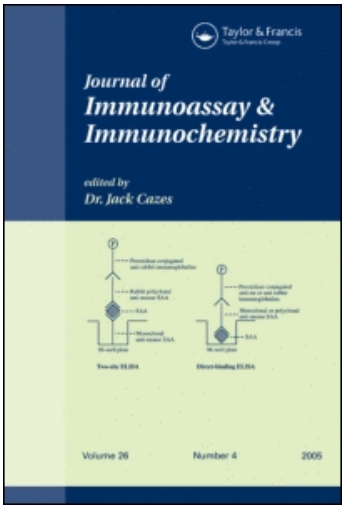

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INTERACTION BETWEEN ANTIBODIES AND HAPTEN-PROTEIN CONJUGATES OF DIFFERENT COMPOSITION: THEORETICAL PREDICTIONS AND EXPERIMENTAL DATA

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ABSTRACT

Mathematical models of competitive ELISAs with labelled antibody and with labelled antigen taking into account bivalent interactions between antibodies and hapten-protein conjugates were developed and analyzed. It was shown that in the kinetic model of the immunochemical reaction the conjugate composition influenced the amplitude of detected signal but not ELISA sensitivity. In the equilibrium model decreased sensitivity correlated with bivalent complexes formation.

The predictions were tested experimentally using 2,4-dichlorophenoxyacetic acid (2,4-D) and testosterone as haptens. It was confirmed that increasing of the hapten : protein ratio resulted in formation of bivalent complexes with antibodies. The equilibrium binding constants for these complexes were two orders of magnitude higher than for monovalent ones. Optimal conjugate compositions have been chosen for ELISA of these haptens.

(KEY WORDS: immune complexes, affinity of antibodies, hapten-protein conjugates, ELISA, testosterone, 2,4-dichlorophenoxyacetic acid)

INTRODUCTION

Development of quantitative descriptions for interactions between hapten-carrier conjugates and antibodies is extremely important for many fields of immunochemistry. Synthetic conjugates are suitable models of antigens with repeating determinants, which are widely presented in biological objects (1,2), and these conjugates are increasingly used as immunogens and reagents for immunoassays (3).

Composition and surface density of antigenic determinants (coupled haptens) are the main characteristics of the conjugates. Change in composition influences antigen-antibody reaction parameters. This is primarily caused by an increased likelihood of bivalent interactions for conjugates with high hapten density. At the present time this problem is described only phenomenologically. Selection of conjugates optimal for analytical systems is based on experimental testing rather than detailed theoretical predictions (4-6).

The purpose of our work was to elucidate and compare the theoretical and experimental dependences of the interaction between antibodies and synthetic hapten-protein conjugates of different composition. 2,4-Dichlorophenoxyacetic acid (2,4-D) and testosterone were used as haptens. Antibodies against these haptens were obtained earlier and characterized in our previous studies (7,8).

MATERIALS AND METHODS

1. Chemicals and Supplies

The following chemicals were used in this work: 2,4-dichlorophenoxyacetic acid (*Serva*), testosterone and testosterone-(3-O-carboxymethyl)oxime (both obtained according to (8)), bovine serum albumin (BSA, *Sigma*), soybean trypsin inhibitor (STI, *Reanal*), horseradish peroxidase with $RZ=A_{403}/A_{280}=3.0$ (HRP, *Biolar*, Latvia), haemocyanine from crab *Paralithodes camtschatica* (HC, *Alexander*, Russia), protein A from *Staphylococcus aureus* (*Vostok*, Russia), L-lysine (*Reanal*), poly-L-lysine hydrochloride (PL, M_w 4-15 kDa and 30-70 kDa, *Sigma*), N-methylmorpholine (*Serva*), isobutylchloroformiate (*Serva*), N-hydroxysuccinimide (*Sigma*), dimethylformamide (DMFA, *Serva*), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (*CalBiochem*), polyethylene glycol (M_w 6 kDa, *Serva*), sodium dodecylsulfate (*Sigma*), L-cysteine (*Reanal*), 2,4,6-trinitrobenzenesulfonic acid (*Chemapol*), sodium ethylenediaminetetraacetate (EDTA, *Serva*), Tween-20 (*Serva*), o-phenylenediamine (*Fluka*).

Polysterene plates (*Dynatech*) were used as solid phase for measurements of constants of antigen-antibody reactions and for ELISA of the haptens.

2. Synthesis and Purification of Hapten-Protein Conjugates

The testosterone-BSA (immunogen), testosterone-STI and testosterone-HRP conjugates were synthesized

according to the following procedure (7). Testosterone-(3-O-carboxymethyl)-oxime was diluted of 20 mg in 0.5 mL of DMFA, cooling down to -20°C followed by addition of 5 μL N-methylmorpholine and 10 μL isobutylchloroformiate to activate the hapten. The reaction mixture was incubated for 30 min with stirring and then added to a 5 % protein solution in water. In the case of immunogen synthesis, the hapten:protein molar ratio was 200:1, in the case of STI the ratio was 10:1, while for conjugates with HRP it was ranged from 2:1 to 200:1. Further DMFA was added dropwise to the resultant mixture (in 1:2 volume ratio) at 4°C, pH was corrected to 8-8.5 by 0.1 M $NaHCO_3$ and the mixture was incubated overnight at 4°C.

The conjugates of 2,4-D with BSA (immunogen), HRP, STI, HC and PL were synthesized as described previously (7,9). At first hapten was activated: to 6.5 mg 2,4-D in 1 mL of DMFA 30 mg 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate and 8 mg N-hydroxysuccinimide were added and incubated for 2 h at room temperature with stirring. The prepared 2,4-D derivative was then added to 1 % protein solution in water. In the case of immunogen synthesis the hapten:protein molar ratio was 200:1, while for other conjugates it was ranged from 2:1 to 200:1.

The conjugates synthesized were separated from low molecular weight compounds by dialysis and/or by

gel-filtration on Sephadex G-25 (*Pharmacia*) in 0.05 M K-phosphate buffer with 0.1 M NaCl, pH 7.4 (PBS).

3. Determination of Hapten-Protein Conjugate Composition

The compositions of the conjugates were determined by comparing the number of surface amino groups in the original protein with the protein conjugated with hapten. Amino groups were detected using a modified 2,4,6-trinitrobenzenesulfonic acid method (10,11). Solutions of protein and hapten-protein conjugates in water (1 mg/mL) were prepared. 50 μL aliquots of these solutions and water (blank sample) were dispensed in plate. 50 μL of saturated $NaHCO_3$ solution and 50 μL of 2,4,6-trinitrobenzenesulfonic acid solution (2 mg/mL) in water were then added. The mixture obtained was incubated for 2 h at 37°C. Then 25 μL of 10 % sodium dodecylsulfate water solution and 25 μL of 0.5 M HCl were added. Optical density was measured at 405 nm using a vertical photometer (MR-580, *Dynatech*).

The titration results were compared with calculations based on changes in ultraviolet spectra of the proteins after incorporation of the hapten groups. These two approaches give similar values for the composition, the titration experiments having higher accuracy.

4. Immunization, Antibody Separation and Testing

Chinchilla rabbits weighing 3-4 kg were immunized with testosterone-BSA and 2,4-D-BSA according to the following procedure. Immunogen dissolved in PBS (1 mg/mL) was emulsified with an equal volume of Freund's complete adjuvant (*Difco*). On days 1, 15 and 29, 1 mL of prepared mixture was injected intracutaneously at multiple sites on the back from scapula to sacrum. On the 89th day the first cycle of reimmunization was carried out: the rabbits were boosted intravenously with 0.3 mL of immunogen dissolved in PBS (1 mg/mL), and were bled 7 days later. This boosting/bleeding procedure was repeated 2-4 times on a monthly basis.

Antisera were collected by allowing the blood samples to stand for 12 h at 4°C. Upper layers were collected, divided into aliquots and stored at -20°C.

The antisera were tested by an indirect ELISA technique (3), using immobilization of STI-hapten conjugates.

The IgG were twofold precipitated from antisera by 20 % polyethylene glycol in 1:1 volume ratio (12).

Monovalent IgG derivatives were produced by the following technique based on reduction of S-S bonds (13). Solutions of cysteine (150 μL, 2 mg/mL), EDTA (75 μL, 2 mg/mL) and $Na_2S_2O_4$ (100 μL, 17.5 mg/mL) in water were added to the IgG solution (1.0 mL, 3 mg/mL) in 0.1 M K-phosphate buffer, pH 7.0. The

mixture was incubated for 2 h at 37°C. Low molecular weight compounds were removed by dialysis. Full conversion of IgG molecules into the monovalent fragments under these conditions had been shown previoulsy by gel-filtration on Sephadex G-100 (*Pharmacia*).

IgG and protein A were conjugated with horseradish peroxidase using a technique based on periodate oxidation of the enzyme (14). Conjugates were separated from low molecular weight compounds and unreacted proteins by gel-filtration on Sephadex G-200 (*Pharmacia*).

5. Determination of the Binding Constants for Reaction between Hapten-Protein Conjugates and Immobilized Antibodies

100 μL aliquots of IgG in PBS (0.1-5 μg/mL) were dispensed in plate wells and incubated for 1.5 h at 37°C. The wells were washed 4 times with PBST (PBS with 0.05% Tween-20). 2,4-D-HRP (or testosterone-HRP) conjugate was titrated from 50 μg/mL to 25 ng/mL (with step 2), its 100 μL aliquots were added to the wells and incubated for 2 h at 37°C. Prior to the washing step, 20 μL aliquots were transferred into another plate to determine final conjugate concentrations in liquid phase. The plate with immobilized IgG was washed, peroxidase activity was determined in both plates and the concentrations of bound and unbound molecules calculated.

In some experiments, antibodies were immobilized via reaction with protein A. The protein A solution (5 μg/mL in PBS) was incubated for 1.5 h at 37°C in plate wells. After washing, IgG (or antiserum) diluted in PBST was added and incubated for 45 min at 37°C, and then processed as described above.

6. Determination of the Binding Constants for Reaction between Antibodies and Immobilized Hapten-Protein Conjugates

100 μL aliquots of conjugates in PBS (0.1-5 μg/mL) were dispensed in plate wells and incubated for 1.5 h at 37°C. The wells were washed 4 times with PBST. Antiserum was titrated from 1:100 to 1:200 000 dilutions (with step 2), added to the wells and incubated for 2 h at 37°C. After washing, 100 μL aliquots of protein A-HRP conjugate were dispensed in the wells (at HRP concentration 0.8 μg/mL, in PBST), incubated for 30 min at 37°C. The wells were washed and peroxidase activity was determined.

7. ELISA of 2,4-D

Competitive ELISA of 2,4-D was carried out by the following two techniques (9).

7.1. ELISA with Immobilized Antibodies

Antibodies were immobilized via reaction with protein A as described above. After washing, 50 μL of

2,4-D sample (the concentrations varied from 50 pg/mL to 1 μg/mL) and 50 μL of 2,4-D-HRP conjugate (at HRP concentration 0.25 μg/mL, in PBST) were added. The plate was incubated for 45 min (or 2 h) at 37°C, washed and the peroxidase activity was determined.

7.2. ELISA with Immobilized Hapten-Protein Conjugates

2,4-D-STI or 2,4-D-HC conjugate (5 μg/mL or 20 μg/mL respectively, in PBS) was immobilized for 16 h at 4°C. After washing, 2,4-D sample (at the same range of concentrations) and the specific antiserum (1:5 000 in PBST) were added to the wells and incubated for 1 h at 37°C. The wells were washed and protein A-HRP conjugate (at HRP concentration 0.8 μg/mL, in PBST) was added and incubated for 45 min at 37°C. After washing, peroxidase activity was determined.

8. ELISA of Testosterone

ELISA of testosterone was carried out using technique 7.1 described above (antiserum dilution 1:15 000), the testosterone concentration in the samples varied from 10 pg/mL to 1 μg/mL, and the testosterone-HRP concentration was 0.2 μg/mL.

9. Detection of Peroxidase Activity

Solution of *o*-phenylenediamine (0.4 mg/mL) in 30 mM Na-acetate buffer, pH 4.5, containing 1.8 mM of H_2O_2, was used as a peroxidase substrate (12). Plates

were incubated for 15 min in the dark at room temperature and the reaction was stopped by adding 2 M H_2SO_4. Optical density was measured at 490 nm using a vertical photometer MR-580.

10. Mathematical Programs Used

Distributions of immunochemical interactions according to equilibrium binding constants (these distributions will be named as "spectra") were determined using the program described previously (15).

RESULTS AND DISCUSSION

DEVELOPMENT AND ANALYSIS OF THE MATHEMATICAL MODEL

We have developed models of antibody-antigen interactions describing systems with immobilized reagents. Such systems underlie ELISA techniques. In contrast to reactions in solution the reactions on the solid phase exclude aggregation processes, hence determination of the binding constants becomes more accurate.

In our models either antibodies or hapten-protein conjugates are immobilized on the solid phase. In both cases, the models deal with reversible reaction of bivalent antibody molecule (Ab) with:

- conjugate (Con) - protein molecule coupled with one or more hapten molecules (in the latter case the conjugate is polyvalent), and
- free monovalent hapten (Hap).

1. Reaction with Immobilized Antibodies

1.1. Choice of Model

The immobilization technique generally used in ELISA is adsorption on polysterene. In this case, surface density of the immobilized molecules on the solid phase is relatively low - usually less than 10^3 on μm^2 (16-18). Thus we ignore binding of several antibodies with the same conjugate molecule as this process has a low probability. The following complexes can be formed:

Ab—Hap, Hap—Ab—Hap, Hap—Ab—Con, Con—Ab—Con,
Ab—Con (single bonded complex) and
Ab═Con (double bonded complex, in which two active sites of the antibody are bound with two different hapten molecules, connected to the same protein molecule).

Their possible sequential transformations are shown in Fig. 1. The reactions include association and dissociation of chemical bonds belonging to three kinds:

1) antibody - free hapten:

$$Ab + Hap \leftrightarrow Ab—Hap \quad [1];$$

$$Ab—Hap + Hap \leftrightarrow Hap—Ab—Hap \quad [2];$$

$$Ab—Con + Hap \leftrightarrow Hap—Ab—Con \quad [3];$$

2) antibody - conjugated hapten (conjugate), the first bond:

$$Ab + Con \leftrightarrow Ab—Con \quad [4];$$

$$Ab—Con + Con \leftrightarrow Con—Ab—Con \quad [5];$$

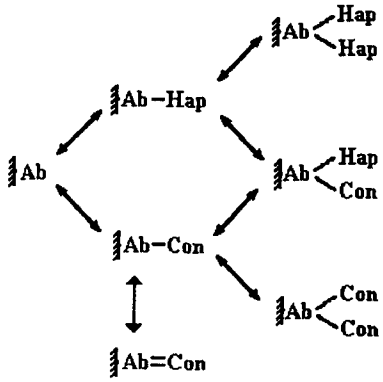

FIGURE 1

The scheme of sequential transformations of immune complexes for the model describing the interaction between immobilized antibodies and hapten-protein conjugate.

Ab - immobilized antibody, Hap - hapten, Con - hapten-protein conjugate.

$$Ab—Hap + Con \leftrightarrow Hap—Ab—Con \quad [6];$$

3) antibody - conjugated hapten (conjugate), the second bond:

$$Ab—Con \leftrightarrow Ab{=}{=}Con \quad [7].$$

In the simplest case of monoclonal antibodies these three kinds of reactions ([1]-[3], [4]-[6] and [7]) are described each by only one pair of association and dissociation constants.

Consider which stages of the immunochemical process depend on the conjugate composition. The number of haptens (N) coupled with protein influences the

association constants (k_+) for reactions [4]-[7]. However for reactions [4]-[6] N changes only effective concentration of reactive groups, and for the reaction [7] the association rate is determined by the hapten density on the carrier surface. It should be noted that the Ab=Con dissociation constant does not depend on conjugate composition, because this reaction is first order.

The analysis of the relationship between N and k_+ for the reaction [7] is based on the assumption that random hapten distribution occurs on the conjugate surface. Prior to reaction [7], an antibody has already been bound to the conjugate by one of its binding sites. Then, k_+ is proportional to the number of hapten groups accessible to the second binding site of the antibody. The likelihood of the case when i hapten groups are accessible is:

$$P_i = (N-1)!/(i!\cdot(N-1-i)!)\cdot(S_{ac}/S_t)^i\cdot(1-S_{ac}/S_t)^{N-1-i},$$

where S_{ac} is the square of the conjugate surface accessible to the second binding site of antibody, and S_t is the total square of the conjugate surface.

Summing elementary association constants for all cases (when i varies from 0 to N-1), we obtain:

$$k_+ = A(1-S_{ac}/S_t)^{N-1}\sum_{i=0}^{N-1} i\cdot(N-1)!/i!/(N-1-i)!\cdot(S_t/S_{ac}-1)^{-i},$$

where A is a coefficient of proportionality.

The equation above consists of coefficients of derivative by x for a binomial distribution $(1+x)^{N-1}$.

So there is a common formula for the result of this summing up. After comparison of the derivative's spreading and unspreading forms, the equation can be transformed to the following formula:

$$k_+ = A \cdot (N-1) \cdot S_{ac}/S_t.$$

The approach given above permits to analyze different conjugate structures. Although the result proportionality between k_+ and (N-1) may be obtained from common considerations (without detailed mathematical study), the proposed approach is suitable for different assumptions about spatial hindrance of the binding on the conjugate surface. We have found that the results presented below hold true both for random hapten distribution on the conjugate surface and for other distributions (e.g. uniform hapten distribution).

Calculations cited are based on the assumption that the conjugate has a strictly defined composition. In reality one is forced to deal with a mixture of conjugates having diverse compositions. Therefore it is necessary to average given formulas for conjugates with different N. The simplest case is random binding of the hapten molecules to the carrier sites during conjugate synthesis. Then relative share of the conjugate containing i hapten groups is $e^{-BN} \cdot (BN)^i/i!$, where B is a constant coefficient.

Properties of conjugates depend not only on hapten surface density but also on the nature of the protein. It influences k_+ value for the reaction [7],

but the relationships between k_+ and the conjugate molecular weight (M) are distinguished for different kinds of protein structure.

Variation of M changes S_t while S_{ac} is kept constant. If M is high enough, k_+ becomes directly proportional to:

$M^{-2/3}$ - when the conjugate is globular, and sites accessible to the second bond with antibody are situated at a defined distance from the first binding point (this distance accords to the most energetically favorable angle between F_{ab}-regions of antibody);

$M^{-1/3}$ - when the conjugate is globular, and the sites accessible to the second bond with antibody are situated at a defined range of distances from the first binding point;

M^{-1} - when conjugate is linear (fibrillar).

The influence of hapten density is due to changes in the rate of Ab=Con formation. The relationship between N and k_+ for this reaction does not depend on the kind of hapten distribution, but depends on the conjugate molecular weight.

1.2. Initial Conditions for the Analysis

Analyzing the models, we set different values for kinetic constants and calculated how the concentrations of reacting molecules and their complexes changed with time.

The results obtained were presented as competitive ELISA curves, i.e. the dependences between $(Hap)_o$ - the initial concentration of free hapten, and C - the final concentration of immune complexes detected on the solid phase:

$$C = (Hap{-}Ab{-}Con) + (Ab{-}Con) + (Ab{=}Con) + 2(Con{-}Ab{-}Con).$$

The competitive curves were compared for conjugates with different compositions at different times in the reaction. Hapten detection by ELISA is based on comparison of C values for hapten presence and absence. Therefore the competitive curves should have equal C values at $(Hap)_o=0$.

Because the purpose of our modeling is primarily to compare different conjugates and assay conditions rather than to predict absolute amplitudes of measured signals, description of the enzymatic stage of the ELISA has not been included in the models. It is assumed that the optical density of colored product is strictly proportional to C value, without analysis of diffusion peculiarities and transformation for particular substrates.

1.3. Results of the Analysis

It was found that the influence of the conjugate composition on the course of the competitive ELISA curves depends essentially on how the reaction approaches the equilibrium.

The typical curves for the kinetic model are given in Fig. 2, and these are independent of the conjugate composition.

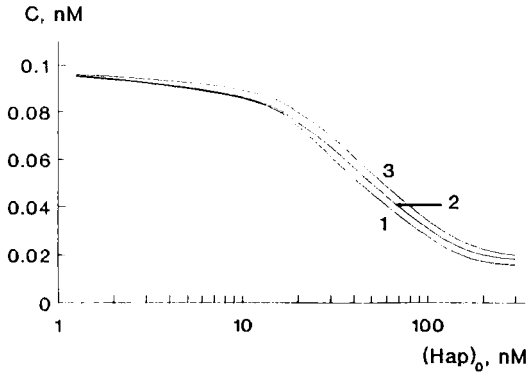

FIGURE 2

Theoretical curves for interaction between immobilized antibodies and labelled conjugate (hapten-enzyme conjugate) determined at the kinetic model. Hapten:protein molar ratios are 2:1, 5:1 and 20:1 for the curves 1-3 respectively. Conjugate concentrations (based on the enzyme) ensuring the equal C value at $(Hap)_o=0$ are $3.45 \cdot 10^{-8}$ M, $1.4 \cdot 10^{-8}$ M and $5.0 \cdot 10^{-9}$ M. The rate constants for forward and reverse antibody-antigen reactions are $1.0 \cdot 10^4$ $M^{-1}s^{-1}$ and $1.0 \cdot 10^{-5}$ s^{-1}. The interaction time - 60 s.

X axis - initial concentration of free hapten, Y axis - concentration of detected antibody-conjugate complexes.

This is due to the fact that few of Ab—Con complexes reacted to form Ab=Con complexes. As the conjugate composition only influences the association constant of the reaction [7], the differences in k_+ do not result in C differences.

However, as the reaction approaches equilibrium, the curves begin to differ and there is a significant

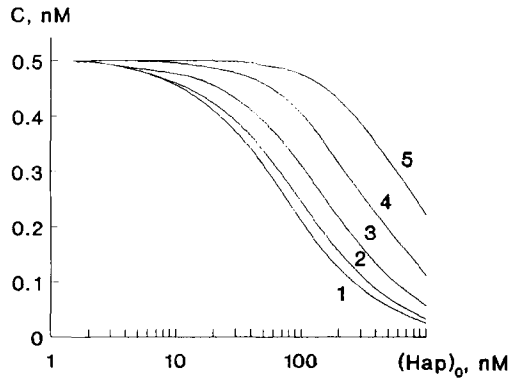

FIGURE 3

Theoretical curves for the interaction between immobilized antibodies and labelled conjugate (equilibrium model). Hapten:protein molar ratios are 1:1, 2:1, 5:1, 12:1 and 30:1 for the curves 1-5 respectively. The interaction time - 3600 s. Rate constants, X and Y axes are the same as for Fig. 2.

lowering of the free hapten detection for high N values (Fig. 3).

This is due to increases in the concentration of Ab═Con complexes. Usually the equilibrium constant of bivalent antibody binding is 2-3 orders of magnitude higher than for monovalent binding (1,2), therefore, the bivalent binding impedes the binding of free hapten with antibodies significantly.

ELISA sensitivity begins to decrease when N declines. At low values of N the medium-weighted number of hapten groups in accessible zone of the conjugate surface (N_{ac}) is low and the majority of Ab—Con

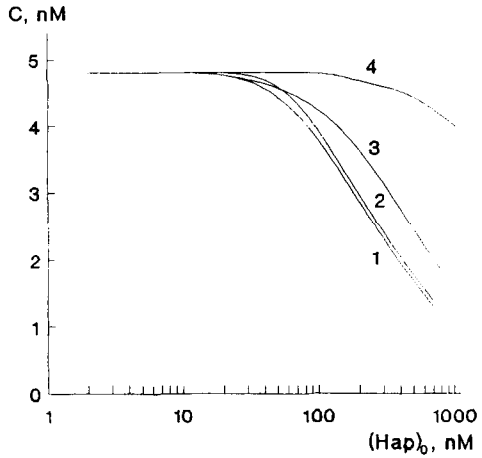

FIGURE 4

Theoretical curves describing changes of the competitive curves for ELISA with immobilized antibodies and labelled conjugate with time. Interaction times are 5 s, 60 s, 300 s and 3600 s for the curves 1-4 respectively. Hapten:protein molar ratio is 10:1. Rate constants, X and Y axes are the same as for Fig. 2.

complexes cannot reach further to form bivalent complexes. Therefore, when N_{ac} is significantly less than 1, the concentration of Ab=Con complexes is low and the competitive curves do not change.

If N is high, an increase in T (if C values at $(Hap)_0=0$ are equal) causes a decline in ELISA sensitivity (Fig. 4). It was shown that the Ab=Con concentration strongly correlated with the assay sensitivity.

The models do not deal with heterogeneity of antibodies according to affinity. They are adequate for ELISA using monoclonal antibodies. However the models may be applied for comparison of conjugates in ELISA with polyclonal antibodies. Since the assay sensitivity is defined by the course of the competitive curve at low $(Hap)_o$ concentrations (19-22), the conditions defining the sensitivity of the assay can be described with reasonable accuracy by one binding constant of the immunochemical reaction.

2. Reaction with Immobilized Hapten-Protein Conjugate

In this case the immobilized conjugate competes with free hapten for binding sites of antibodies. Detection of antibody-conjugate complexes is achieved by enzyme labelling of antibodies.

For this reaction it is necessary to take into account the possibility of one conjugate molecule interacting with several antibody molecules. We compared variants of the model that differed in the number of antibodies (one, two or three) bound to the conjugate molecule.

No differences between the second and the third variants was found, and therefore, the variant in which conjugate reacts with no more than two antibodies was selected (scheme of sequential transformations of the immune complexes is shown in Fig. 5).

For this case increase of the ELISA sensitivity with the growth of N was also established, when the assay conditions approached equilibrium.

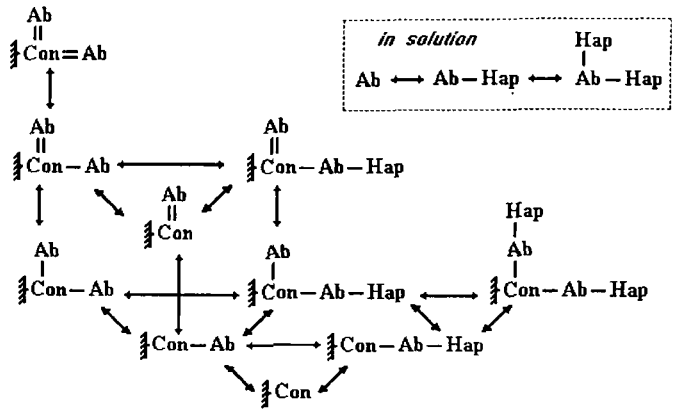

FIGURE 5

Scheme of sequential transformations of immune complexes for the model describing interactions between immobilized hapten-protein conjugate and antibodies.

Abbreviations - see Fig. 1.

EXPERIMENTAL RESULTS

According to the models, the increase of hapten surface density in conjugates results in ELISA sensitivity decreasing at the equilibrium assay conditions. This effect is determined by the increased concentration of Ab=Con complexes. Hence, for experimental verification of the models the following should be demonstrated:

1) differences in binding constants for the conjugates,
2) the dependence of these differences on bivalent binding,

3) the influence of the conjugate composition on the sensitivity of competitive hapten ELISA.

3. Determination of Equilibrium Constants for Antibody-Conjugate Interactions

The interaction distributions according to the equilibrium binding constants (referred to as "spectra") were determined experimentally for two systems: (i) titration of immobilized antibodies with hapten-enzyme conjugate and (ii), titration of immobilized hapten-protein conjugates with labelled antibodies.

In both cases it was shown that the curves obtained could be approximated by the model with two types of interactions, each with a different binding constant (affinity). The deviations of real binding curves and "spectra" from the theoretical ones were less than 10 % (for all analyzed conjugates with 2,4-D and testosterone).

It was established that an increase of surface hapten density in peroxidase conjugates was associated with an increase in the number of interactions of the type with higher affinity (Fig. 6).

This was true both for the antisera against 2,4-D and against testosterone obtained from different rabbits at different cycles of immunization (50-200 fold differences in equilibrium constants). These data are in good agreement with results from earlier investigations (1,2,23-26).

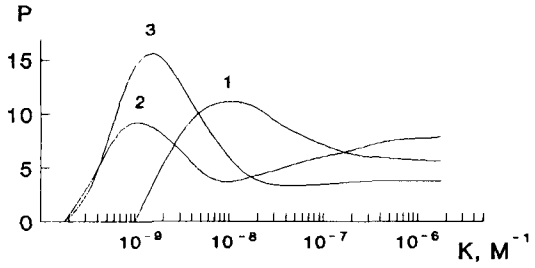

FIGURE 6

"Spectrum" analysis of the binding data for the interaction between 2,4-D-HRP conjugates and immobilized antibodies against 2,4-D. The interaction time - 2 h. 2,4-D:HRP molar ratios are 1.5:1, 5:1 and 15:1 for the curves 1-3 respectively.

Curves were obtained through the approximation of experimental data for titration of the antibodies with the 2,4-D-HRP conjugates. 11 dilutions were analyzed (n=8). The "Affinogen" program (15) was used to calculate the optimal approximation.

K - equilibrium constant of conjugate-antibody binding, P - relative amount of interactions with appropriate equilibrium constant.

The same effects were observed for the systems with conjugate immobilization (Fig. 7) regardless of a carrier conjugated with hapten. This confirmed that the effects obtained were general rather than dependent on the individual properties of a protein. Note that for the conjugates with high surface hapten density no low affinity immune interactions were observed.

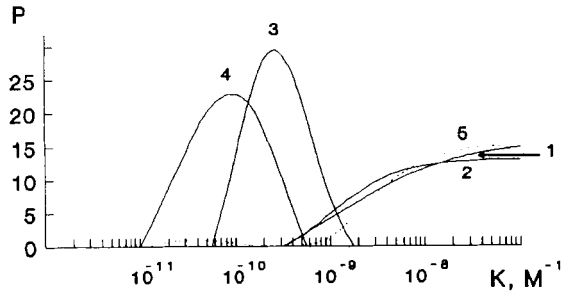

FIGURE 7

"Spectrum" analysis of the binding data for the interaction between immobilized 2,4-D-HC conjugates and antibodies against 2,4-D. The interaction time - 2 h. 2,4-D:HC molar ratios are 5:1, 20:1, 95:1 and 165:1 for the curves 1-4 respectively. Curve 5 indicates the interaction between the immobilized 2,4-D-HC conjugate with 165:1 molar ratio and monovalent fragments of the antibodies. Calculation technique, X and Y axes are the same as for Fig. 6.

To check that the increase in the interactions with high binding constants (about 10^{-9} M^{-1}) was mainly due to the formation of Ab=Con complexes, the "spectra" of conjugate interactions with native IgG molecules and their monovalent fragments were compared. We used fragments obtained by reducing S-S bonds instead of the widely used proteolytic F_{ab}- or $F_{ab'}$-fragments. The advantage of these fragments is the presence of the F_c-region that can react with immobilized protein A. In was found that interactions with higher affinity were absent in the reaction of these fragments with peroxidase conjugates but were

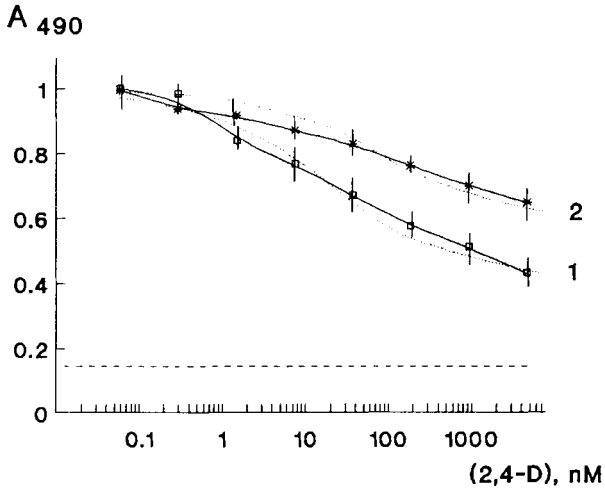

FIGURE 8

Competitive ELISA of 2,4-D with immobilized antibodies. 1,2 - experimental curves (mean values of and standard deviations) for conjugates with 2,4-D:HRP ratios 1.5:1 and 15:1 respectively. Dashed line is the initial optical density of the peroxidase substrate. Dotted lines are the theoretical approximations.

present in the reaction of these conjugates with native antibodies (see Fig. 7, curve 5).

4. ELISA with the Conjugates of Different Composition

We also studied systems in which antibodies reacted competitively with free hapten and hapten-protein conjugate. According to the model predictions the sensitivities of 2,4-D and testosterone ELISAs depended on the conjugate composition. The limit of

free hapten determination correlates with the number of interactions having high affinity: conjugates having a peak in the "spectra" at approximately 10^{-9} M^{-1} gave inferior ELISA sensitivity. These effects were observed both for peroxidase conjugates in ELISA with labelled antigen (Fig. 8) and for haemocyanine conjugates in ELISA with labelled antibodies.

The data obtained indicates that the choice of conjugate composition is a very important step in the development of ELISA for low molecular weight antigens. The assay sensitivity is significantly decreased if a conjugate having high hapten : protein ratio forms bivalent complexes with antibodies. So the absence of a peak at high binding constants in the "spectra" of antigen-antibody interactions is an effective criterium of increasing ELISA sensitivity.

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