UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

FACULTAD DE QUIMICA

DIVISION DE ESTUDIOS DE POSGRADO

ESTUDIOS SOBRE LA REACCION ANTIGENO - ANTICUERPO :

1. ESPECIFICIDAD
2. COOPERATIVIDAD

TESIS PARA OBTENER EL GRADO DE

MAESTRO EN CIENCIAS QUIMICAS (BIOQUIMICA)

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RESUMEN

En esta tesis se incluyen dos trabajos acerca de la natura-- leza bioquímica de la reacción antígeno-anticuerpo.

En el primero de ellos se hace una evaluación experimental - indirecta de la hipótesis de multiespecificidad de los anticuerpos. Para ello se determinó la frecuencia con que ocurren reacciones con cierta -- afinidad cuando un hapteno se enfrenta a una muestra grande de inmunoglo bulinas. Los resultados obtenidos concuerdan con las predicciones hechas por un modelo teórico de multiespecificidad y por lo tanto, apoyan la - idea de que los anticuerpos son multiespecíficos.

En el segundo trabajo se presentan una serie de experimentos tendientes a investigar la presencia de cooperatividad en la fijación de hapteno a anticuerpos anti-hapteno bivalentes. Nuestros resultados apoyan la idea de que la fijación de antígeno a algunos anticuerpos bivalentes ocurren en forma cooperativa (al menos en algunos sistemas).

Se incluye una discusión general acerca de la interacción -- antígeno-anticuerpo.

ABSTRACT

This thesis includes two works about the biochemistry of the antigen-antibody interaction.

In the first one, the hipothesis of multispecificity of antibodies is experimentally evaluated. For this, we determine the frequency with which reactions with certain affinities ocurr when a large sample of immunoglobulins are assayed against a single hapten. Anti-DNP activity was found in normal human immunoglobulins with affinities and frequencies that correlate well with those expected from multispecific binding sites. Therefore, our results support the idea that antibodies are multispecific.

The other work deals with cooperativity in the binding of univalent hapten to bivalent anti-hapten antibodies. The binding of DNP-lysine by heterogeneous and by two homogeneous anti-DNP antibodies was studied by fluorescence quenching. Scatchard plots of the titrations of heterogeneous and of one homogeneous antibodies showed ascending limbs, which are diminished or abolished when Fab fragments were assayed. The results are consistent with cooperative binding.

A general discussion about some aspects of antigen-antibody interactions is included.

Esta tesis se desarrolló en su mayor parte en el laboratorio del Dr. Carlos Larralde en el Departamento de Inmunología del Instituto de Investigaciones Biomédicas, UNAM. Una parte del trabajo experimental del artículo II se hizo en el laboratorio del Dr. Israel Pecht, en el Instituto Weizmann de Ciencias, en Israel.

Agradezco a Ruy Pérez Montfort su generosa colaboración y apoyo en la preparación de esta tesis y del artículo II. Agradezco también la ayuda de la Srita. Esperanza Ruíz, de la Srita. Violeta Aguilar y del Sr. José Avilés. Agradezco la amistad y el apoyo de todos mis compañeros del IIBM. Por último, agradezco a Carlos Larralde la confianza, el apoyo y la paciencia que me ha brindado.

Introducción

La reacción de asociación entre un determinante antigénico y el sitio activo de un anticuerpo es de fundamental importancia en la Inmunología. Esta reacción tiene lugar en puntos críticos de la red de eventos cuyo fin último es la eliminación del antígeno del organismo.

El estudio riguroso de la naturaleza bioquímica de esta reacción es importante tanto para obtener una visión clara de algunas ideas en que se sustenta la inmunología como disciplina, así como por la gran potencialidad que tienen los anticuerpos como herramienta diagnóstica y terapéutica.

En esta tesis se incluyen dos trabajos sobre dos aspectos de la reacción antígeno-anticuerpo: la especificidad y la presencia de interacciones. Estos dos trabajos forman parte de un esfuerzo que se lleva a cabo en el Departamento de Inmunología del Instituto de Investigaciones Biomédicas, UNAM, para comprender mejor el funcionamiento bioquímico de los anticuerpos en particular, y del fenómeno de reconocimiento molecular en general.

Es bien conocida la alta especificidad de un suero inmune (Landsteiner, 1945; Kabat, 1976). Esta especificidad se entiende como la capacidad del suero de reaccionar únicamente con el antígeno que indujo su formación y ocasionalmente con sustancias parecidas-

estructuralmente al antígeno original. A partir de estas observaciones, se ha inferido que los anticuerpos constituyentes de ese suero son igualmente selectivos para el antígeno que indujo su formación.- Este paradigma surgió de la idea de que para que un anticuerpo reaccionara con un determinante antigénico debía existir entre ambos una complementaridad estructural perfecta.

La idea de anticuerpos monoespecíficos (capaces de reaccionar con un solo determinante antigénico), resulta insostenible cuando se considera el número total de determinantes antigénicos distintos que pudieran existir (casi infinito) y el número total de anticuerpos con sitios activos diferentes que un individuo es capaz de sintetizar (limitado por el número de clonas linfocíticas que posee). Si cada especie de moléculas de anticuerpos fuese capaz de reaccionar con un solo determinante antigénico (o con unos pocos parecidos entre sí), no podría explicarse el hecho de que un animal sea capaz de producir anticuerpos contra casi cualquier sustancia (aún contra sustancias artificiales) cuando se le inmuniza adecuadamente. Por ejemplo, el ajolote, que solo posee alrededor de 10^6 linfocitos, es capaz de montar una respuesta específica contra el hapteno 2,4 dinitrofenol (DNP) (Haimovich y Du Pasquier, 1973). Estos resultados, y otros similares (Williamson, 1973; Quattrochi, 1969) no se pueden explicar fácilmente suponiendo que los anticuerpos son monoespecíficos: no hay suficientes clonas para responder a todos los antígenos diferentes con que un individuo pudiera llegar a estar en contacto.

Talmage, en 1959, propuso que los anticuerpos deben ser multiespecíficos, es decir, capaces de reaccionar con un número grande de sustancias diferentes entre sí. De acuerdo a Talmage, para que ocurra una reacción antígeno-anticuerpo no es necesaria una complementaridad estructural perfecta. Un complejo estable se formará siempre que entre las dos moléculas puedan establecerse interacciones no covalentes que disminuyan la energía libre del sistema. El grado en que la interacción disminuya la energía libre estará reflejado en la constante de afinidad. Ahora bien, la multiespecificidad de los anticuerpos no disminuye la alta especificidad de un suero inmune. Un suero inmune contiene una mezcla de anticuerpos diferentes que son capaces de reaccionar con el antígeno con diferentes afinidades. Ya que las afinidades son diferentes, los sitios activos de cada familia de anticuerpos son diferentes, y por lo tanto es lógico pensar que el conjunto de antígenos con los que cada familia puede reaccionar sea diferente. Esto trae como resultado que las reactividades contra otros antígenos quedará diluída a niveles imperceptibles, y la reactividad común contra el antígeno inmunizante predomina (Figura 1).

La hipótesis de Talmage ha encontrado apoyo experimental. Estudios sobre la estructura tridimensional de algunas inmunoglobulinas homogéneas con un hapteno fijado (Segal et al, 1974; Poljack et al, 1974), demuestran claramente que el requisito de perfecta complementaridad estructural es innecesario, y que bastan establecerse algunas interacciones atómicas para que un ligando se fije al sitio activo con afinidad considerable. Esto puede verse esquemá-

ANTI-A CON DISTINADOS DE HETEROGENEIDAD Y SMA CONCENTRACION DE ANTI	NUMERO DE INTERACCIONES ENTRE EL SUERO ANTI-A y DISTINTOS ANTIGENOS (A-M)												
	A	B	C	D	E	F	G	H	I	J	K	L	M
D (A F G) (A H I) (A J K) (A L M) 3 4 5 6	++ ++ ++	+	+	+	+	+	+	+	+	+	+	+	+
B (A D E) (A F G) (A H I) (A J K) 2 3 4 5	++ ++ ++	++	++	+	+	+	+	+	+	+	+		
A B C (A B C) (A D E) (A F G) (A H I) 1 1 2 3 4	++ ++ ++	++ +	++ +	+	+	+	+	+	+				
A B C (A B C) (A B C) (A D E) (A F G) 1 1 1 2 3	++ ++ ++	++ ++	++ ++	+	+	+	+						
A B C (A B C) (A B C) (A B C) (A B C) 1 1 1 1 1	++ ++ ++	++ ++ ++	++ ++ ++										

1.- Especificidad de un suero inmune con distintos grados de heterod.

2,3,4,5 y 6, moléculas distintas de anticuerpos multiespecíficos. Toas reaccionan con el antígeno A, pero las especificidades adiciona-- M) son diferentes para cada una. Si el suero inmune anti-A está - - uído por distintas clases de moléculas, las reacciones con los gru-- implicarán pocas interacciones y serán imposibles de detectar. A - que la heterogeneidad del antisuero disminuye, el número de interaccon antígenos distintos de A aumenta. Si el suero inmune es homogéonocerá a los antígenos B y C tanto como a A. Así, es claro que laicidad aumenta con la heterogeneidad dado que haya un límite de de-- dependiente del número de interacciones en cada sistema.

sólo una representación esquemática; tanto el número de especies de rpos que reaccionan con un mismo antígeno como el número de determiantigénicos diferentes con que una especie de anticuerpos puede reac se cree que son mucho mayores (13, 30).

ticamente en la Figura 2. También se ha demostrado experimentalmente que un sitio activo puede reaccionar con ligandos diferentes (Edmundson et al, 1974; Rosenstein et al 1972), y más importante aún, que las reacciones de diferentes ligandos con un mismo anticuerpo pueden desencadenar funciones biológicas efectoras tales como fijación de complemento, o proliferación y diferenciación de linfocitos B (Cameron y Erlanger, 1977; Varga et al, 1973).

Inman (1974,1978), basado en las ideas de Talmage, ha dado una descripción cuantitativa de la hipótesis de multiespecificidad. En base a ésta desarrolló un modelo probabilístico de la frecuencia con que ocurrirían reacciones con cierta constante de afinidad, si se probase una inmunoglobulina contra un conjunto grande de haptenos diferentes.

El modelo probabilístico de Inman es importante porque hace predicciones sobre el grado de multiespecificidad de las inmunoglobulinas, y porque en base a este modelo, Inman demuestra matemáticamente que: a) anticuerpos multiespecíficos pueden constituir sueros inmunes altamente específicos; b) la heterogeneidad de un suero inmune es en gran parte responsable de su alta especificidad-a mayor heterogeneidad mayor especificidad-, y c) con un número relativamente pequeño de sitios activos multiespecíficos diferentes (entre 10^5 y 10^6), un individuo puede asegurar su capacidad de responder a cualquier determinante antigénico que se le presente con un grado de especificidad adecuado;

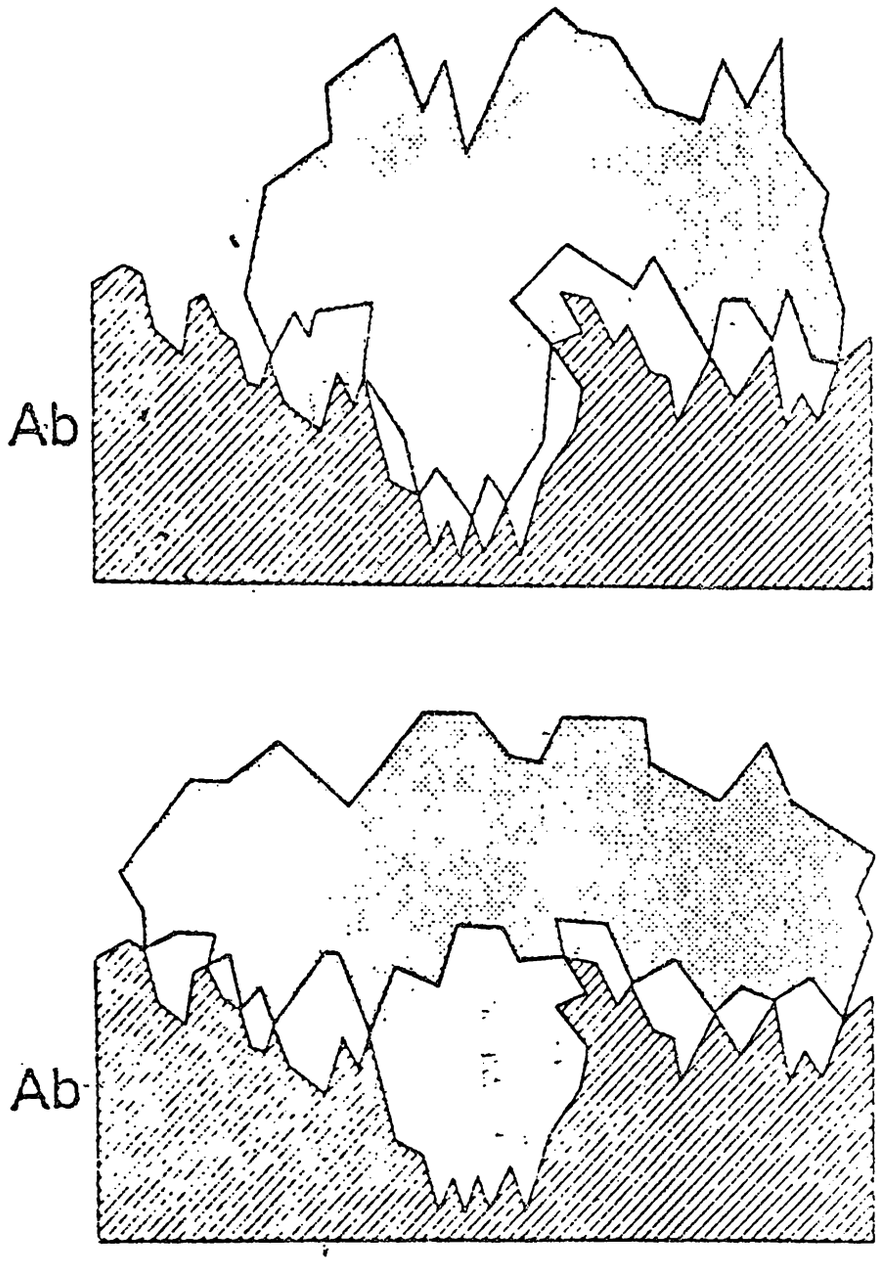

Figura 2. Representación esquemática de dos haptenos, cuyas estructuras y forma tridimensional son distintas, unidos a un mismo sitio activo de un anticuerpo.

En el artículo I (Natural DNP-binding Immunoglobulins and Antibody Multiespecificity) se hace una evaluación experimental del modelo de Inman. Se determinó la frecuencia con que ocurren reacciones con cierta afinidad cuando un hapteno (DNP) se enfrenta a una muestra grande de inmunoglobulinas (de sueros humanos). Los resultados obtenidos concuerdan con el modelo de Inman y por lo tanto, apoyan la hipótesis de multiespecificidad de los anticuerpos.

Los anticuerpos cumplen su función biológica porque la fijación del determinante antigénico al sitio activo desencadena una serie de eventos conducentes a la eliminación del antígeno, tales como activación del sistema del complemento, degranulación de células, fagocitosis, etc. Los mecanismos moleculares responsables de éstos efectos no se conocen con precisión, aunque se sabe que están involucrados los dominios constantes de las cadenas pesadas (porción Fc).

Existen algunos estudios recientes que empiezan a delinear más exactamente las regiones involucradas (Pérez-Montfort y Metzger, 1982). Dado que la reacción en el sitio activo provoca reacciones mediadas por la porción Fc, los anticuerpos pueden considerarse proteínas alostéricas (Hoffmann, 1976). Los mecanismos por los cuales se transmite la señal del sitio activo a la parte Fc se desconocen. Se ha visto que la reacción antígeno anticuerpo produce cambios conformacionales en el anticuerpo (Lancet y Pecht, 1976; Vuk-Pavlovic et al, 1974; Kaivarainen et al, 1981). Es muy probable que estos cambios traigan consigo la aparición de las funciones efectoras.

Los anticuerpos son moléculas que poseen dos sitios de combinación con el antígeno, los que se presume sean estructuralmente idénticos. Por lo tanto, existe la posibilidad de que al ocurrir la fijación de un determinante antigénico a uno de los sitios activos, el cambio conformacional producido se transmita al segundo sitio activo, aumentando su afinidad por el antígeno (cooperatividad), o reduciéndosela (interacción negativa).

Aunque existen algunas evidencias de cooperatividad en la reacción antígeno-anticuerpo, ninguna de ellas es totalmente concluyente (ver introducción al artículo II: Cooperativity in the Reaction of DNP-lysine With Anti-DNP Antibodies). Fenómenos cooperativos han sido reportados en gran número de sistemas de fijación como: hemoglobina-oxígeno (Perutz, 1968, 1970); enzima-sustrato (Levitzky y Koshland, 1972); receptor-hormona (Hope y Walti, 1975); DNA-proteína (Alberts y Frey, 1970); etc.

La cooperatividad ha sido vista como un fenómeno molecular que da a los sistemas de fijación la capacidad de regularse por la concentración de ligando (Gabler, 1977). El hecho de que haya tantas moléculas con características de fijación cooperativa, sugiere que este fenómeno debe traer consigo alguna ventaja adaptativa (Koshland, 1976). La cooperatividad podría ser importante para el funcionamiento de los anticuerpos, tomando en cuenta que para la activación de las funciones efectoras es necesario que los dos sitios activos hayan reaccionado con el antígeno.

En el artículo II se presentan una serie de experimentos tendientes a investigar la presencia de fijación cooperativa de hapteno (DNP) a anticuerpos anti-DNP bivalentes. Nuestros resultados apoyan fuertemente la idea de que la fijación de antígeno a algunos anticuerpos bivalentes ocurra en forma cooperativa (al menos en algunos sistemas).

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NATURAL DNP-BINDING IMMUNOGLOBULINS AND ANTIBODY MULTISPECIFICITY

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Abstract—Anti-DNP activity was found in normal human immunoglobulins with average affinities (10^4–10^6 l/mole) and frequencies (15–2%) that correlate well with those expected from multispecific binding sites.

INTRODUCTION

Antibody multispecificity is defined as the ability of an individual immunoglobulin to bind alternatively at its active site two or more distinct antigenic determinants. Multispecificity is of theoretical interest because it explains how a limited repertoire of antibody combining sites would be capable of reacting with, and discriminating among, an immense number of antigenic determinants (Talmage, 1959; Inman, 1974; Richards and Konigsberg, 1973). In fact, Talmage (1959) explains the higher specificity of an antiserum compared to that of is constituent antibodies on the basis of multispecificity and heterogeneity.

The idea of antibody multispecificity is supported by some experimental observations. The clearest indications come from studies using myeloma immunoglobulins binding different haptens (Richards *et al.*, 1974; Eisen *et al.*, 1970; Tolleshang and Hannestad, 1975, Rosenstein *et al.*, 1972) but there is evidence indicating that normally raised antibodies also are multispecific (Varga *et al.*, 1973; Michaelides and Eisen, 1974; Cameron and Erlanger, 1977). Also in favor of multispecificity is the frequency with which myeloma immunoglobulins bind conventional haptens such as DNP,† phosphorylcholine and a series of carbohydrate moieties (Potter, 1977).

In this paper we present observations of a different nature that support the idea of antibody multispecificity. The observations deal with the probability of encountering an association constant greater than a given value in a set of normal immunoglobulins when tested with a given hapten, DNP-lysine in our case. The results approximate to Inman's (1978) theoretical predictions very closely; so close in fact that his physical model of the active site–hapten interaction and the very important conclusion derived from it—that a given hapten will be capable of reacting with about 1% of all immunoglobulins with affinity constants equal to or greater than 10^4 l/mole—appear essentially valid.

MATERIALS AND METHODS

Experimental design

Two different methods were employed in the determination the mean association constant (K_o) for the DNP determinant: one was employed in a large and the other in a small sample of normal human sera. In the small sample, K_o was determined by directly assaying by fluorescence quenching the affinity with which DNP-binding immunoglobulins purified from each serum bound DNP-lysine. The cumulative frequency distribution of K_os found in this sample was compared to that predicted by Inman (1978). In the large sample, K_o was estimated indirectly by assaying the anti-DNP activity in the serum by hemagglutination (HA) of human erythrocytes coupled with $DNP_{32}HSA$. The HA titer was then expressed as the ΔK_o between sera by use of the linear correlation between HA titer, antibody concn and K_o, as built with purified sheep anti-DNP antibodies. Then, the final K_o corresponding to each titre was assigned by fitting the curve of frequencies of ΔK_o to that of the frequencies of K_o as estimated by florescence quenching.

This design is based on the assumption that the pool of different immunoglobulins in our sets of normal sera constitutes a representative sample of the entire immunoglobulin repertoire of the donor (human) species (Inman, 1974; Williamson, 1976).

Purification of DNP-binding immunoglobulins from normal human sera

A set of 36 sera from healthy donors were used to purify DNP-binding immunoglobulins. Between 5 and 7 ml of each serum were passed through a 5-ml column of Sepharose 4B–DNP-lysine immunoadsorbent prepared by the method of March *et al.* (1974). The column was washed with PBS until the

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†Abbreviations: DNP, 2,4-dinitrophenol; DNP-lysine, ε-(NH_2)-2,4-dinitrophenyl-lysine; $DNP_{32}BSA$, bovine serum albumin with 23 moles of DNP/mole of protein; DNBS, 2,4-dinitrobenzensulfonic acid sodium salt; PBS, 0.01 *M* NaH_2PO_4–Na_2HPO_4, 0.15 *M* NaCl, pH 7.4; HSA, human serum albumin.

eluate contained no protein ($O.D._{280} < 0.02$). Eight millilitres of DNP-OH (10^{-1} *M*) were passed through the column, and the eluate was directly chromatographed in a 10-ml column of Dowex 1-8X, to eliminate DNP-OH. Fractions (2.5 ml each) were collected and those containing protein were pooled and concentrated to 2 ml final vol (Minicon B-15, Amicon Corp.). IgM and IgG concns in each sample were then determined with LC-Partigen radial immunodiffusion plates (Behring) using human IgM and IgG standards (Hyland).

K_o *of purified DNP-binding immunoglobulins from humans*

The K_os of purified human DNP-binding immunoglobulins for DNP-lysine were determined by fluorescence quenching in an Aminco–Bowman spectrophotofluorometer at 25°C. Excitation wavelength was 295 nm; emission wavelength, 345 nm. Ligand concns ranged from 10^{-7} to 10^{-4} *M*. The % quenching values were calculated by correcting each fluorescence value for solvent fluorescence, dilution and non-specific quenching, and normalizing the resulting value, taking initial fluorescence as 100%. Correction for non-specific quenching was done using the titration of a solution of DL-tryptophan (Sigma) under the same conditions.

The molar concns of binding sites were calculated from the IgM and IgC concns determined, assuming a 900,000 mol wt and valence or 10 for IgM and a 150,000 mol. wt and valence 2 for IgG.

Calculations were made using a Q_{max}-value of 50 for all the sera, because, due to the low affinities, saturation could not be reached with the hapten concns used. Higher concns are not recommended since the correction needed is too large [see Eisen and McGuigan (1971) for a complete description of the mathematical treatment of the data]. K_o was calculated from Sips plots of the data.

Purification of sheep anti-DNP antibodies

Antiserum against DNP was obtained from a sheep that had been hyperimmunized with DNP-BSA for a period of over 2 years using Freund's complete adjuvant (DIFCO) on a number of occasions. $DNP_{23}BSA$ (5 mg/ml PBS) was injected intramuscularly and 7 days later the animal was bled from the jugular vein. The anti-DNP antiserum was separated from the clot and the γ-globulin fraction was obtained by precipitating 3 times with 33% $(NH_4)_2SO_4$.

Anti-DNP antibodies were isolated from the γ-globulin fraction using a Sepharose 4B–DNP-lysine immunoadsorbent (March *et al.*, 1974). The γ-globulin fraction (1200 mg in 30 ml) was incubated with 25 ml of immunoadsorbent for 18 hr at 25 C and then the mixture was filtered on a sintered glass funnel and the immunoadsorbent thoroughly washed with PBS to remove proteins not specifically adsorbed. Anti-DNP antibody fractions with different K_o were sequentially eluted from the immunoadsorbent with a discontinuous gradient of increasing DNP concn. This was carried out by incubating the immunoadsorbent with an equal vol of DNP-OH (Merck) (stock solution 0.1 *M* in 0.1 *M* $NaHCO_3$), diluted to a given concn with PBS, for 1 hr at room temp. with occasional stirring. The DNP concns used in the discontinuous gradient ranged between 10 and 10^{-1} M. After each incubation the mixture was filtered on a sintered glass funnel and washed once with 25 ml of DNP-OH at the same concn. Each filtrate was concentrated by ultrafiltration (Amicon membrane XM 100) to an approx vol of 10 ml. To remove free DNP each conc. filtrate was passed through a Dowex 1-8X (Sigma Chemical Co.) column (20 ml).

K_o *of purified sheep anti-DNP antibodies*

The K_o of each antibody fraction for DNP-lysine was measured by fluorescence quenching as described for human antibodies, except that: (a) ligand concns ranged from 2×10^{-8} to 2×10^{-4} *M*; (b) the molar concn of binding sites was calculated from the protein concn [determined as described by Lowry *et al.* (1951)], assuming a 160,000 mol. wt and valence of 2; and (c) the value of Q_{max} was obtained at high ligand concns ($Q_{max} = 40$). The K_o of each fraction was obtained from Sips' plots.

Sensitization of erythrocytes

Human O Rh^- blood was obtained from the blood bank of the Centro Medico La Raza (Instituto Mexicano del Seguro Social). After the erythrocytes had been separated from the blood by centrifugation and had been washed 4 times with PBS they were fixed by incubation in a 1% solution of glutaraldehyde (Merck) in PBS for 1 hr at room temp with gentle stirring. After incubation, the erythrocytes were washed 4 times with PBS.

One half of the packed cells (fixed but unsensitized) were then resuspended in an equal vol of PBS and were used either for adsorption of the test human sera or as controls in the HA assay (see later). The other half of the packed cells were sensitized with $DNP_{32}HSA$ which had been prepared from HSA (Sigma Chemical Co.) and DNBS (Eastman Kodak Co.) as described by Eisen (1964). A 5% suspension of cells was incubated in a solution of $DNP_{32}HSA$ (12 mg/ml) in PBS for 18 hr at 4 C with slow stirring. The cells were then washed with PBS 4 times and resuspended in a equal vol of PBS. The cells were stored in PBS with 0.02% sodium azide at 4°C until used.

The cells were not agglutinated by anti-A, anti-B or anti-D commercial antisera (Johnson & Johnson) either before or after either treatment.

HA assays

HA assays were performed on Microtiter V plates (Cooke Laboratories Inc.) using 0.025-ml micro-

pipettes and microdilutors. To test the specificity for the DNP hapten in the positive reactions, the following criteria were applied: (1) the serum should not agglutinate fixed, unsensitized cells; and (2) the agglutination of sensitized cells should be inhibited by soluble $DNP_{32}HSA$ but not by soluble HSA. Therefore, each serum was assayed in three different tests: the agglutination of fixed unsensitized cells, and the agglutination of sensitized cells in the presence of either soluble HSA (2 mg/ml) or soluble $DNP_{32}HSA$ (6 mg/ml in the first two wells, and serially diluted thereafter). In all cases, 1% suspensions of cells were used with PBS (with 2 mg/ml HSA) as diluent. HA plates were incubated for 18 hr at 4°C and the results were read the next day. To be sure that the sensitivity of the HA system remained constant throughout the experimentation, a standard anti-DNP antiserum was included each time the HA assays were performed.

The HA titers of purified sheep anti-DNP antibodies were determined exactly as described for human sera, starting from a concn of approximately 1 mg of protein/ml, on four different days. All titers correspond to the last dilution showing positive agglutination.

Four hundred and ninety-two human sera from healthy donors were tested for anti-DNP HA activity. Prior to any assay, 0.1 ml of each serum was adsorbed with an equal vol of packed fixed cells at 4°C for 1 hr to eliminate natural antibodies against erythrocyte antigens.

RESULTS

DNP-binding immunoglobulins from human sera

From the set of 36 sera from which DNP-binding immunoglobulins were purified, a protein peak that eluted from the Sepharose-DNP-lysine with soluble DNP-OH was detected in 26 cases. The concns of DNP-binding M and G immunoglobulins, in the original vol of serum, ranged from 0.023 and 0.006 mg/ml serum for IgM (mean = 0.0125, not detected in five of the 26 cases) and from 0.027 to 0.005 mg/ml serum for IgG (mean = 0.0134, detected in all 26 cases).

The purified immunoglobulins obtained were titrated by fluorescence quenching for determination of K_o for the DNP-lysine hapten. A K_o-value could be calculated for 10 of them, as 16 showed no appreciable quenching when comparing their titration with that of a tryptophan solution at the hapten concns used.

The frequencies with which a K_o equal to or greater than a given value (K_{min}) were obtained are plotted in Fig. 1, along with the curve obtained by Inman (1974) from his probabilistic model. Briefly, about 27% of human sera (10/36) had anti-DNP activity with a K_o greater than $10^{3.6}\ M^{-1}$, a proportion that consistently decreased towards higher K_o values, the maximal K_o being $10^{4.8}\ M^{-1}$ in 2.7% (1/36) of the sera.

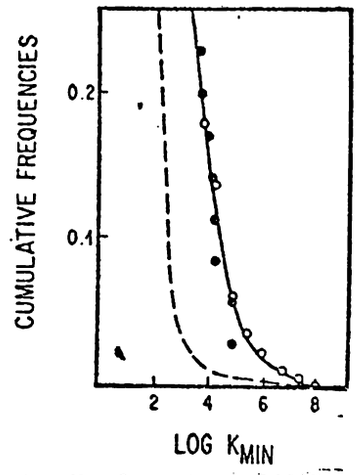

Fig. 1. Frequencies of occurrence of binding association constants equal to or greater than a certain given value (K_{min}) found for anti-DNP activity in normal sera. The filled circles (●) were obtained from the direct determination of K_o of the DNP-binding immunoglobulins purified from human sera; the empty circles (○) were obtained from the frequencies of HA titers (Table 2) and placed so as to have the maximal overlapping with the filled circles. The dashed line was obtained by Inman (1978) from his probability model and represents the binding of DNP-lysine by a population of mouse myeloma immunoglobulins (Eisen *et al.*, 1970).

K_o *and HA titer of purified sheep anti-DNP antibodies*

Table 1 shows the DNP concns used to elute each anti-DNP antibody fraction from the immunoadsorbent, the K_o calculated, and also the HA titers obtained with each fraction at the concns shown. All the seven affinity fractions precipitated with $DNP_{32}HSA$ but not with HSA.

Correlation of HA titer with K_o *and concn of antibodies*

On the basis of the mean HA titer and protein concn for each affinity fraction shown in Table 1 the correlations between the starting concn of purified anti-DNP antibodies and the HA titer for each K_o-class were constructed (Fig. 2), assuming a linear correlation between HA titer and anti-DNP antibody concn. From Fig. 2 the HA titer that each affinity class would have at concns of 0.031, 0.13, 0.5 and

Table 1. Purified anti-DNP fractions sequentially eluted from the Sepharose–DNP-lysine with a discontinuous gradient of DNP concns

DNP molar concn used for elution	Log K_o	Protein concn (mg/ml)	Observed titers (log_2)	Mean HA titer
1×10^{-4}	5.00	0.84	5, 5, 5, 5	1:32
5×10^{-4}	5.35	1.05	6, 5, 5, 6	1:48
1×10^{-3}	5.54	1.05	6, 6, 6, 6	1:64
5×10^{-3}	5.94	0.98	6, 6, 6, 7	1:80
1×10^{-2}	6.21	0.97	7, 6, 6, 7	1:96
5×10^{-2}	6.34	1.10	7, 7, 7, 7	1:128
1×10^{-1}	6.51	0.79	8, 7, 7, 8	1:196

Log K_o values were calculated from a Sip's plot of fluorescence quenching titrations. Protein concns were determined by the method of Lowry *et al.* (1951) using BSA as standard. The HA titer of each fraction was determined 4 times at the protein concn shown, and the resulting titers were averaged.

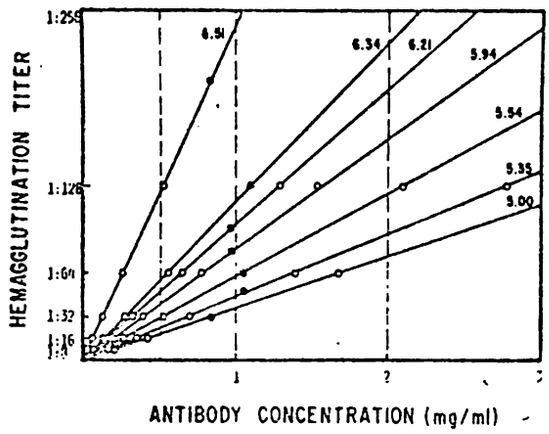

Fig. 2. Correlation between HA titer and concn of purified anti-DNP antibodies with different K_o-values. The number on each line is the log K_o of each fraction. The mean titer (●) for each fraction at one antibody concn was determined experimentally from four replicates. The remaining points (○) were calculated assuming a linear correlation between HA titer and antibody concn. From the regression we can calculate the theoretical titers that each fraction would show if its antibody concn were 0.03, 0.12, 0.5 or 2 mg/ml.

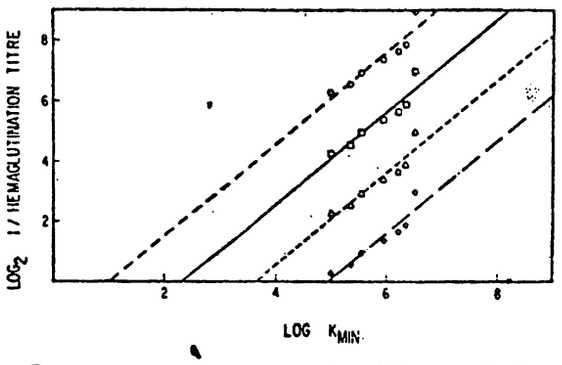

Fig. 3. HA titer as function of K_o, for four different antibody concns: 0.03 (◇), 0.12 (△), 0.5 (□) and 2 (○) mg/ml. The values of the points were derived from Fig. 2. These standard curves were constructed by plotting the $\log_2$ of the reciprocal of the titer values against log K_o ($m = 1.526$, $R = 0.95$ for each of the four lines). From the inverse of the slopes, the change in log K_o required for a change in 1 $\log_2$ 1/HA titer unit (for equal antibody concns) was found to be 0.655.

2 mg/ml was calculated. With this data, the correlation between HA titer and K_o was constructed (Fig. 3). From the inverse of the slopes of the lines in Fig. 3, the difference in log K_o necessary to bring up a unit change in $\log_2$ 1/HA titer was calculated and found to be the same for all the antibody concns: 0.655 log K_o units.

Anti-DNP HA activity in normal sera

As means of testing the stability of the dinitrophenylated human erythrocytes, they were tested every day during the experiment against a standard anti-DNP sheep antiserum; 0 in 11 of the 13 days a titer of 1:1024 was found with the sheep antiserum; on the other two days the titer was 1:512.

Of the 492 sera 129 gave a positive reaction with sensitized cells, 22 of which also reacted with fixed unsensitized cells and were not considered further. Of the remaining 107 sera, the agglutination was inhibited by soluble $DNP_{32}HSA$ but not by soluble HSA in 87 cases, which were taken as showing specific anti-DNP activity. The HA titers obtained ranged from 1:2 (21 sera) to 1:256 (one serum) (Table 2). To relate the frequencies of HA titers observed with frequencies of K_o, the correlation between K_o and HA titer for our system (Fig. 3) was used, assuming that the antibodies responsible for the agglutination of sensitized cells were at the same concn in all sera.* Relating the value of 0.655 log K_o units required for a change of 1 $\log_2$ 1/HA titer unit (that is one titer), with the observed frequencies of each titer, a curve of cumulative frequencies of K_os could be constructed, but its position along the log K_o axis could not be defined from these data alone. Therefore, we placed the frequency curve as to have the maximal overlapping with the frequencies of K_o found for the DNP-binding immunoglublins purified from the small set of sera (Fig. 1). As can be seen, the overlapping between the data obtained with both samples is good, and the shape of the curve is similar to that obtained by Inman (1974), although displaced to higher K_{min}-values.

*Although this assumption may at first appear unlikely, we found it reasonable, as the conens of DNP-binding IgG and IgM that were measured in normal sera have no more than a five-fold difference between their minimal and maximal values, and, thus, in an HA assay this difference would mean at most 2 $\log_2$ 1/HA titer units. Therefore, we think that the differences in HA titers within the sera were due mainly to different affinities and not to differences in concn of DNP-binding immunoglobulins.

DISCUSSION

Anti-DNP activity was found in normal human sera. By an immunoadsorption procedure followed by fluorescence quenching, 10 out of 36 sera were shown to contain DNP-binding immunoglublins with

Table 2. Frequencies of anti-DNP activity in normal human sera

	HA titer							
	1:256	1:128	1:64	1:32	1:16	1:8	1:4	1:2
Sera with anti-DNP activity	1	2	3	5	6	12	37	21
Frequency (%)	0.2	0.4	0.6	1.0	1.2	2.4	7.5	4.2
Cumulative frequency	0.2	0.6	1.2	2.2	3.4	5.8	13.3	17.5
Δ log K_o	0.0	−0.66	−1.31	−1.97	−2.62	−3.27	−3.93	−4.58

Frequencies were calculated dividing the number of sera showing each titer by the total number of sera tested (492). The values of Δ log K_o were calculated from Fig. 2.

measurable affinity ($K_o > 10^{3.6} M^{-1}$) for the hapten DNP-lysine. Besides, 87 out of 492 sera (17.5%) showed hemagglutinating activity specific for the DNP determinant. These high frequencies of anti-DNP activity indicate that the fraction of immunoglublins capable of binding DNP in the entire antibody repertoire must be high, supporting the idea that antibodies are multispecific.

Figure 1 shows the curve of frequency of anti-DNP activity found in normal human sera as a function of K_{min}, and the curve given by Inman [Fig. 3 of Inman (1978)] as derived from his mathematical model of multispecificity. Data obtained by two different methods were used to construct the curve of frequency of K_{min} for the DNP determinant of immunoglobulins from normal human sera. The direct determination of K_o is the best way of testing the validity of Inman's model, but it would be practically impossible to assay by this method a very large sample of sera. On the other hand, the data obtained from the HA assays has the advantage that the shape of the curve could be better defined, specially in the zone of low frequencies, as a larger sample of sera was used. The curve obtained from our data shows that the frequency of each K_{min} decreases as the value of K_{min} increases; the frequency of $K_o > 10^5$ was as high as 5%. Our curve is similar in shape to that of Inman's, although it shows a displacement to higher frequencies. The main point is that the frequency of anti-DNP activity among human sera with $K_o > 10^5$ l mole^{-1} is of the order of 1–10%, regardless of reasonable variations in antibody concn. We take these high frequencies to be consistent with the hypothesis of antibody multispecificity as posed by Inman.

The displacement of our experimental curve from the theoretical one is to be expected because of a number of reasons. Firstly, our frequencies are higher because our definition of frequency (the fraction of sera which bind hapten above a given K_{min}-value) differs from that of Inman (the fraction of all individual immunoglobulins that interact with the hapten with affinities greater than K_{min}). The serum-count frequencies are higher simply because a serum contain a set of many different immunoglobulins, the chances of encountering a positive reaction being higher when testing a mixture of different immunoglobulins than when testing them individually. That Inman's frequencies are indeed lower than serum counts can be seen from the serum concns of the DNP-binding immunoglobulins relative to total IgG and IgM concns. For IgG, the mean concn found was 0.0134 mg/ml, that is, about 0.001% of the mean IgG concn in serum; for IgM, this figure is about 0.009%.

Also, because of the immunoadsorption technique employed to isolate DNP-binding immunoglobulins, we are in some way selecting those immunoglobulins with affinities for DNP greater than a certain value. Thus, we measured the average affinity of the subpopulation isolated, and not of all the immunoglubulins in the serum that may react with DNP, albeit with very low affinities. Thus, our experimental curve is probably displaced to K_{min}-values higher than the real average affinities.

A minor contribution to the displacement of the curve may come from the determination of K_o of DNP-binding immunoglobulins purified from human sera. For an exact affinity determination by fluorescence quenching, good estimates of Q_{max} and the molar concn of binding sites (S_o) are required. As stated earlier, the Q_{max} was not determined experimentally, but a value of 50 was used for all the titrations, and the S_o was determined from the IgG and IgM concns in the eluate from the immunoadsorbent, though it is not really known if all the active sites remained active and if the IgM antibodies used their 10 active sites in binding. However, errors in Q_{max} and S_o are less important with low affinity antibodies, as our's are (Eisen and McGuigan, 1971). For example, the same data for the antibodies purified from serum 1009, calculated using different Q_{max}-values, ranging from 30 to 70, yielded values for log K_o that differed by less than 1 log K_o unit. Further, decreasing by half the S_o-value used changed log K_o only by 0.20 units.

We feel that none of these uncertainties weaken the main observation that there is a significant fraction of non-immune immunoglobulins capable of binding DNP with appreciable affinity. Thus, it seems reasonable to propose that a number of antibodies directed against different natural antigens bind DNP. Furthermore, the close similarity of shapes of our experimental frequency curve of K_{min} with that of Inman's theoretical model for antibody active sites strengthens the notion of multispecificity.

Surely more experimental data are needed to better determine the exact position of the curve, that is, the actual degree of multispecificity, which, as pointed out by Inman, may be different for each particular immunoglobulin (tested against several haptens) or each particular hapten (tested with many different immunoglobulins).

The presence of anti-DNP activity in normal sera has been previously reported in humans (Terry *et al.*, 1980; Farah, 1973; Brandriss, 1977; Tuset *et al.*, 1978) and rabbits (Haimovich *et al.*, 1970), but the percentages of sera with anti-DNP activity are not similar; discrepancies probably arising from differences in the sensitivity of the screening methods employed. Our results are original in that they express anti-DNP activity in terms of K_a and are, therefore, the only ones useful in testing the validity of Inman's model of multispecificity.

Other possible explanations of our results alternative to antibody multispecificity need consideration. The existence of anti-DNP antibodies in the sera of normal individuals could be due to previous exposure to the hapten. We feel such exposure is unlikely since DNP is not present in our actual environment in

concentrations sufficiently high to immunize the species in which natural anti-DNP activity has been found. Also, DNP is a synthetic substance that could not have been the immunogen for the appearance of antibodies capable of reacting with it in species existing long before man. The possibility remains that the presence of natural anti-DNP antibodies is due to a cross-reaction with an unknown but very common antigen that holds, in some part of its molecule, structural similarities with DNP. This possibility also seems improbable to us since the DNP-anti-DNP system has been widely studied and cross-reactions with common antigens have seldom been reported (Michaelides and Eisen, 1974), and since the haptens with which homogeneous DNP-binding immunoglobulins react in addition to DNP are different for each particular immunoglobulin (Eisen *et al.*, 1970; Cameron and Erlanger, 1977). Thus, we conclude that DNP is capable of reacting with many different immunoglobulins whose synthesis could have been induced by a variety of antigens with which the invididual has been in contact.

If experiments with other haptens should indicate that multispecific binding is general, we would then have to consider the significance of a role but seemingly unnecessary genetic diversification of immunoglobulin active sites.

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COOPERATIVITY IN THE REACTION OF DNP-LYSINE WITH ANTI-DNP ANTIBODIES

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ABSTRACT

The binding of DNP-lysine by heterogeneous and by two monoclonal anti-DNP antibodies was studied by fluorescence quenching. Scatchard plots of the titrations of heterogeneous and of one homogeneous antibodies showed ascending limbs, which are diminished or abolished when Fab fragments were assayed. The results are consistent with cooperative binding.

INTRODUCTION

The binding reaction of antibodies with antigens has long been considered to occur in a non-cooperative manner (Metzger, 1974, 1978; Kelly et al., 1971; Nisonoff et al., 1975; Skubitz et al., 1977). However, some evidence has been reported that there is cooperativity in the binding of some hormones by anti-hormone antibodies (Matsukura et al., 1971; Matsuyama et al., 1971; Weintraub et al., 1973; Carayon & Carella, 1974), and in the binding of multivalent dinitrophenylated antigens by anti-DNP antibodies coupled to a solid phase (Celis et al., 1977). Furthermore, it has been shown that the light chain dimer of a myeloma immunoglobulin binds two DNP groups per dimer in a cooperative manner (Lancet et al., 1977, Zidovetzki et al., 1979). (Light chains dimers have been proposed as models for primitive antibodies (Edmundson et al., 1974). An ascending limb in Scatchard plots (Scatchard, 1949) consistent with cooperativity has also been observed in the binding of a univalent hapten (^{3}H-DNP-lysine) by anti-DNP antibodies elicited by conventional immunization and assayed by equilibrium dialysis (Pérez-Montfort, 1979). This latter work has been criticized because of the large scatter of experimental points in the region of low saturation (a common feature of equilibrium dialysis experiments) and because of the use of heterogeneous preparations of antibodies.

In order to see if the ascending limb, in the Scatchard plot is a real event and not the result of any artifact, we studied the same system (DNP-lysine and anti-DNP antibodies) using a completely different experimental technique: fluorescence quenching (FQ), and used both mono-

clonal and polyclonal (heterogeneous) antibodies. Scatchard plots with ascending limbs were consistently obtained when preparations of polyclonal antibodies and of one monoclonal antibody (Mo) were assayed. Plots of another monoclonal immunoglobulin (MOPC-315) or of Fab fragments from Mo showed no ascending limbs, and those of Fab fragments of polyclonal antibodies showed a significantly diminished or absent ascending limb.

In view of these results, the possibility of the ascending limb of Scatchard plots arising from an experimental artifact and/or because of the heterogeneity of the antibodies used in previous studies may be discarded and the notion that at least some antibodies can bind their ligand cooperatively must be seriously considered.

MATERIALS AND METHODS

Heterogeneous anti-DNP antibodies and their Fab fragments:

Antiserum against DNP was obtained from a sheep that had been hyperimmunized with DNP-BSA for a period of over two years. DNP_{23}-BSA (5 mg/ml PBS) was injected intramusculary and seven days later the animal was bled from the yugular vein. The anti-DNP serum was separated from the clot and the gammaglobulin fraction precipitated with 33% $(NH_4)_2SO_4$ three times.

Anti-DNP antibodies were isolated from the gamma-globulin fraction using Sepharose-4B-lysine- DNP immunoadsorbent which was prepared by the method of March et al. (1974) from Sepharose 4B (Pharmacia) and ϵ-NH_2- dinitrophenyl-L-Lysine (Sigma Chemical Co.). The gammaglobulin

fraction was incubated with the immunoadsorbent for 18 hrs. at 25°C with gentle shaking. The mixture was filtered on a sintered glass funnel and thoroughly washed with PBS. The anti-DNP antibodies were eluted from the immunoadsorbent with 0.0025 M DNP. This was carried out by incubating the immunoadsorbent with an equal volume of DNP (Merck), for one hour at room temperature. After the incubation time the mixture was filtered on a sintered glass funnel and washed once with 25 ml of DNP at the same concentration. The filtrates were pooled and concentrated by ultrafiltration (Amicon membrane XM 100) to an aproximate volume of 10 ml. To remove DNP the concentrated filtrate was cromatographed on a 20 ml Dowex 1-8X column (Sigma Chemical, Co.).

Fab fragments of anti-DNP antibodies were prepared as follows: a) To a solution of antibody (18 mg in 10 ml PBS), we added 1:100 (w/w) of papain (Sigma), EDTA and β-mercaptoethanol to final concentrations of 0.002 M and 0.01 M respectively. b) The mixture was incubated for 3 hrs. at 37°C with occasional shaking. c) Iodoacetamide, 0.013 M, was added, and the pH was raised to 8.2 with Tris-HCl, 2 M. d) After 30 minutes of stirring at room temperature, the mixture was cromatographed on a Sepharose-lysine-DNP column and was thoroughly washed. e) The adsorbed protein was eluted with 0.1 M DNP and the eluate was cromatographed through a Dowex 1-8X column.

The presence of unigested antibodies in Fab preparations was assesed by SDS-PAGE and by gel filtration on a Sephadex G-75 superfine column. SDS-PAGE was performed in 5%/7.5% gel (Laemmli, 1970) stained with Coomasie Brillant Blue.

Mild reduction and alkylation of antibodies (3 mg in 3 ml PBS) were carried out with dithiothreitol (Sigma) 0.001 M in Tris buffer 0.1 M, pH 8.2. After incubating one hour at room temperature, Iodoacetamide, 0.004 M was added, and the mixture was incubated for 30 min. and dyalized exhaustively with PBS.

Preparation of anti-DNP hybridoma (Mo)

Cell lines and media.

Dulbecco's modified minimal essential medium (DMME) with L-glutamine and glucose (GIBCO) was used for the preparations of other media. Incomplete medium (IM) was prepared from DMME, $NaHCO_3$ (0.24 M) and HEPES (N-2hydroxyethyl-piperazine-N-2-ethanesulfonic acid) 0.08 M, pH 7.4. Complete medium (CM) contained sodium piruvate (0.1 M), L-glutamine (0.002 M), non-essential aminoacids (0.001 M), penicillin (1 IU/ml), streptomycin (1 µg/ml) and 15% heat inactivated fetal bovine serum. HAT medium was prepared from CM and a solution of hypoxantine, aminopterine and thymidine (Littlefield, 1964).

The enzyme deficient, non secreting myeloma cell line Sp2/0-Ag 14 (Schulman et al., 1978) was cultured in CM containing 8-azoguanine, at 37°C in a humid atmosphere with 5% CO_2, at concentrations of up to 5×10^4 cell/ml.

Immunization

Male BALB/c mice (6 to 8 weeks old) were immunized intraperitoneally with 0.5 mg of DNP-KLH (prepared from Keyhole Lympet Hemo-

cyanin, Calbiochem, and 2,4-dinitrobenzen-sulfonic acid, Eastman Kodak) in 0.1 ml of Freund's complete adjuvant (GIBCO). Thirty days later they were immunized in the same manner but using Freund's Incomplete Adjuvant. Thirty days after the second immunization, they were injected intravenously with 0.1 mg of DNP-KLH in isotonic saline. Four days later, the spleens of two animals were removed, and the cells were dissaggregated, and washed with IM.

Cell fusion and cloning

10^7 washed tumor cells were added to 10^8 washed lymphocytes, and centrifuged. 0.2 ml of a 50% polyethyleneglycol (1000 MW, Sigma) solution in IM were added to the cells. The cells were shaken gently for 6 min, washed and resuspended in CM at a concentration of 4×10^5 cells/ml, and distributed in microculture plates. They were then cultivated for 3 weeks in selective HAT medium, and the supernates were assayed for anti-DNP activity by passive hemagglutination. Cells from positive wells were cloned by limiting dilution and cultured in monocyte layers (2×10^5 cells/ml) for 8 days. After this, anti-DNP activity was assayed in the supernates by passive hemagglutination. One of the positive clones was selected and expanded *in vitro* prior to it's injection into BALB/c mice.

Purification of monoclonal antibody (Mo)

BALB/c mice were injected intraperitoneally with 3×10^4 hybrid cells one week after injection of 0.5 ml Pristane. Mo immunoglobulin was purified from the ascites fluid of these animals by affinity cromatography using a Sepharose-lysine-DNP column. Either ascites fluid

diluted 1:2 with PBS, or the gamma-globulin fraction of the ascites obtained after three precipitations with 33% $(NH_4)_2SO_4$, was directly passed through the column. After exhaustive washing of the column with PBS, the antibody was eluted with 0.1 M DNP. The eluate was directly cromatographed in a Dowex 1-8X column to remove DNP.

The purity of the preparations of Mo was assesed by a) SDS-PAGE in 5/7.5% gels (Laemmli, 1970); b) Double immunodiffusion against rabbit anti-mouse IgA, IgM, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 (Miles Laboratories), and c) analytical isoelectrofocusing as described by O'Farrell (1975).

Fab fragments of Mo were prepared and purified as described for heterogeneous antibodies, but using a ratio of 1:25 of mercury-papain (Sigma) to antibody.

MOPC-315 immunoglobulin

Monoclonal immunoglobulin MOPC-315, purified from ascites fluid by the method of Goetzl and Metzger (1970), was a kind gift of Dr. Israel Pecht, of the Weizmann Institute of Science.

Fluorescence quenching assays

Fluorometric titrations of purified sheep anti-DNP antibodies and it's Fab fragments were carried out in a Perkin-Elmer MPF-44A Fluorescence Spectrophotometer. All measurements were done at 24°C. The hapten solution (ϵ-NH_2- 2,4, dinitrophenyl-L-lysine from Sigma Chemical Co.) was automatically and continously added to a cuvette containing the

antibody solution. Immediate and complete mixing of the reactants was provided by a motor driven plastic agitator that was inmersed in the solution. The fluorescence values were collected in an oscilloscope attached to the spectrofluorometer, that can store more than 1000 values for each titration. The data were transferred from the oscilloscope to a magnetic tape and from there to a computer, which carried out all the calculations.

Fluorescence quenching titrations of MOPC-315, Mo, and it's Fab fragments were done in an Aminco Bowman Spectrophotofluorometer. Measurements were done at 20°C. The hapten was added in aliquots of 4 or 5 µl from DNP-lysine solutions of different concentrations. The solution was stirred with a small magnetic bar. Stable fluorescence values were obtained in less than 30 seconds.

All calculations were done esentially as described by Eisen and McGuigan (1971). For the heterogeneous antibodies, correction for non-specific quenching was done using the titration of a monoclonal mouse immunoglobulin which does not bind DNP. Both Q_{max} and the association constants were calculated by a curve fitting procedure using the saturation vs. free ligand curve and assuming homogeneity in affinities. The molar concentration of protein was calculated by O.D. at 280 nm assuming $E_{280}^{1\%}$ of 14. The solid lines given in the figures are simulated Scatchard plots of a population of bivalent homogeneous antibodies, with no interactions between sites, with affinity constant equal to those given for each curve. Due to the presence of hétérogeneity in affinities,

estimates of cooperativity (that is, the ratio K2/K1) cannot be obtained, as there is no mathematical model which can estimate the site constants if the distribution of affinities is not known.

For the titrations of homogeneous antibodies, correction for non-specific quenching was done using the titration of a solution of DL-tryphotophane (Merck) done under the same conditions as the titrations of antibodies. Q_{max} values were obtained adding a very high concentration of hapten (10^{-4} M). The molar concentration of antibody was calculated spectrophotometrically using a value for $E_{280}^{1\%}$ of 15.

Association constants for the titrations of MOPC-315, Mo and Fab fragments of Mo, were calculated by an iterative fitting procedure using the regression of bound ligand vs. free ligand. The equation used to calculate the best fit was (Rodbard and Bertino, 1973):

$$B = \frac{(K1\ Ao\ H) + (2\ K1\ K2\ Ao\ H^2)}{1 + (K1\ H) + K1\ K2\ H^2)} \qquad (1)$$

were B and H represent the molar concentration of bound and free ligand, Ao the molar concentration of antibody and K1 and K2 represent the stoichiometric association constants for the first and second site respectively (Klotz, 1953; Fletcher et al., 1970, Rodbard and Bertino, 1973). Values for K1, K2 and Ao were sequentially modified until the sum of squares of the differences between B values of experimental points and B values calculated from equation 1 was brought to a minimum. First guesses for the iterative procedure were calculated from the mean association constant estimated from a Sips plot of the experimental data.

A simulated curve using the values obtained by the fitting procedure was drawn back in the Scatchard coordinates along with the experimental points. The simulated curves were drawn according to the equation:

$$H = \frac{B - Ao + \sqrt{((Ao - B)^2 + K2/K1\ B\ (2\ Ao - B)}}{2\ K2\ (2\ Ao - B)} \qquad (2)$$

which is obtained from equation 1, solving for H and taking the positive sign of the root so that H = 0 when B = 0. Values for H were calculated for every B value and the ratio B/H was plotted against B values. It is important to note that K1 and K2 in this model correspond to the stochiometric association constants and not to the intrinsic association constants. Thus, if no interactions occur and the sites are identical with respect to intrinsic affinity for the hapten, then K1 = 4 K2, and $\beta = 4\ K2/K1 = 1$. $\beta > 1$ implies positive cooperativity and $\beta < 1$ implies negative cooperativity.

Equilibrium dyalisis

Equilibrium dyalisis were performed in a lucite multidyalisis apparatus constructed by the Centro de Instrumentos of the Universidad Nacional Autonoma de Mexico. The hapten used was ε-3,5-^{3}H-2,4-DNP-lysine, purchased from New England Nuclear. Purified antibody concentration was 0.07 mg/ml (9×10^{-7} M). Hapten concentrations ranged from 3×10^{-8} to 8×10^{-6} M. After 72 hours at 4°C samples were taken from both sides of each chamber and radioactivity was counted in Bray's scintillation liquid. The values of bound and free hapten obtained were used for the curve fitting procedure described above for calculating K1 and K2.

RESULTS

Binding of DNP-lysine by heterogeneous anti-DNP antibodies ant it's Fab fragments

Figures 1 a, c, and e show Scatchard plots of the binding of DNP-lysine by purified sheep anti-DNP antibodies, showing an ascending limb in the region of low saturation, and the typical descending limb. In plot a, which corresponds to a complete titration, the descending limb shows an upward curvature, characteristic of heterogeneity in affinities (titrations c-f were not carried out to complete saturation in order to have more points in the regions of low saturation). These types of Scatchard curves were consistently obtained when antibodies with different affinities and from different donor animals were used. Plots b, d, and f of Figure 1 show Scatchard plots of the binding of DNP-lysine by Fab fragments prepared from the purified antibodies used for plots a, c, and e. Each pair of titrations (a and b, c and d, e and f) were done under the same experimental conditions, using the same hapten solution, and the data were handled in the same way. One may readily appreciate that the ascending limb is diminished (d) or not present at all (b,f).

Figure 2 shows a Scatchard plot of the titration of the same preparation of antibodies used for figure 1, but after mild reduction and alkylation. Again, the ascending limb has been greatly reduced.

Binding of DNP-lysine by homogeneous antibodies

We assayed by FQ to DNP-binding monoclonal immunoglobulins: MOPC-315 (mouse IgA secreted by a myeloma), and Mo (mouse IgG_3 secreted by an hybridoma produced in our laboratory) as well as Fab fragments of Mo.

The SDS-PAGE patterns of the prèparations assayed are shown in Figure 3. Fab fragments of Mo and of heterogeneous antibodies showed two bands of 45000 and 25000, and no appreciable contamination with undigested molecules. Fab fragments of Mo and of heterogeneous antibodies appeared as a single band of 25000 MW in a reducing SDS gel. The Fab preparation of heterogeneous antibodies appears as a single peak in a column of Sephadex G-75, the elution volume of this peak corresponding to a MW of about 50000 (data not shown).

The results we obtained for MOPC-315 (Fig. 4) agree with previous reports in Q_{max}, affinity for DNP-lysine and the almost linear Scatchard plots. When titrations of MOPC-315 were subjected to out fitting procedure for estimating K1 and K2, values of β (4K2/K1) close to 1 were obtained, that are consistent with abscence of interactions and homogeneity in affinity (Table 1).

The Scatchard plots of Mo (Fig. 5) showed a very obvious ascending limb. Plots 5 a and b are from different preparations of Mo, assayed separately. Each time Mo was titrated, a titration of MOPC-315 was done and both were calculated with the same titration of DL-tryptophan for correction. Ascending limbs in the Scatchard plots of Mo titrations were consistently obtained. This type of curves have been also obtained with equilibrium dyalisis experiments (Figure 7). The estimated values of K1, K2 and β are similar (Table 1).

FQ titrations of Fab fragments of Mo (Fig. 6) showed no ascending limb and, when subjected to the fitting procedure, β values close

to 1 were obtained. Plots 6a and b are of Fab fragments prepared from two different preparations of Mo.

In FQ assays, the molar concentration of antibody binding sites (S_o) should be known in order to calculate, from the fractional quenching, the concentrations of bound and free ligand at each point. In order to estimate how errors in the assumed S_o could affect the shape of the Scatchard plots, we processed the same data of a titration of Mo, asumming different S_o values. As can be seen in Figure 8, the ascending limb is present regardless of the S_o assumed over a tenfold range.

DISCUSSION

It is clear, from many theoretical studies, that ascending limbs in Scatchard plots are indicative of cooperative binding (Koshland and Neet, 1968; Rodbard and Bertino, 1973; Dahlquist, 1975; Celis et al., 1977; Thakur and Rodbard, 1979; José and Larralde, 1983).

Here we report ascending limbs in the Scatchard plots of the binding of the hapten DNP-lysine with both homogeneous and heterogeneous anti-DNP antibodies.

These unusual types of plots have been observed previously in the DNP-system using different experimental techniques (Celis et al., 1977, Pérez Montfort, 1979). These results were taken as non-conclusive because of the use of multivalent antigen in one case, and because equilibrium dyalisis, which was used to study the binding of monovalent

hapten, yields a very large scatter of points. In this study we used monovalent hapten and a different experimental technique (Fluorescence quenching) using both heterogeneous and homogeneous anti-DNP antibodies.

The observed ascending limbs in the Scatchard plots of heterogeneous anti-DNP antibodies, obtained with FQ, corroborate previous studies with the same system studied by equilibrium dyalisis (Pérez Montfort, 1979). Plots of two different monoclonal immunoglobulins, when assayed under the same experimental conditions, and the data calculated in the same way, consistently gave different types of plots. That only one of them shows an ascending limb, is a strong evidence that the ascending limb is not an artifact of the technique employed. Furthermore, very similar ascending limbs were obtained for Mo with two different experimental techniques.

We suggest that the ascending limbs observed here are indicative of cooperativity in the binding, since the hapten used is monovalent, the effect is seen with both homogeneous and heterogeneous antibodies, and, in the case of homogeneous preparations, the experimental curve could be very well reproduced theoretically assuming cooperative binding. Furthermore, the ascending limb is diminished to a considerable exent in the binding of hapten to Fab fragments, when compared with the assays of intact antibodies (Figures 1 and 5-6).

Upon comparing the plots of homogeneous with those of heterogeneous antibodies, it is apparent that the shapes of the plots with ascending limb are different. In the case of Mo, the maximum is not so

near the Y axis as in the case of heterogeneous antibodies, and the points seemed to intercept with the X axis in a precise point. The second difference argues in favor of Mo being truly homogeneous. The fact that in plots of heterogeneous antibodies the maximum is very near the Y axis could be because not all the antibodies present are cooperative. This is not suprising, as we found with homogeneous immunoglobulins that MOPC-315 does not present cooperative binding. In fact, the proportion of antibody molecules capable of cooperative binding seems to be low, as there is no other bivalent monoclonal antibody reported to show this behaviour. (Other explanations for the absence of reports of antibodies binding ligand cooperatively are that regularly the lowest ligand concentrations used are above the ones used here, and/or that the points of very low saturation are discarded because they are considered to arise from experimental variation. Also, it has been demonstrated that the graphical effect of cooperativity could be masked by heterogeneity in affinities (José and Larralde, 1983).

So far, we have no idea about the molecular mechanisms that may be involved in cooperative binding. We have noted that by reducing and alkylating disulfide bridges in bivalent molecules, the ascending limb is diminished. It is interesting that L_2 315 (the light chain dimer of MOPC-315, which has been shown to bind ligands cooperatively) also shows a more pronounced cooperativity when both chains are covalently linked by a disulfide bridge, than when they are held together only by non-covalent interactions (Zidovetzky et al., 1979). Therefore, the disulfide bridges could be important but non-essential for transmiting the signal. It is known that conformational changes are induced in the anti-

body molecule when hapten is bound (Lancet and Pecht, 1976; Vuk-Pavlovic et al., 1979; Käivärainen et al., 1981) so, beside the functional roles adscribed to these changes (complement fixation, binding to cell receptors, etc.), they could also be functioning to transmit a signal to the other antigen binding site so that a conformational change takes place there that increases the affinity for the ligand. These changes need not to be large as a very subtle difference in the fine tridimensional conformation of the active site could significantly affect the affinity for the hapten. Obviously, a thorough study of the reaction of Mo with DNP-lys would be required to establish the molecular events responsible for the phenomenon, and to explain why it is not present in all the antibody molecules.

The biological relevance of cooperativity in antibodies should also be examined, as cooperative systems could be regulated by the concentration of ligand (Gäbler, 1977; Ghosh, 1981). Since the primary event in many immunologic phenomena is the antigen-antibody reactions, the role that cooperativity could be playing in making the system capable of responding to low antigen concentrations, could in fact be very important, and deserves to be investigated. Obviously, the demonstration of the phenomena in other Ag-Ab systems is necessary to establish cooperativity as a general property of antibodies and not a very special case without biological significance.

TABLA I

ESTIMATES OF K1, K2 AND β FOR FQ TITRATIONS OF HOMOGENEOUS ANTIBODIES AND ITS FAB FRAGMENTS

Antibody preparation	$S_o \times 10^7$ (mol/lt)	K1 $\times 10^{-6}$ (lt/mol)	K2 $\times 10^{-6}$ (lt/mol)	β (4K2/K1)	Figure
MOPC-315	7.3	6.35	1.35	0.85	4a
MOPC-315	7.3	9.30	1.94	0.83	4b
Mo	6.5	2.32	13.20	18.00	8d
Mo+	18.0	1.40	6.10	17.42	7
Mo	5.2	1.54	8.68	22.25	5a
Mo	3.0	1.52	9.84	25.89	5b
Mo Fab	7.14	6.20	2.57	1.60	6a
Mo Fab	5.08	7.65	2.76	1.44	6b

+ Equilibrium dyalisis

The values of S_o, K1 and K2 were obtained by the fitting procedure described under Materials and Methods. The S_o was originally calculated from $O.D._{280}$ and during the fitting it was no substantially modified. The values of K1, K2 and S_o were used to simulate a Scatchard plot that was drawn along with the experimental poins.

LEGENDS TO FIGURES

Figure 1. Scatchard plots of F.Q. titrations of purified sheep anti-DNP antibodies (a,c,e) and Fab fragments of them (b,d,f). The solid lines are simulated Scatchard plots of populations of homogeneous and non-interacting bivalent antibodies with affinity constans of: $2.86 \times 10^6\ M^{-1}$ (a), $2.58 \times 10^6\ M^{-1}$ (b), $3.28 \times 10^6\ M^{-1}$ (c), $1.59 \times 10^6\ M^{-1}$ (d), $3.46 \times 10^6\ M^{-1}$ (e), and $1.66 \times 10^6\ M^{-1}$ (f). These values were obtained from a fitting procedure using the saturation vs. free ligand curve.

Figure 2. Scatchard plot of the F.Q. titration of the same antibodies in figure 1 (a,c, and e) but after mild reduction and alkylation.

Figure 3. Non reducing SDS-PAGE of the antibodies and their Fab fragments. Electrophoresis were run at 15 mA, in 7.5 mm thick, 7.5% poli-acrylamide gels. Samples were boiled with 1% SDS for 5 minutes before the run. Lane 1: molecular weight markers (Bio-Rad), lane 2: Fab fragments of Mo; lane 3: Mo; lane 4: MOPC-315; lane 5: purified sheep anti-DNP antibodies; lane 6: Fab fragments of sheep anti-DNP antibodies; lane 7: bovine gamma globulin (Pentax).

Figure 4. Scatchard plots of F.Q. titrations of MOPC-315. The solid lines are simulated Scatchard curves drawn according to the K1, K2 and S_o values obtained from the fitting procedure described under Material and Methods and given in Table 1.

Figure 5. Scatchard plots of F.Q. titrations of Mo, the solid lines are simulated Scatchard curves drawn according to the values gives in Table 1.

Figure 6. Scatchard plots of F.Q. titrations of Fab fragments of Mo. The solid lines are simulated Scatchard curves drawn according to the values given in Table 1.

Figure 7. Scatchard plot of an equilibrium dyalisis experiment with Mo. The solid line is a simulated Scatchard curve draw according to the values given in Table 1.

Figure 8. Effect of the assumed S_o on the shape of a Scatchard plot showing ascending limb. The S_o calculated from $O.D._{280}$ was 6.5×10^{-7} M. The same F.Q. titration of Mo was calculated assuming different S_o values: 7.5×10^{-7} M (a), 9×10^{-7} M (b), 1×10^{-6} M (c), 6.5×10^{-7} M (d), 5×10^{-7} M (e) and 3.5×10^{-7} M (f).

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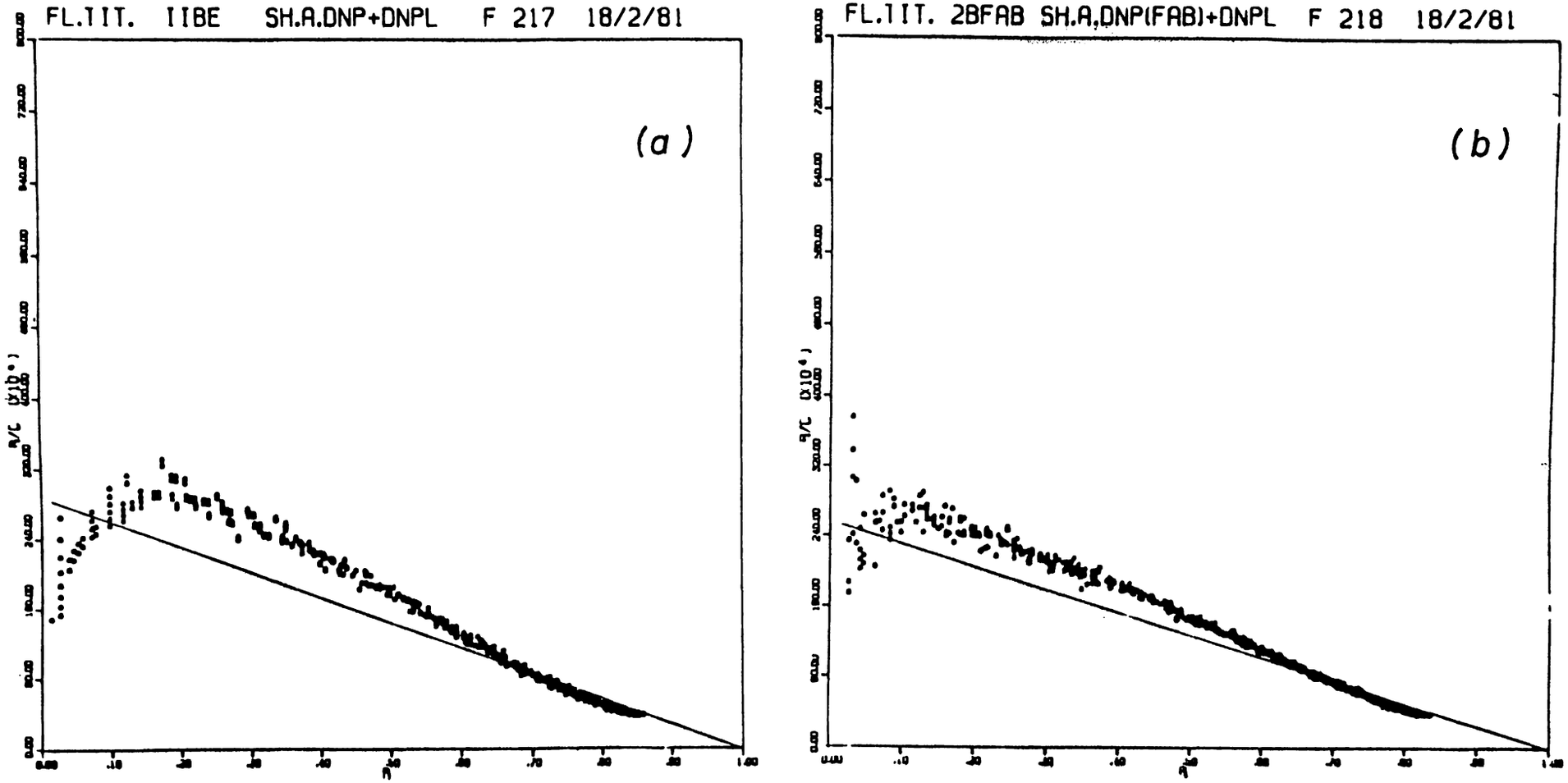
FL.TIT. IIBE SH.A.DNP+DNPL F 217 18/2/81
(a)
FL.TIT. 2BFAB SH.A.DNP(FAB)+DNPL F 218 18/2/81
(b)

FIGURE 1

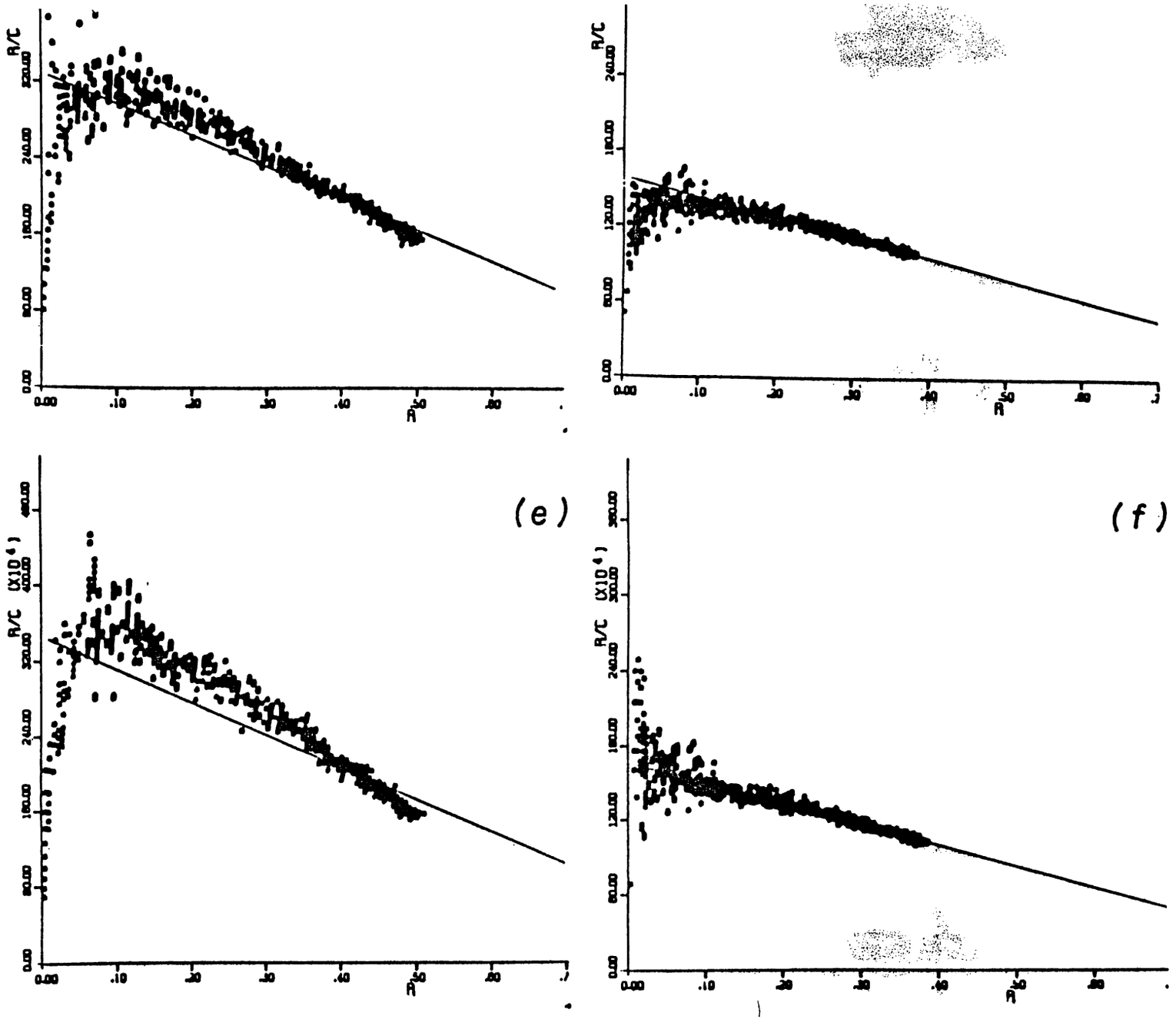

FIGURE 1
(cont.)

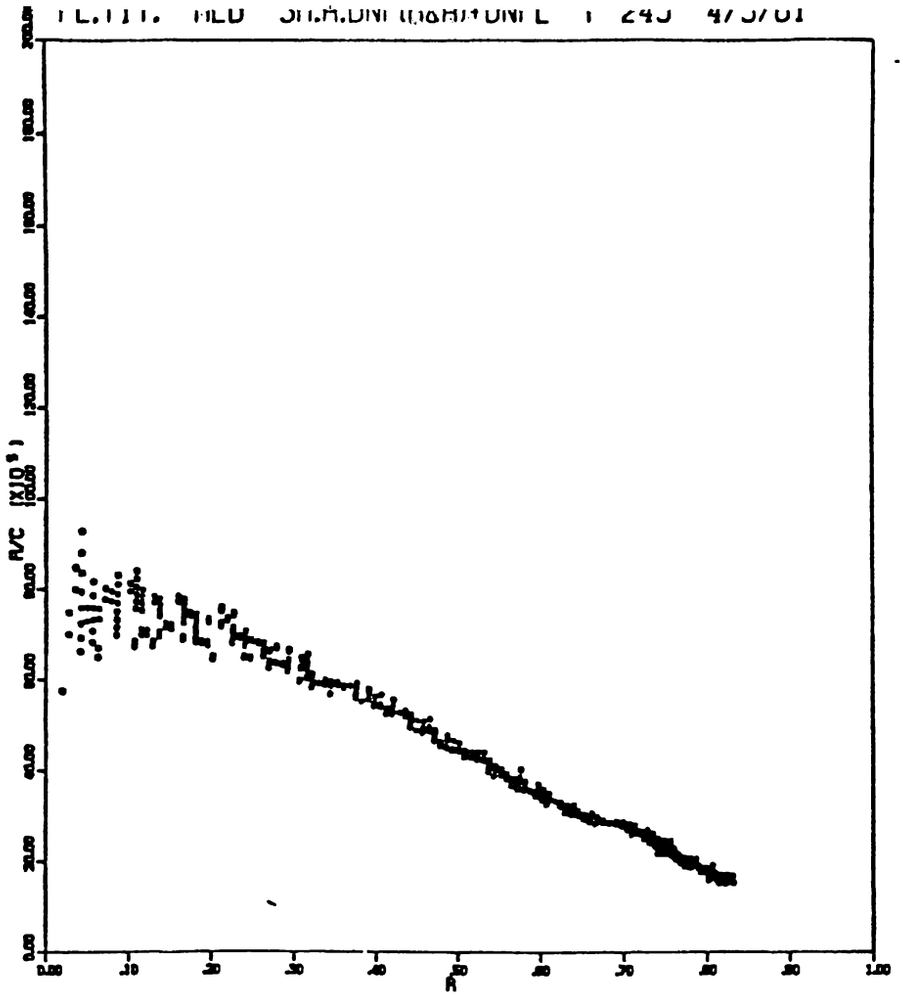

FIGURE 2

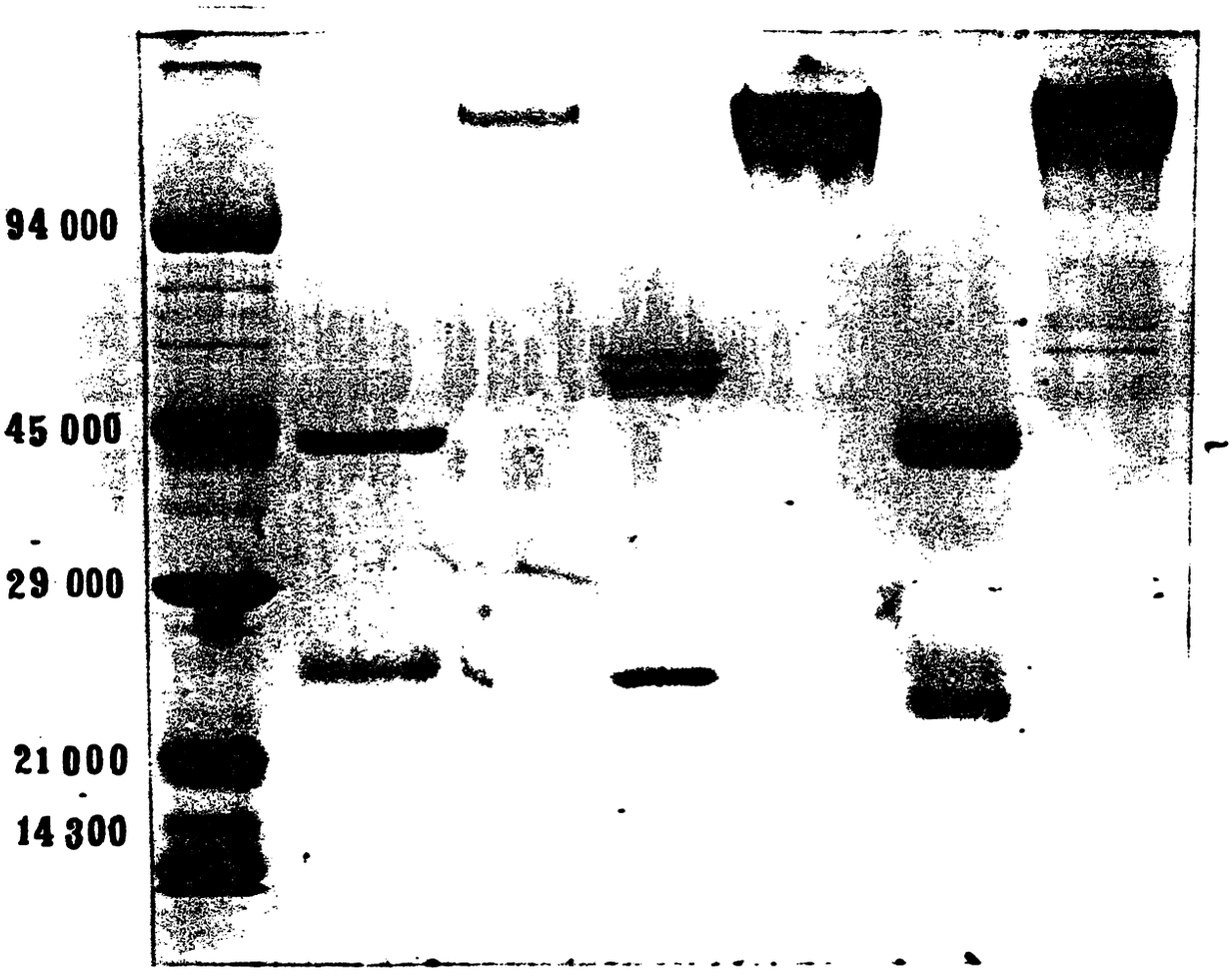

FIGURE 3

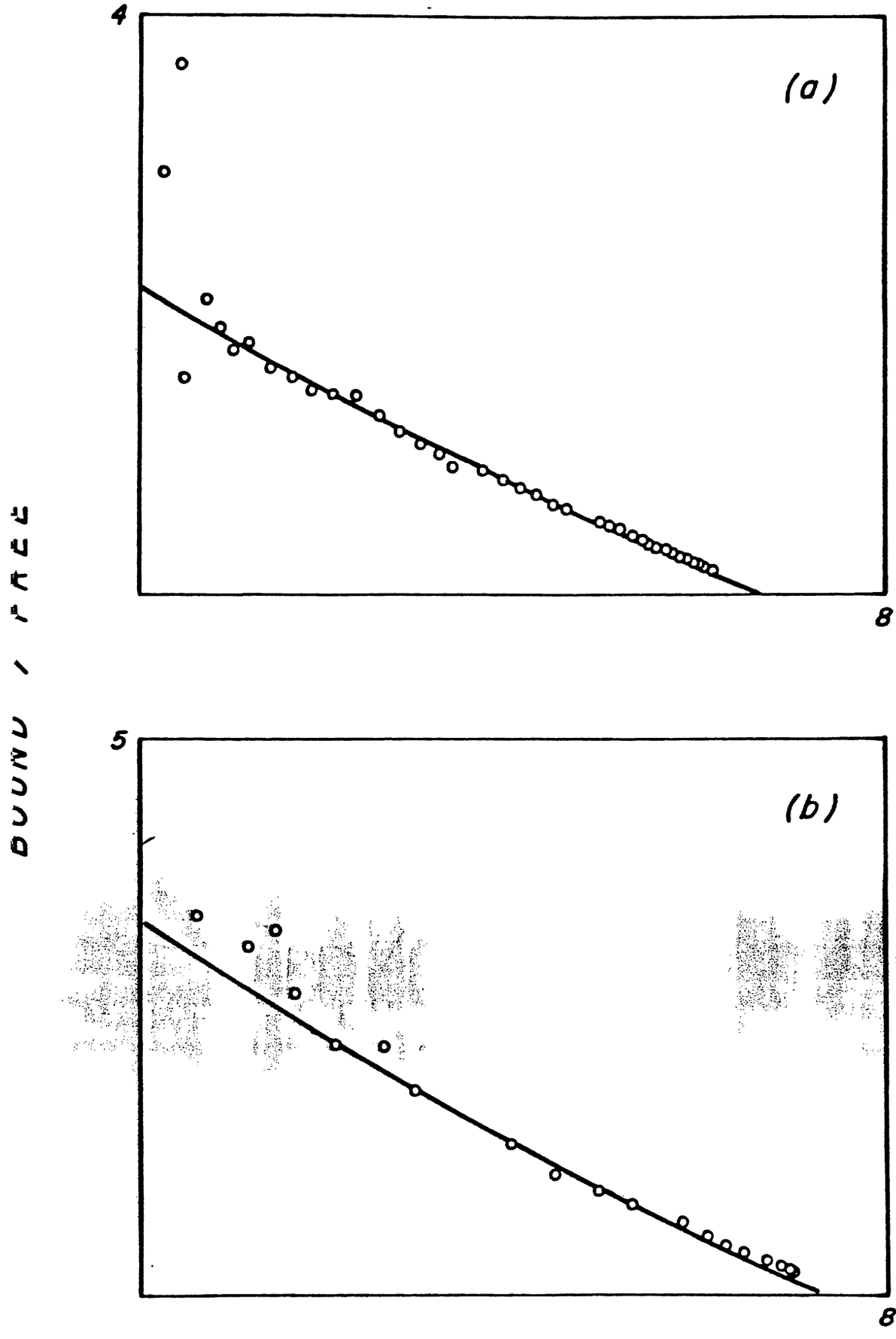

FIGURE 4

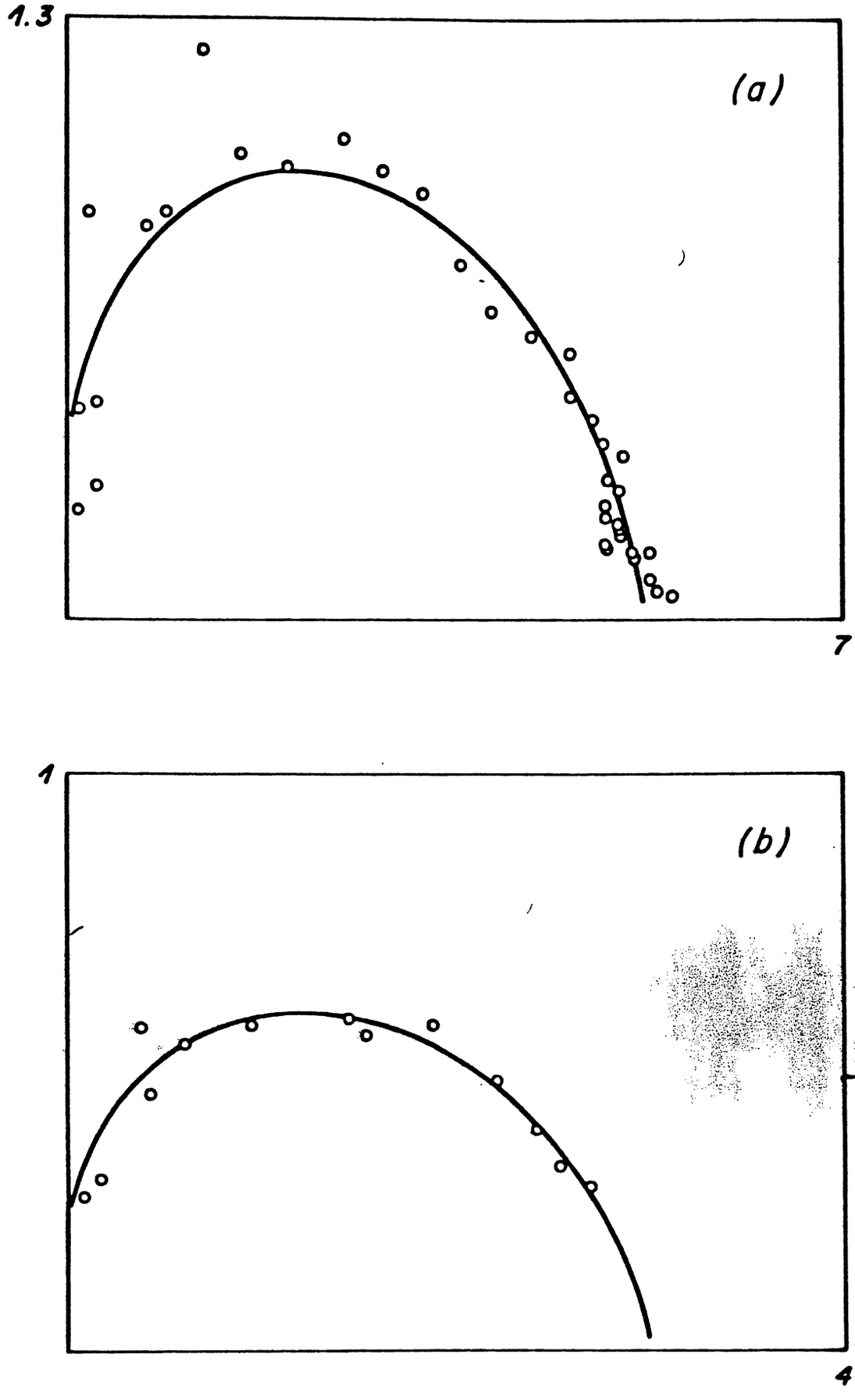

FIGURE 5

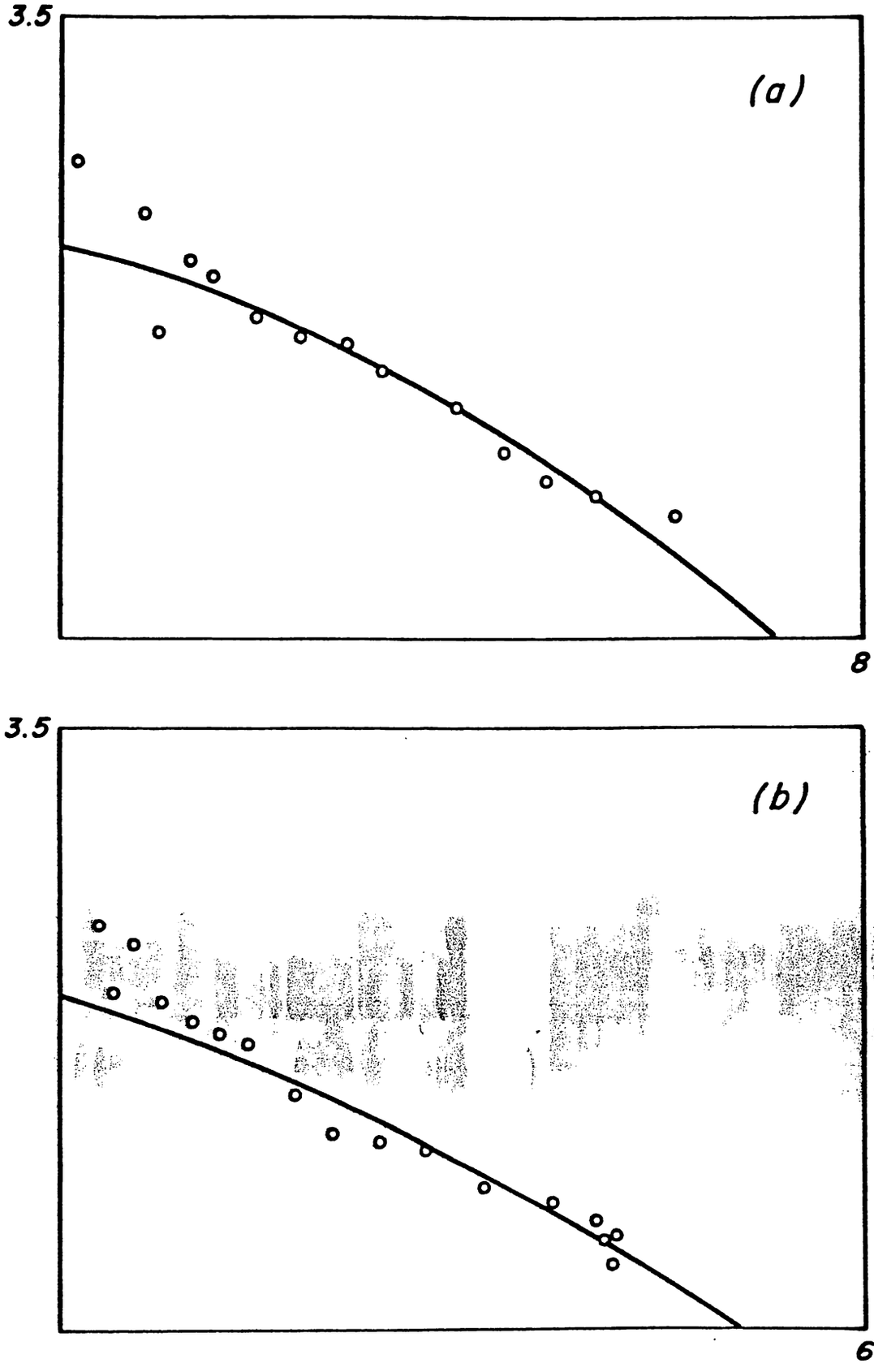

FIGURE 6

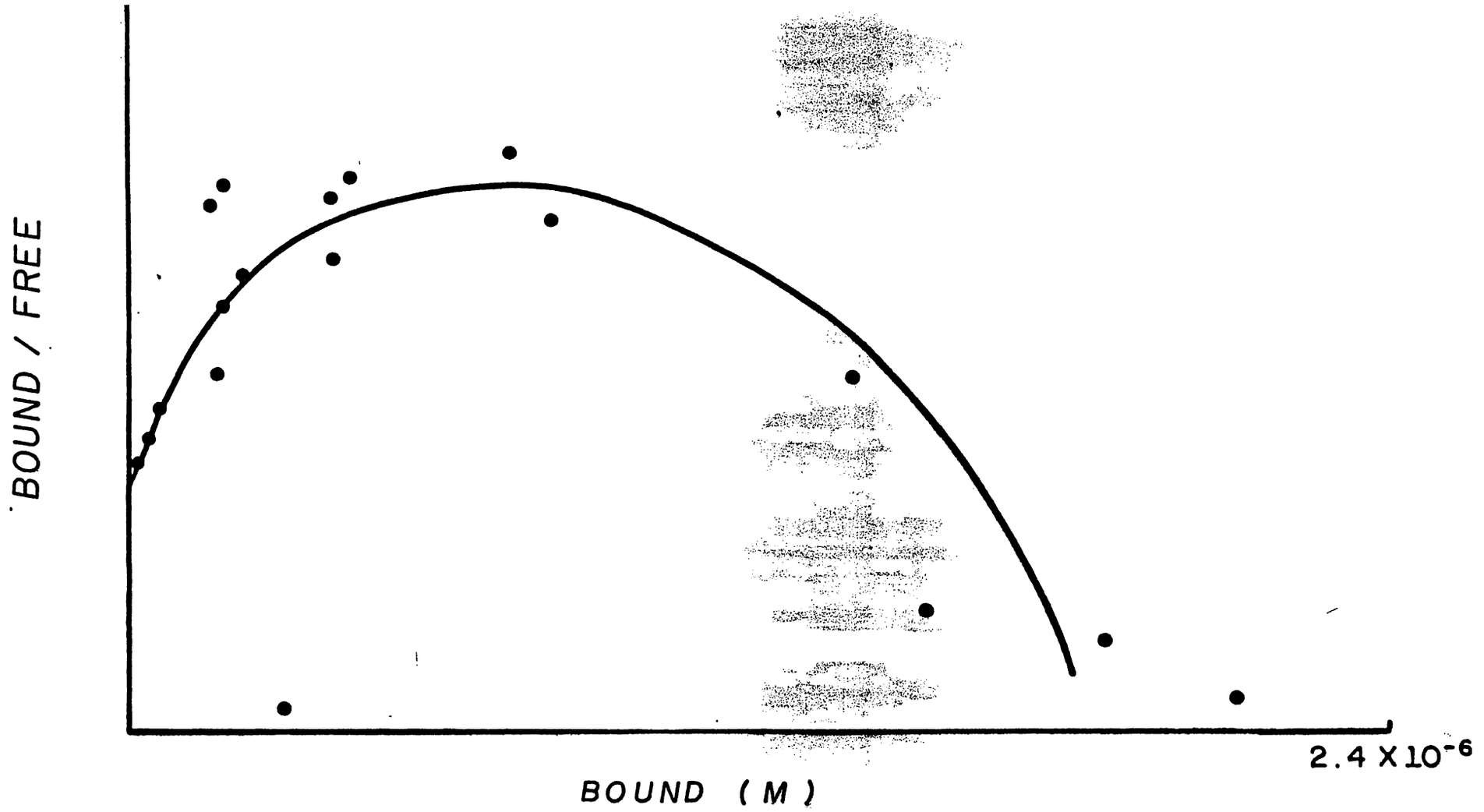

FIGURE 7

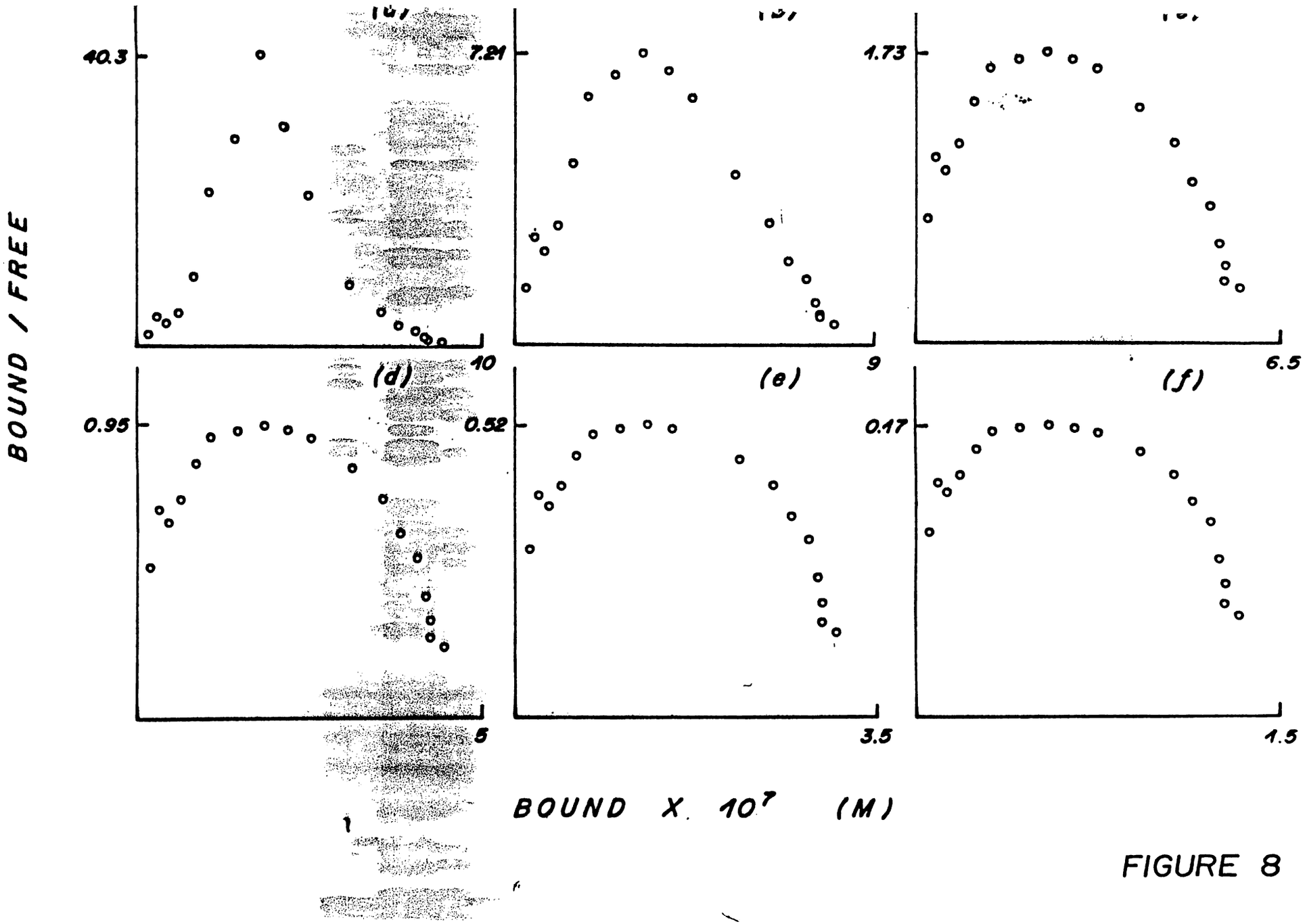
BOUND / FREE
40.3
7.21
10
(d)
0.95
0.52
5
1
9
(e)
3.5
1.73
6.5
(f)
0.17
1.5
BOUND X 10⁷ (M)

FIGURE 8

Discusión

Dada la estructuración de ésta tesis, cada uno de los trabajos que la integran contiene la discusión sobre los puntos más relevantes. Por lo tanto, en esta parte quisiera presentar algunas ideas sobre la interacción antígeno-anticuerpo que, por razones de espacio, no se incluyen en el texto de los trabajos. Estas ideas surgen de los trabajos presentados, y se espera que generen nuevos experimentos y nuevas concepciones.

Los puntos a tratar son:

1. Especificidad y Afinidad
2. Heterogeneidad y Multiespecificidad
3. Cooperatividad

1. Especificidad y Afinidad

De acuerdo a Inman (1978): "Un anticuerpo reacciona con cualquier determinante antigénico EN UN CIERTO GRADO". Este grado - no es otra cosa que la constante de afinidad. Según ésto, un anticuerpo cualquiera reaccionará con afinidades altas (10^8-$10^9 M^{-1}$)con un grupo -- muy restringido de determinantes antigénicos. A medida que el límite de afinidad se desplaze a afinidades menores el número total de determinantes antigénicos con los que el anticuerpo puede reaccionar es mayor. Si el límite se coloca en afinidades muy bajas (cercanas a 0), un anticuerpo reaccionará con la mayoría de los determinantes antigénicos (Figura 1).

De acuerdo a ésto, cualquier definición de especificidad deberá tener en consideración la afinidad de las interacciones. - Karush (1978) introduce el término selectividad para describir la capacidad de un anticúerpo para discriminar entre dos antígenos debido a -- que las afinidades son tales que solamente la afinidad por uno de ellos está arriba del umbral de detección del sistema experimental dado.

Por lo tanto la definición de un anticuerpo como específico para tal o cual antígeno es arbitraria, y depende del límite - de sensibilidad de nuestra técnica de medición*.

Pero más importante que nuestra dificultad para de

* No hay que perder de vista, sin embargo, que para cuestiones prácticas los anticuerpos son una herramienta valiosa para detectar o cuantificar antígenos, ya que el porcentaje de reacciones con otros determinantes que ocurran con afinidades detectables será despreciable comparado con la reacción con el antígeno común.

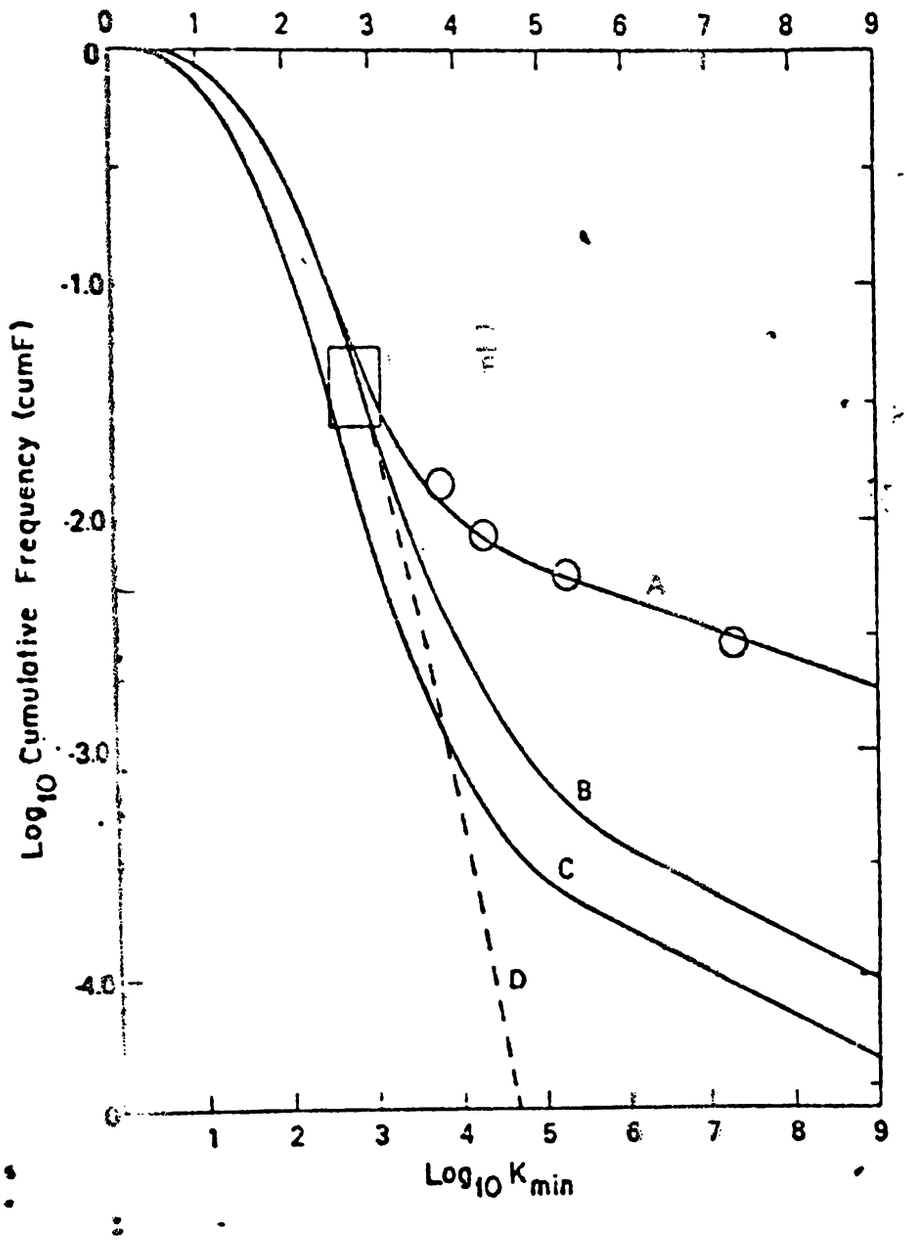

Figura 1. Frecuencias acumuladas esperadas de la ocurrencia de reacciones con constantes de afinidad mayores o iguales a Kmin, de un experimento en que se probasen un conjunto de inmunoglobulinas diferentes con un conjunto de haptenos diferentes. (Cada ensayo tomando un anticuerpo y un hapteno). La curva B representa los resultados esperados si la selección de anticuerpos y haptenos -- fuese enteramente al azar. (La curva A corresponde a la curva - dada en la figura 1 del artículo I).

finir la reactividad de un anticuerpo, es la cuestión de la operatividad biológica, En otras palabras: ¿Hay algún límite de afinidad para que una reacción antígeno-anticuerpo desencadene las funciones efectoras? ¿O es más bien necesaria la interacción con ciertas subregiones del sitio activo independientemente de la afinidad total de la reacción?

Hay indicios de que ligandos diferentes, al reaccionar con el sitio activo de una inmunoglobulina, pueden activar las funciones efectoras (Varga et al, 1973; Cameron y Erlanger, 1977). En el trabajo de Cameron y Erlanger se vió que la naturaleza química de la fijación de dos haptenos (menadiona y monofosfato de adenosina) a una inmunoglobulina era diferente, ya que la modificación de la afinidad al variar la temperatura fué diferente. La fijación de ambos ligandos fué, pues, cualitativa y cuantitativamente distinta, pero ambas pudieron activar el sistema del complemento.

Si aceptamos la hipótesis de que las funciones efectoras son activadas por cambios conformacionales producidos en el anticuerpo al fijarse el antígeno (Huber et al 1976), es obvio que los diferentes ligandos, aunque interaccionando en forma diferente, deben producir en la porción Fc el mismo efecto, cualquiera que éste sea. Esta conclusión debe ser considerada en cualquier hipótesis acerca de los mecanismos de activación de las funciones efectoras.

2. Heterogeneidad y Multiespecificidad

Es bien conocido que un animal, cuando se le inmuniza con un hapteno simple, responde produciendo una familia de anticuerpos que difieren en su afinidad por el hapteno. Una explicación razonable acerca del significado biológico y la importancia de esta heterogeneidad en la respuesta, puede brindarla la teoría de multiespecificidad de los anticuerpos.

De acuerdo a ésta teoría, la heterogeneidad de la respuesta no solo es explicable, sino necesaria. La heterogeneidad confiere a un suero inmune una mayor selectividad, puesto que disminuye la intensidad de las reacciones con antígenos diferentes (Ver figura 1 de la introducción).

Por otro lado, se han hecho experimentos encaminados a dilucidar el papel que juega la heterogeneidad en el funcionamiento biológico de los anticuerpos, usando mezclas de anticuerpos monoclonales, como modelos de sueros heterogéneos.

Recientemente se ha reportado que puede existir cooperatividad intermolecular entre moléculas de anticuerpos. La mezcla de dos anticuerpos monoclonales anti-gonadotropina coriónica humana puede presentar una mayor afinidad por el antígeno que cualquiera de los dos por separado (Ehrlich y Moyle, 1983). Se ha visto también que algunas mezclas de anticuerpos monoclonales pueden actuar sinérgica

mente para lisar eritrocitos (Howard et al, 1979). Haber y colaboradores (1974) desarrollaron un ensayo radioinmunométrico usando dos anticuerpos monoclonales, y vieron que el ensayo presentaba una mayor selectividad que si se usase cualquiera de los dos anticuerpos por separado.

Estos resultados indican que la heterogeneidad podría ser muy importante para el fenómeno de la respuesta inmune. En vista de éstos resultados es claro que la reactividad de un antisuero heterogéneo difícilmente será simplemente la suma de las reactividades individuales de los anticuerpos que lo componen. Es posible que la heterogeneidad de un suero esté de tal manera coordinada que produzca una mayor eficiencia, no nadamás en cuanto a especificidad (como propone la hipótesis de multiespecificidad) sino a una mejor potenciación de los efectos biológicos.

Una mejor comprensión sobre las ventajas que la heterogeneidad reporta al sistema podrá obtenerse a partir de estudios experimentales del tipo de los arriba citados. Sobre las ventajas que la multiespecificidad de los anticuerpos aporta al sistema, podemos enumerar las siguientes:

a) El número de genes V_H y V_L necesarios para que un individuo asegure su capacidad de responder a cualquier antígeno es mucho menor que si los anticuerpos fuesen monoespecíficos.

b) La multiespecificiad confiere al individuo (o - la especie) la capacidad de responder adecuadamente a antígenos que -- nunca hayan aparecido en la historia de la especie. Asímismo, se pre-- serva la capacidad de responder a antígenos que hayan desaparecido del medio ambiente y eventualmente pudieran reaparecer.

La multiespecificidad explica también la presencia de anticuerpos "naturales" así como la alta frecuencia con que se ha- encontrado fijación de antígenos por proteínas de mielomas. (Si, como se cree, la transformación neoplásica ocurre al azar, solo la multiespecificidad puede explicar porqué un alto porcentaje de proteínas de mielomas diferentes, fijan haptenos como dinitrofenol, fosforilcolina, carbohidratos, etc.) (Potter, 1977).

3. Cooperatividad

Por último, quisiera referirme a una posible ex-- plicación del hecho de que no todos los anticuerpos presenten cooperatividad. Como se indica en el artículo II, probablemente esta capacidad no sea una característica general de las inmunoglobulinas. Se sabe que las distintas clases y subclases de inmunoglobulinas llevan a- cabo funciones diferentes (véase Turner, 1977) de tal modo que para - algunas de ellas la capacidad de fijar al antígeno cooperativamente - podría ser muy importante.

A la luz de la multiespecificidad, puede darse una hipótesis alternativa: el que el cambio conformacional en el segundo - sitio provocado por la fijación del antígeno al primer sitio, afecte - la fijación de algunos ligandos y no de otros, porque interactúen con- diferentes subregiones del sitio. Así, un anticuerpo podría fijar cooperativamente a un hapteno y no a otros, aunque tenga afinidad por los últimos

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