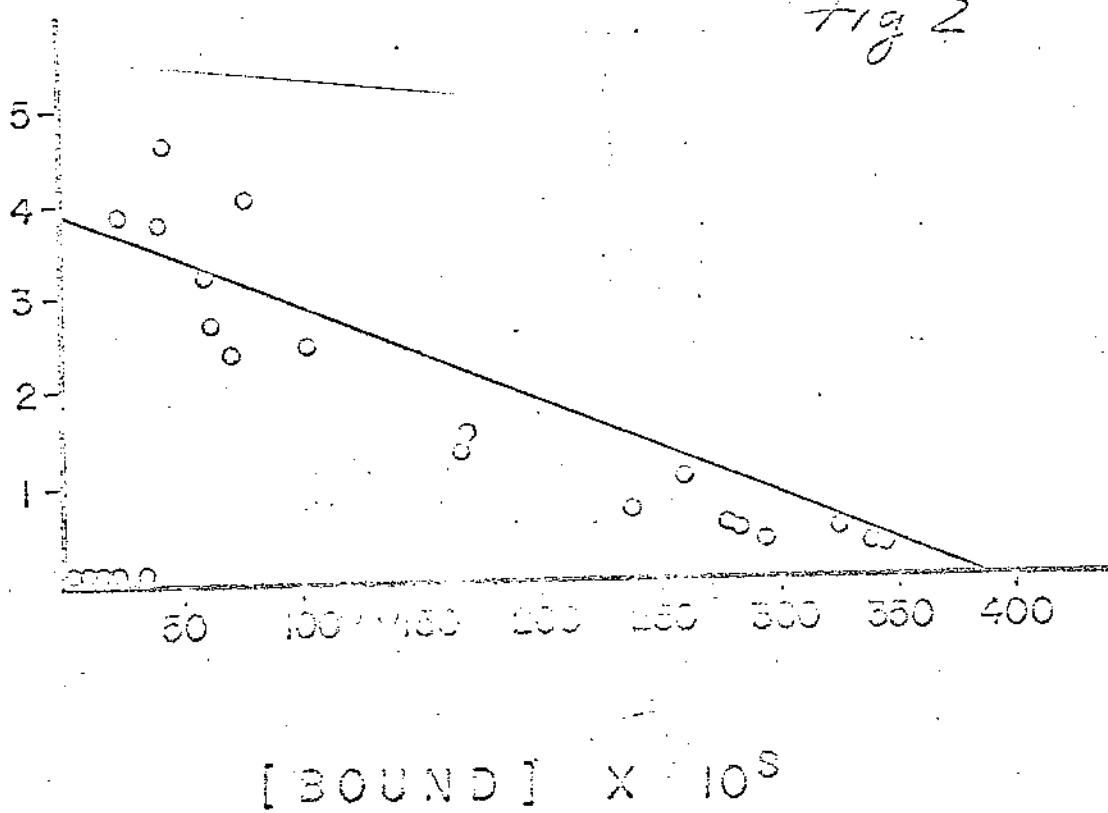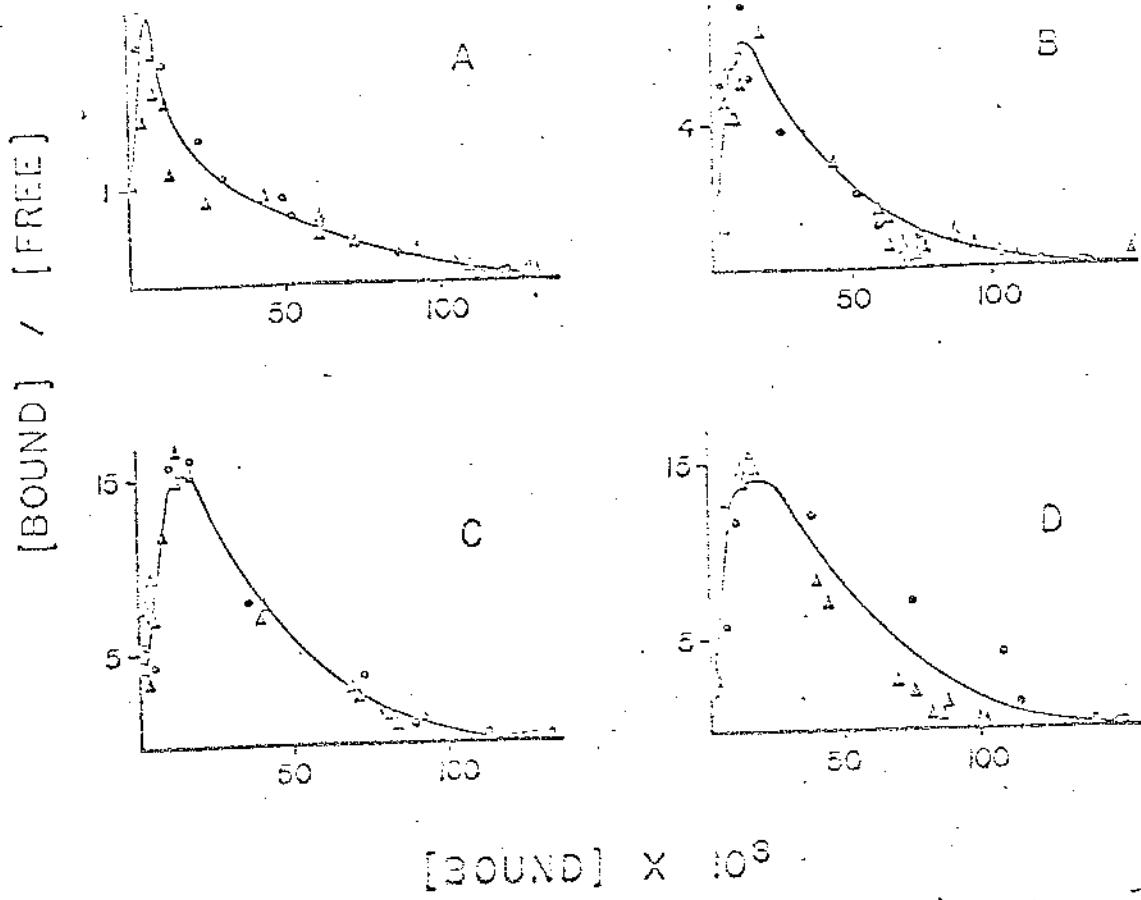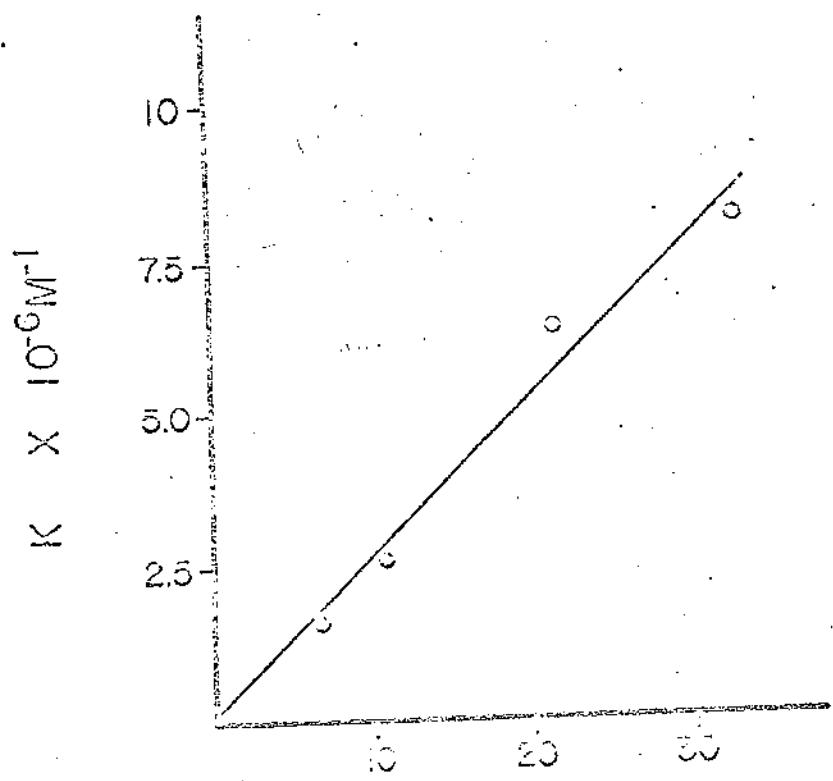
Fig 3

Fig 4
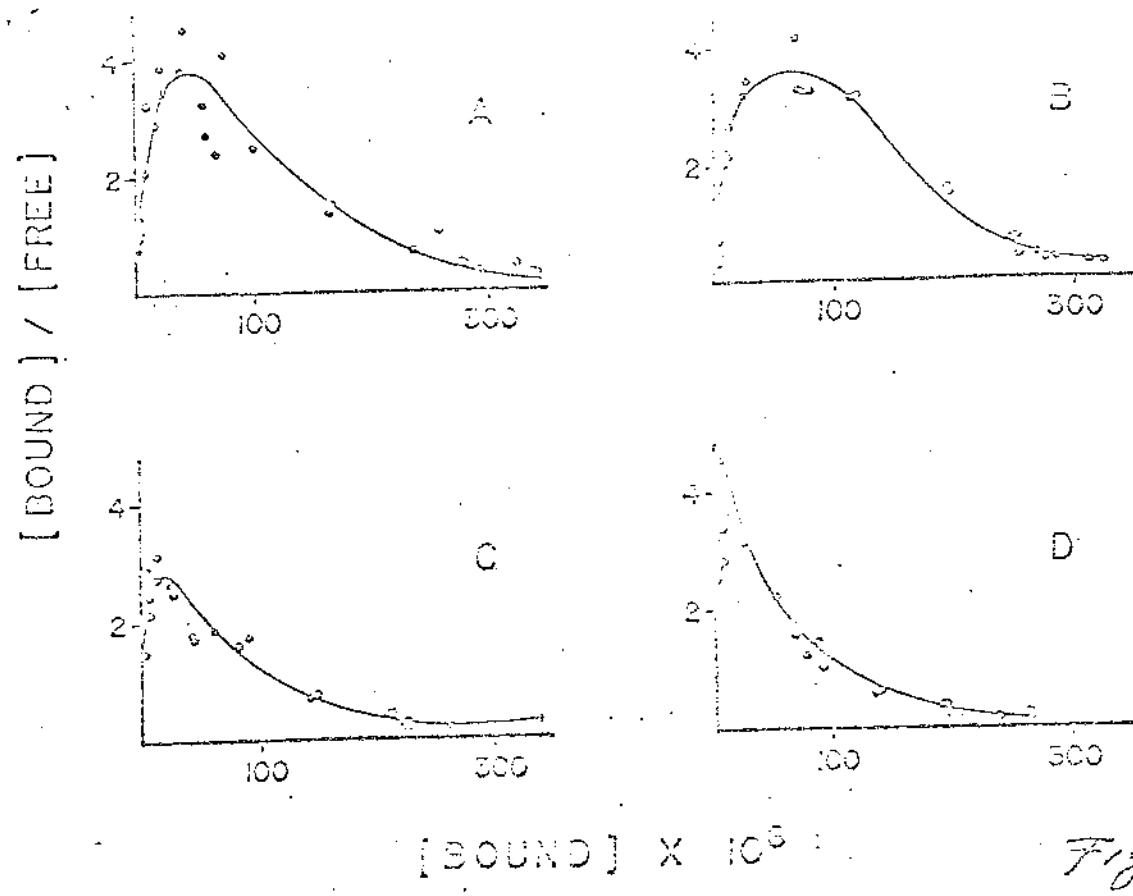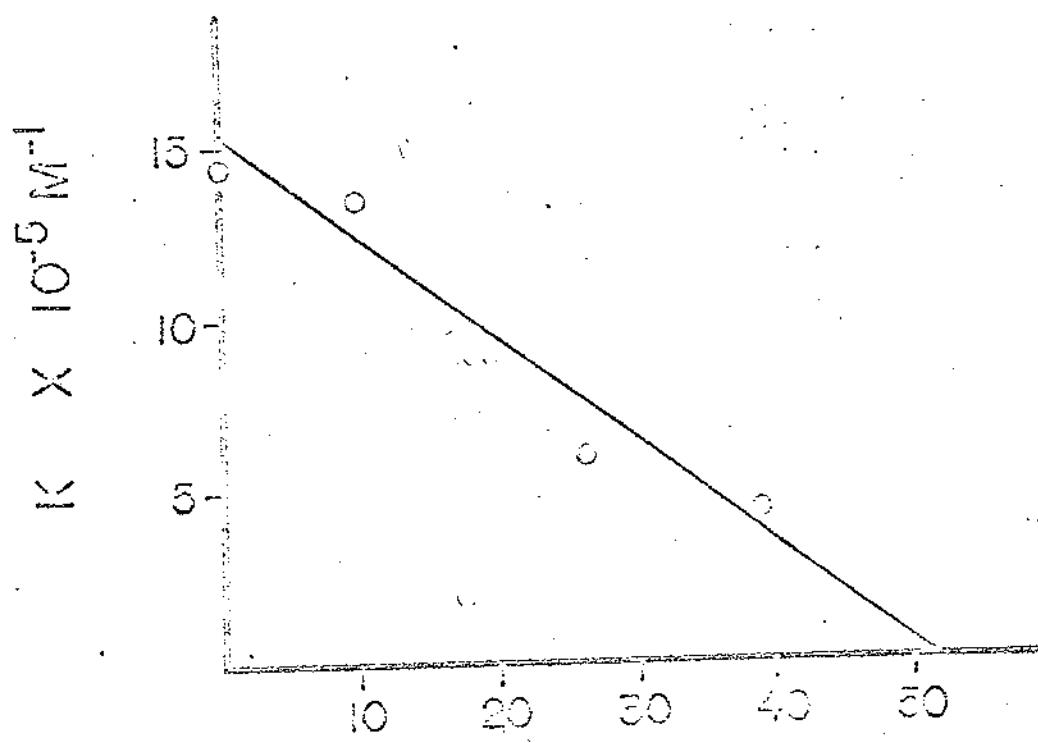
Fig 5
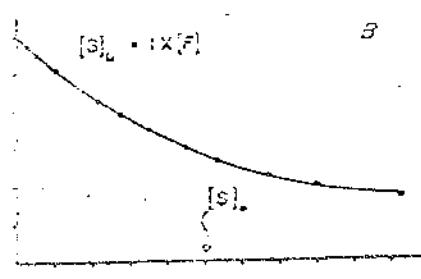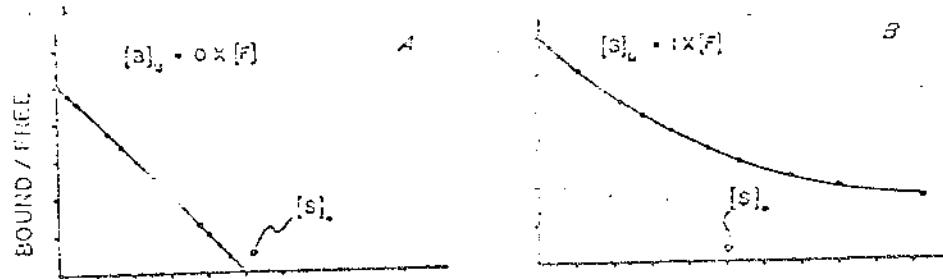
DNP·HSA MOLAR RATIO Fig. 6

SINGLE POPULATION OF BINDING SITES

$[S]_o = 5 \times 10^{-7}, \quad K = 1 \times 10^{-7}$

$[B]_u = 0 \times [F] \quad A$

$[B]_u = 1 \times [F] \quad B$

BOUND / FREE

$[S]_o$

$[S]_o$

TWO POPULATIONS OF BINDING SITES

$_1[S]_o = 1 \times 10^{-7}, \quad _1K = 1 \times 10^{8}$

$_2[S]_o = 1 \times 10^{-7}, \quad _2K = 1 \times 10^{6}$

$[B]_u = 0 \times [F] \quad C$

$[B]_u = .3 \times [F] \quad D$

BOUND / FREE

$[S]_o$

$[S]_o$

[BOUND]

[BOUND]

Fig 7

SINGLE POPULATION OF BINDING SITES

$[S]_o = 5 \times 10^{-6}, \quad K = 3 \times 10^6 + 7 \times 10^6 (1-e^{-[B] \times 10^5})$

$[B]_u = 0 \times [F] \quad A$

$[B]_u = 3 \times [F] \quad B$

BOUND / FREE

K

$[B]$

K

$[B]$

$[S]_o$

$[S]_o$

TWO POPULATIONS OF BINDING SITES

$_1[S]_o = 1 \times 10^{-7}, \quad _1K = 3 \times 10^7 + 8 \times 10^7 (1-e^{-[B] \times 3 \times 10^5})$

$_2[S]_o = 1 \times 10^{-7}, \quad _2K = 3 \times 10^5 + 7 \times 10^5 (1-e^{-[B] \times 10^5})$

$[B]_u = 0 \times [F] \quad C$

$[B]_u = .3 \times [F] \quad D$

BOUND / FREE

$[B]$

$_1K$

$_2K$

$[B]$

$[S]_o$

[BOUND]

[BOUND]
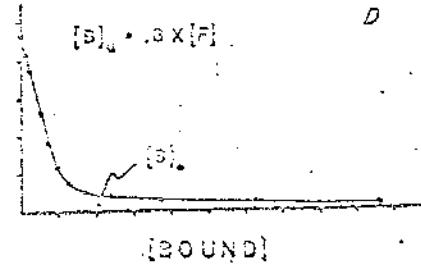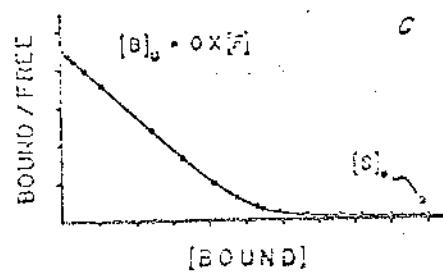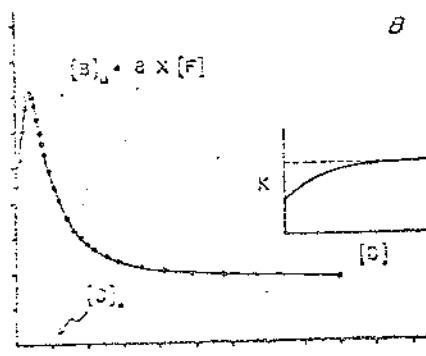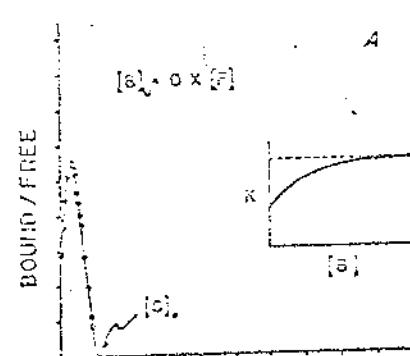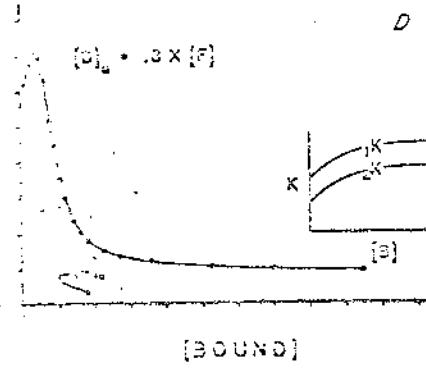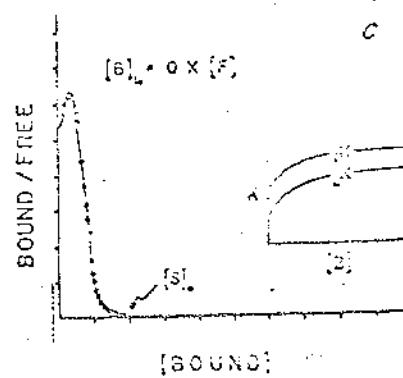
Fig 8

TABLE II

	SCATCHARD PLOT						STPS PLOT		
	Descending Limb			$(B/F)_{max}$	Ascending Limb				
	$[S]_o \times 10^6 M$	r	df		r	df	$K \times 10^{-6} M^{-1}$	r	df
$DHP_{6.8}^{HSA-3}H$	1.16	0.860	21	1.63	0.534	7	1.57	0.976	23
$DHP_{10.7}^{HSA-3}H$	1.06	0.831	27	4.78	0.724	15	2.60	0.976	29
$DHP_{21.4}^{HSA-3}H$	1.06	0.920	19	13.51	0.877	14	6.34	0.967	26
$DHP_{32.5}^{HSA-3}H$	1.23	0.886	21	12.78	0.912	12	8.10	0.857	17

TABLE III

	SCATCHARD PLOT						S... PLOT		
	Descending Limb			$(B/F)_{max}$	Ascending Limb				
	$[S]_0 \times 10^6$ M	r	df		r	df	$K \times 10^{-6}$ M^{-1}	r	df
$DNP_0 HSA-{}^3H$	3.80	0.940	16	3.701	0.923	10	1.46	0.983	22
$DNP_{10.1} HSA-{}^3H$	2.30	0.980	15	3.698	0.812	10	1.35	0.943	25
$DNP_{26.4} HSA-{}^3H$	2.57	0.950	16	2.497	0.636	9	0.59	0.995	23
$DNP_{38.8} HSA-{}^3H$	3.07	0.930	19	3.014	0.456	5	0.44	0.994	13

TABLE I

Agarose Antibodies	Ligand	SCATCHARD PLOT			SIPS PLOT		
		$[s]_0$	r	df	K	r	df
...-α...	ε-2,4-DNP-Lysine-^{3}H	2.9×10^{-7}M	0.853	35	6.6×10^5M^{-1}	0.942	30
	HSA-^{3}H *	—	—	—	—	—	—
...-HSA	ε-2,4-DNP-Lysine-^{3}H *	—	—	—	—	—	—
	HSA-^{3}H	3.8×10^{-6}M	0.940	18	1.5×10^6M^{-1}	0.983	23

*No apparent binding of these ligands was observed.

(Legends to Figures, Cont.)

Fig. 8. Theoretical Scatchard plots with positive interactions between binding sites consistent in an exponential increase of the affinity constant K, with increments in the concentrations of bound ligand: A) Single population of binding sites and no unspecific binding; B) Single population of binding sites with unspecific binding of eight times the concentration of free ligand; C) Two populations of binding sites with different initial affinities and no unspecific binding; D) Two populations of binding sites with different initial affinities and unspecific binding of 0.8 times the concentration of free ligand. Note that such exponential increase in K during the progression of the reaction does indeed generate an ascending limb in the Scatchard plots in the presence or absence of initial heterogeinity of binding sites and unspecific binding.

(Legends to Figures, Cont.)

Fig. 4. The effect of the antigens' valence on the apparent affinty constant, K, of the reaction of anti-DNP agarose with four differently DNP substituted and tritiated human serum albumin ($DNP_\alpha HSA-^3H$).

Fig. 5. Scatchard plots of the binding reactions of anti-HSA agarose with differently substituted $DNP_\alpha HSA-^3H$: A) $DNP_0 HSA-^3H$; B) $DNP_{10.1} HSA-^3H$; C) $DNP_{26.4} HSA-^3H$; D) $DNP_{38.9} HSA-^3H$.

Fig. 6. The effect of the antigens' valence on the apparent affinity constant, K, of the reaction of anti-HSA agarose with four differently DNP substituted and tritiated human serum albumin ($DNP_\alpha HSA-^3H$).

Fig. 7. Theoretical Scatchard plots with no interaction between sites to show the effects of unspecific binding ($[B]_u$) and those of heterogeinity in affinity of binding: A) Single population of binding sites and no unspecific binding; B) Single population of binding sites with unspecific binding equal to the concentration of free ligand; C) Two populations of binding sites with different affinities and no unspecific binding; D) Two population of binding sites with different affinities and unspecific binding of 0.8 times the concentration of free ligand. Note that either or both unspecific binding and heterogeneity among the specific binding sites can curve the plot downwards but neither generate an ascending limb.

tally determined maximal value of BOUND/FREE. The value of $(B/F)_{max}$
was estimated as the intercept of the two linear regressions
of each Scatchard plot, one built with the points in the ascen-
ding limb and the other constructed with the points in the
descending limb.

(Legends to Figures)

Fig. 1. Scatchard plots of the binding reactions of ε-2,4-
DNP-L-Lysine-^{3}H with anti-DNP (•-•) and anti-HSA (o-o) antibo-
dies coupled to agarose. No binding of ε-2,4-DNP-L-Lysine-^{3}H
by the anti-HSA agarose was apparent.

Fig. 2. Scatchard plots of the binding reactions of HSA-^{3}H
with anti-HSA (•-•) and anti-DNP (o-o) antibodies coupled to
agarose. No binding of HSA-^{3}H by the anti-DNP agarose was
apparent.

Fig. 3. Scatchard plots of the binding reactions of anti-DNP
agarose with differently DNP substituted $DNP_x HSA-^3H$: A)
$DNP_{6.6}HSA-^3H$; B) $DNP_{10.7}HSA-^3H$; C) $DNP_{21.4}HSA-^3H$; D)
$DNP_{32.5}HSA-^3H$. Two separate experiments (o and ∆) made under
the same conditions are shown for each antigen.

(Legends to Tables)

Table I. Antigen binding capacity, $[S]_0$, and apparent affinity constants, K, of the binding reactions of agarose coupled antibodies with the ligands ε-2,4-DNP-L-Lysine-^{3}H and HSA-^{3}H, shown with the respective linear correlation coefficients (r) and the degrees of freedom (df).

Table II. Antigen binding capacity, $[S]_0$, and apparent affinity constants, K, of the reaction of anti-DNP antibodies coupled to agarose with differently substituted $DNP_\alpha HSA$-^{3}H, shown with their respective linear correlation coefficients (r) and degrees of freedom (df). Also, statistics on the ascending limbs are presented: the linear correlation coefficients (r) were estimated with only those points lying between the Y intercept and the experimentally determined maximal value of BOUND/FREE. The value of $(B/F)_{max}$ was estimated as the intercept of the two linear regressions of each Scatchard plot, one built with the points in the ascending limb and the other constructed with the points in the descending limb.

Table III. Antigen binding capacity, $[S]_0$, and apparent affinity constants, K, of the reaction of anti-HSA antibodies coupled to agarose with differently substituted $DNP_\alpha HSA$-^{3}H, shown with their respective linear correlation coefficients (r) and degrees of freedom (df). Also, statistics on the ascending limbs are presented: the linear correlation coefficients (r) were estimated with only those points lying between the Y intercept and the experimen-

to estimate its importance in the immune system. Cooperation in the antigen-antibody reaction could be a central event in immunology if envisaged as the first magnification signal of the finding of an antigen in the general strategy of the immune response to rid the organism of foreign organic material. And, if more than molecular "contortions" may bring about cooperation, geometry of the immune complex for example, one could think of intermolecular effects and synergism between antibody populations with different intrinsic affinities.

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