

Conjugation of Haptens

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Abstract

Many naturally occurring proteins, peptides, carbohydrates, nucleic acids, and lipids, as well as synthetic peptides, are successful immunogens. To elicit an immune response, a compound must contain an *antigenic determinant* or *epitope* and must be of sufficient size to initiate lymphocyte activation necessary for an antibody response. In practice, small chemical compounds (haptens) are generally not good immunogens. However, when attached to macromolecules (carriers), they can become immunogenic. An immunogen must have epitopes that can be recognized by antigen-presenting cells and a T-cell receptor, and it must be degradable.

Haptens and corresponding hapten-carrier conjugates have been essential to the development of sensitive quantitative and qualitative immunoassays. In the design of hapten conjugates, consideration must be given to the hapten, the carrier, the coupling strategy, and the hapten density because the amount of hapten attached to the carrier influences the strength of the immune response directed toward the newly created antigenic determinant. Hence the haptenic density of the conjugate is also important in the development of immunoassays. The optimal epitope density of a conjugate to elicit either a strong immune response or provide the best immunoassay is dependent on the structure of the epitope and the nature of the immunoassay.

The aim of this chapter is to describe the diverse techniques used to couple haptens to carriers and provide guidance in the selection of the most appropriate procedure for a particular hapten.

Key Words: Epitope; hapten; carrier; immunogen.

1. Introduction

Many naturally occurring proteins, peptides, carbohydrates, nucleic acids, and lipids, as well as synthetic peptides, are successful immunogens. To elicit

an immune response, a compound must contain a distinct structural component referred to as an *antigenic determinant* or *epitope* and must be of sufficient size to initiate lymphocyte activation necessary for an antibody response. In practice, small chemical compounds 700–1500 Da are generally not good immunogens. However, when attached to macromolecules, they can become immunogenic. The small molecule is referred to as a *hapten* and the macromolecules that render haptens immunogenic are referred to as *carriers*. The hapten can be thought of as an exogenous epitope attached to a carrier. The essential features of an immunogen are (a) it must have epitopes that can be recognized by antigen-presenting cells and a T-cell receptor and (b) it must be degradable (1).

Haptens have diverse uses in immunology. They have been employed to evaluate the structure of epitopes and the valence, specificity, affinity, and heterogeneity of antibodies. They have also been employed in antibody purification, production of specific monoclonal antibodies, and investigation of biological properties of antibodies. Haptens and corresponding hapten–carrier conjugates have been essential to the development of sensitive quantitative and qualitative immunoassays (2–4).

In the design of hapten conjugates, consideration must be given to the hapten, the carrier, the coupling strategy, and the hapten density, i.e., the hapten:carrier molar ratio (*H/P*) (5). The amount of hapten attached to the carrier influences the strength of the immune response directed toward the newly created antigenic determinant (6). The haptenic density of the conjugate is also important in the development of immunoassays. The optimal epitope density of a conjugate to elicit either a strong immune response or provide the best immunoassay is dependent on the structure of the epitope and the nature of the immunoassay. For example, several conjugates of hexamethylene diisocyanate (HDI) with HSA were prepared in our laboratory. The molar ratio ranged from 4:1 (hapten:carrier) to 49:1. The 12:1 conjugate was found to be optimum for stimulating proliferation of lymphocytes from patients with HDI hypersensitivity (7), whereas the 36:1 conjugate was best for use in ELISA to detect IgG antibodies (8), and a 25:1 conjugate was ideal in RAST testing to detect anti-HDI IgE antibodies (8). Others have found a conjugate with *H/P* of 25:1 to yield better binding to ELISA plates when compared with conjugates having *H/P* of 54:1 (9). In our experience with diisocyanate haptens, we use conjugates with *H/P* of 30–40 to immunize animals (10), conjugates with *H/P* of 20–30 for ELISA (11), and *H/P* of 10–20 for RAST.

The aim of this chapter is to describe the diverse techniques used to couple haptens to carriers and provide guidance in the selection of the most appropriate procedure for a particular hapten.

2. Materials

2.1. Preparation of Conjugates

2.1.1. Hardware

1. Hot/stir plates.
2. Centrifuge.
3. Freeze-dryer.

2.1.2. Spontaneous Chemical Reactions

1. Hapten (isocyanates or anhydrides).
2. Carrier protein (*see Table 1*).
3. 0.05 M phosphate buffer (pH 7.4).
4. Monoethanolamine.
5. Whatman paper # 4.
6. 1 and 6 N HCl.
7. 0.1 M ammonium carbonate.
8. Dialysis tubing.
9. 0.15 M saline.

2.1.3. Chemicals that Require Activation

1. Hapten (bearing a carboxyl group).
2. Bovine serum albumin (BSA): 4.2 g in 220 mL 1:1 water–dioxane containing 4.2 mL of 1 M NaOH.
3. Tri-*n*-butylamine.
4. Dioxane.
5. Isobutylchlorocarbonate.
6. 1 M NaOH.

2.1.4. Use of Crosslinking Intermediary Molecules

2.1.4.1. CARBODIIMIDES

1. Hapten.
2. Carrier protein.
3. EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride).
4. Sodium acetate.
5. Phosphate-buffered saline (PBS) (1× conc).
6. Sodium azide.

2.1.4.2. GLUTARALDEHYDE

1. Hapten.
2. Carrier protein.
3. Glutaraldehyde.

4. PBS (1× conc).
5. Sodium borohydride.
6. Sodium azide.

2.2. Characterization of Conjugates

2.2.1. Hardware

1. Spectrophotometer.

2.2.2. Determination of the Extinction Coefficient of Conjugated Haptens (Methylene Diphenyl Diisocyanate)

1. Methylene diphenyl diisocyanate (MDI).
2. Acetone.
3. Glycine.
4. Phosphate buffer, 0.02 M (pH 7.5).

2.2.3. Spectral Analysis of Hapten Conjugates (Toluene Diisocyanate)

1. Toluene diisocyanate (TDI)–HSA conjugate.
2. Human serum albumin (HSA).
3. Phosphate buffer, 0.05 M (pH 7.4).

2.2.4. Chemical Determination of Hapten Adduction

1. Borate–KCl buffer, 0.05 M (pH 9.4). Dissolve 3.09 g boric acid and 3.73 g potassium chloride in 800 mL water, adjust pH to 9.4 with NaOH, and bring to 1 L with distilled water.
2. 0.03 M picryl sulfonic acid (2,4,6-trinitrobenzenesulfonic acid, TNBS).

3. Methods

3.1. Selection of a Carrier

A variety of carriers can be used for coupling with haptens. Selection of a carrier is based on; availability of reactive sites for hapten conjugation, size, solubility after derivatization, potential for immunogenicity, lack of in vivo toxicity, commercial availability, and cost (**4,18**). Proteins are most frequently used, but liposomes (**4**), polysaccharides (AECM-Ficoll, dextran, agar, carboxymethyl cellulose [**12–15**]) and synthetic polypeptides such as poly-L-lysine and poly-L-glutamic acid (**16,17**) have also been employed.

Numerous proteins have been employed as carrier molecules as indicated in **Table 1**. Those most frequently used include serum globulins and albumins (**6,19**), keyhole limpet-hemocyanin (KLH) (**20**), gelatin (**21**), ovalbumin (**22**), casein, hemocyanin (**23**), thyroglobulin (**24**), fibrinogen, and tetanus (**25**), cholera (**26**), or diphtheria toxoid (**2,27,28**). The protein carrier should be immunogenic and have a sufficient number of amino acid residues with

Table 1
Protein Carriers Frequently Used for Conjugation with Haptens

Protein carrier	M _r (kDa)	Functional group per molecule			
		ε-NH ₂ from Lys	-SH from Cys	Phenol from Tyr	Imidazole from His
Keyhole Limpet Hemocyanin (KLH)	4.5 × 10 ² – 1.3 × 10 ⁴	2000 ^a 6.9 ^b	700 ^a 1.7 ^b	1900 ^a 7.0 ^b	8.7 ^b
Human Serum Albumin (HSA)	66.5	61	1	28	16
Bovine Serum Albumin (BSA)	67	59 ^c	1	19	17
Tetanus Toxoid (TT)	150	106	10	81	14
Ovalbumin (OVA)	43	20	4	10	7

Adapted from S. Muller, 1999.

Abbreviations: Da, Daltons; Lys, lysine; Cys, cysteine; Tyr, tyrosine; His, histidine.

^aThe amino acid composition is expressed using a molecular weight average of 5,000 kDa (T. Hermanson, 1996).

^bAmino acid groups are expressed in grams of amino acid containing this functional group/100 g.

^c30–35 of the 59 lysine residues are available for coupling.

reactive side chains for conjugation to the hapten (**29**). When used for production of antibodies, conjugates can be injected into any animal except the animal of origin for the carrier protein. For example, human, mouse, and rabbit serum albumins have been employed for preparation of conjugates for use in guinea pigs (**31**).

Haptens may be reactive or activated to a reactive state. As indicated in **Table 2**, electrophilic haptens react primarily with amino and thiol moieties. The ε-amino group of lysine is a good nucleophile, forming stable bonds above pH 8.0. The α-NH₂ group of N-terminal amino acids are less basic and are reactive around pH 7.0. Thiols (cysteine or methionine) are the most reactive functional group at neutral pH. Under specified conditions, haptens will react with phenols

Table 2
Methods for Coupling Haptens to Proteins

Hapten	Modified amino acid	Notes	Reference
Direct hapten binding			
Diisocyanate ^a	α -NH ₂ , ϵ -NH ₂ Lys, α -COOH, SH-Cys		Karol, 1983
Acid anhydride ^b	α -NH ₂ , ϵ -NH ₂ Lys, α -COOH, SH-Cys		Welinder and Nielsen, 1991
2,4,6 Trinitrobenzene sulfonic acid (TNBS)	α -NH ₂ , ϵ -NH ₂ Lys	Also used to measure the free amino content of proteins	Makela and Seppala, 1986
Aromatic amino acid	Tyr	Aromatic amino acid converted to diazonium group	Makela and Seppala, 1986
Carbohydrate	α -NH ₂ , ϵ -NH ₂ Lys	Coupling can occur through: a) the reducing end, b) carboxyl groups of acidic carbohydrates, or c) via hydroxyl groups	Makela and Seppala, 1986
Cyanogen bromide (CnBr)	α -NH ₂ , ϵ -NH ₂ Lys	Carbohydrates activated by CnBr spontaneously couple with amino groups	Makela and Seppala, 1986
Mixed anhydride	α -NH ₂ , ϵ -NH ₂ Lys	R-COOH converted into anhydride with isobutylchloro-carbonate	Karol and Tanenbaum, 1967
Use of coupling agents			
Glutaraldehyde	α -NH ₂ , ϵ -NH ₂ Lys	Two compounds are linked through their amino groups	S. Muller, 1999

(Continued)

Table 2 (Continued)

Hapten	Modified amino acid	Notes	Reference
Carbodiimides	α -NH ₂ , ϵ -NH ₂ Lys	Carboxylic groups are changed into reactive sites for coupling with free amino groups	Harlowe and Lane, 1988

^aHDI, TDI, IPDI, MDI.^bTMA, PA.

Abbreviations: HDI: hexamethylene diisocyanate, IPDI: isophorone diisocyanate, MDI: methylene diphenyl diisocyanate, PA: phthallic anhydride, TDI: toluene diisocyanate, TMA: trimellitic anhydride

(tyrosine), carboxylic acids (aspartic and glutamic acids), and other amino acid chains (arginine, histidine, and tryptophan) (30).

3.2. Preparation of Hapten Conjugates

Selection of the method for coupling hapten to protein is dictated by the functional groups present on the hapten and the carrier, as well as the desired orientation of the hapten for presentation to the immune system. Hapten conjugation to carriers can be spontaneous when the hapten is a chemically reactive molecule such as isocyanates and anhydrides, or it can occur with the aid of a crosslinking agent, such as glutaraldehyde or a carbodiimide, that has at least two chemically reactive groups. Reagents frequently used to couple nonreactive haptens to proteins are listed in **Table 2**.

Nonreactive haptens may be coupled to carriers using reagents that are either incorporated into the final product or that can activate sites of the carrier protein for subsequent linkage (**Table 2**). When preparing conjugates, inter- and intramolecular crosslinking can occur (32). Several factors that influence the extent of crosslinking in the final conjugate are the ratio of carrier and/or hapten to coupling agent, carrier/hapten concentration, ionic strength of the solution, temperature, and pH (12).

3.2.1. Spontaneous Chemical Reactions

When the hapten is chemically reactive (i.e., isocyanates and acid anhydrides), conjugation to carriers can be spontaneous reactions.

Procedure:

1. Warm 200 mL of 0.05 M phosphate buffer (pH 7.4) to 37°C in a water bath (using a hot/stir plate), add 1 g of carrier protein (see **Notes 1 and 2**).

2. To the middle of the rapidly stirred solution, add dropwise 110 μL of hapten.
3. Stir vigorously for 1 h, then moderately for 3 h (total time 4 h).
4. Place the flask at ambient temperature, add 420 μL of monoethanolamine, and stir moderately for 1 h.
5. Filter the solution using Whatman paper #4.
6. Adjust the pH to 4.0 using 6 N HCl initially. (The starting pH is around 10.0). As the pH nears 7, use 1 N HCl. The solution will come cloudy and protein will precipitate (*see Note 3*). Cover the flask and refrigerate overnight.
7. Centrifuge solution for 20 min at 4°C at 4000 rpm.
8. Retain the supernatant until the conjugate is analyzed, then discard.
9. Dissolve the precipitate in 15 mL of 0.1 M $(\text{NH}_4)_2\text{CO}_3$. Transfer the solution to dialysis tubing and dialyze against 1 L of 0.15 M NaCl (saline) 4 h at 4°C.
10. Dialyze against distilled water for 3–4 d with 3–4 changes of distilled water/d.
11. Lyophilize the solution. Record the yield of protein.
12. Analyze the product for the amount of hapten conjugated to protein (*H/P*).

3.2.2. Chemicals that Require Activation – Mixed Anhydride Reaction

The method has been used for coupling steroids (**33**) and nucleosides (**34**) to proteins.

1. Dissolve 3.05 mmol of hapten (bearing a carboxyl group) and 3.05 mmol of tri-*n*-butylamine in 30 mL dioxane.
2. Cool the solution to 10°C.
3. Add 3.05 mmol of isobutylchlorocarbonate and stir at 4°C for 20 min.
4. Add mixture to a cooled solution of BSA (4.2 g in 220 mL of 1:1 water–dioxane containing 4.2 mL of 1 M NaOH).
5. Stir the mixture for 1 h, add 2 mL NaOH, and stir for another 3 h.
6. The product should contain approximately 30 mol hapten per mol BSA (**35**).

3.2.3. Use of Crosslinking Intermediary Molecules

3.2.3.1. CARBODIIMIDES

Carbodiimides have the general formula $\text{R}-\text{N}=\text{C}=\text{N}-\text{R}'$, where R and R' are aliphatic (i.e., ethylcarbodiimide) or aromatic groups (i.e., diphenylcarbodiimide). Conjugation using carbodiimides requires the presence of α and ε -amino and carboxyl groups. For the most part, lysyl residues of the carrier protein and carboxyl groups donated by the hapten are involved in the reaction. Carbodiimides also react with sulfhydryl and phenolic residues. Although the reaction mechanism is not fully understood, it is proposed that (1) an intermediate product (IP) is formed that can react with an amine to give the desired peptide at low temperature by reaction between the IP and amino groups and (2) there is rearrangement to an acyl urea, which is a side product at elevated temperatures (**3,12**).

The following method typically yields 50–70% conjugation of protein (*see Note 4*) (**1,4,12**).

1. Dissolve the hapten (up to 4 mg/mL) in 1 mL water (*see Note 5*).
2. Add EDC to a final concentration of 10 mg/mL with constant mixing. Adjust the pH to 5 (*see Note 6*).
3. Incubate for 5 min at ambient temperature with gentle agitation.
4. Add an equal volume of carrier protein to yield a final hapten:carrier molar ratio of 20:1–40:1.
5. Stir at ambient temperature for 4 h.
6. Stop the reaction by addition of sodium acetate (pH 4.2) to a final concentration of 100 mM. Incubate at ambient temperature for 1 h.
7. Dialyze the product against 3–4 changes of PBS for 24 h to separate free hapten from the hapten–protein conjugate (*see Note 7*). Store at 4°C in the presence of sodium azide (0.02%) or in aliquots at –20°C.

3.2.3.2. GLUTARALDEHYDE

Glutaraldehyde is a bifunctional coupling reagent that can react with amine groups to create Schiff bases or Michael-type double bond addition products. In general, high pH favors formation of Schiff base intermediates and results in greater conjugation, but also in higher molecular weight conjugates. Varying the pH and the amount of glutaraldehyde added to the reaction allows some control of the yield and the molecular weight of the conjugate. The concentration of glutaraldehyde added generally varies from 0.2 to 2% with sporadic use of very dilute solutions (0.05%) (**4**). The glutaraldehyde crosslink is very stable, but in most cases the glutaraldehyde bridge is recognized as an epitope by the immunized animal (**1,4,12**).

Glutaraldehyde crosslinking can be performed in a one- or two-step conjugation protocol. Most frequently, the single step method is used. This method is recommended for haptens that do not contain internal lysine or cysteine residues (**12**).

1. Prepare a 1 mg/mL solution of the carrier protein in PBS, pH 7.4.
2. Add the hapten to the carrier protein solution using a molar ratio of 20:1–40:1 hapten:carrier protein. Cool to 4°C.
3. Just before use, prepare a 2% (v/v) glutaraldehyde solution in water.
4. Add dropwise, with constant agitation, an equal volume of 2% glutaraldehyde to the hapten–protein carrier solution.
5. After 1 h at 4°C, stabilize the conjugate by addition of sodium borohydride (NaBH_4) to a final concentration of 10 mg/mL.
6. Incubate at 4°C for 1 h.
7. Dialyze against 3–4 changes of PBS for 24 h (*see Note 7*). Store at 4°C in the presence of 0.02% sodium azide (or in aliquots at –20°C).

3.3. Use of Spacers

Hapten-directed immunogenicity may be enhanced through the use of spacers. These molecules function to extend the distance of the hapten from the surface

of the carrier. The length provided by the spacer may provide less steric hindrance to conjugation by the hapten. The nature of the spacer may also govern the hydrophilicity of the hapten. Generally the spacer is attached to the hapten prior to coupling, but may also be attached to the carrier. Spacers may be immunogenic. With small haptens, the actual epitope will include portions of the carrier. In this instance, the spacer will become part of the structurally known epitope, thus reducing the size of the structurally unknown portion. Glycine and alanine residues are the frequently used spacers (12,35).

3.4. Analysis of Conjugates

The degree of haptentation of the conjugate can be determined using radioactive hapten tracers (3), by measurement of UV or visible light-absorbing groups on the hapten, using non-denatured gel electrophoresis (36), amino acid analysis (37), fluorodensitometric methods (38), gas chromatography (39), HPLC (40), or electrospray mass spectrometry (41).

3.4.1. Spectral Analysis of Conjugates

The hapten may have an absorbance spectrum that allows it to be differentiated from the carrier protein. This procedure is especially useful when the hapten has a strong chromophore. The *H/P* determination by spectroscopy is accomplished by first dialyzing the preparation to remove unbound hapten. The absorption of the conjugate is measured at the $\lambda_{\max}$ for the hapten, then the protein concentration of the conjugate is determined from absorbance at 280 nm. The result is corrected for the $A_{280 \text{ nm}}$ because of the hapten. The *H/P* is calculated using Beer's law: $A = \epsilon Cl$, where ϵ is the extinction coefficient, A the molar absorbance, C the molar concentration, and l the path length (6,30). It should be noted that inaccuracy might result if the spectral characteristics of the hapten change when conjugated to the protein (30). The following assumptions are made when hapten conjugates are analyzed by UV absorbance: (1) the solution is free of noncovalently bound hapten, (2) conjugates are soluble in the matrix used for analysis, and (3) chromophores of the carrier protein and hapten do not change on conjugation (6).

3.4.1.1. DETERMINATION OF THE EXTINCTION COEFFICIENT OF CONJUGATED MDI

The extinction coefficient of the free hapten and protein can be obtained from tables or handbooks. That of the conjugated form can be calculated using the following approach (32):

1. MDI (Mondur M, Bayer USA, Pittsburgh, PA) was recrystallized, then dissolved in acetone at 2.1 mg/mL.
2. A total of 10 μL of the solution was added to 0.16–2.8 mM glycine in 0.02 M phosphate buffer, pH 7.5.

Table 3
Determination of the Extinction Coefficient of MDI Following Carbamylation of the Nucleophile

Glycine (mg)	MDI (μg)	λ_{max} (nm)	Absorbance at λ_{max}	Molar extinction coefficient
0.2	21	245.1	0.415	24714
2.0	21	246.8	0.480	28577
20	21	248.8	0.592	35238
200	21	248.8	0.615	36613

Adapted from Jin and Karol, 1988.

3. The absorbances of the solutions were determined at 200–350 nm. *See Table 3.*
4. The extinction coefficients were calculated from Beer's law: $A = \epsilon Cl$ (*see Section 3.4.1.*).

3.4.1.2. SPECTRAL ANALYSIS OF TDI CONJUGATES

When there is overlap in the spectra of the hapten and the carrier, H/P can be determined from a “difference” spectrum (*see Fig. 1*). The hapten can be quantified by subtracting the absorbance of the uncoupled protein carrier at the haptenic λ_{max} from that of the conjugate (**42,43**).

1. Separately dissolve 3 mg of the TDI–HSA conjugate and 3 mg of HSA in 0.05 M of phosphate buffer, pH 7.4 to a final concentration of 600 $\mu\text{g/mL}$.
2. Determine the absorbance from 600 to 190 nm of the following solutions:
 - a. carrier protein vs buffer,
 - b. conjugate vs buffer,
 - c. conjugate vs carrier protein.
3. Calculate H/P using Beer's law with the absorbance value of the conjugate obtained from 2c.

3.4.2. Chemical Determination

When the hapten does not have absorbance in the UV or visible range, the extent of hapten coupling to protein can be estimated from the determination of the number of remaining amino groups on the carrier protein. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) is frequently used for this estimation. Haptenic content of conjugates is determined from absorption of the trinitrophenyl derivative of the protein before and after conjugation (**3,37**).

1. Separately prepare 1% solutions of the carrier protein and conjugate in 0.05 M borate–KCl buffer, pH 9.4.

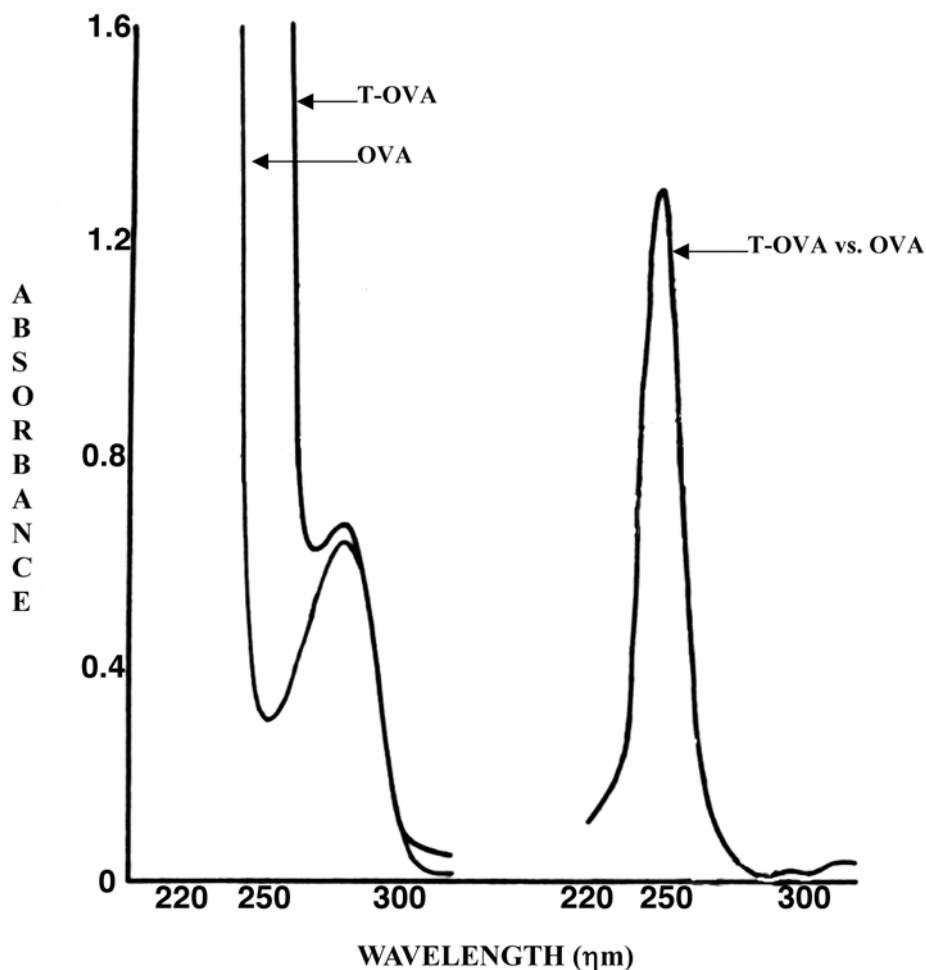


Fig. 1. Ultraviolet absorption spectra. **Left:** Ovalbumin (OVA) and p-tolylureido-ovalbumin (T-OVA) conjugate each 1 mg/ml, 0.05 M phosphate buffer, pH 7.0. **Right:** the difference spectrum of T-OVA versus OVA (T-OVA vs. OVA).

2. Prepare assay tubes according to **Table 4**.
3. Prepare 0.03 M picryl-sulfonic acid (TNBS; Sigma, Cat # p-2297) in distilled water using a glass beaker.
4. Add 50 μ L of TNBS to each tube.
5. Vortex, let sit at ambient temperature for 30 min.
6. Immediately thereafter, read absorbance 420 nm (do not use quartz cuvettes) (*see Note 8*).

Table 4
Set Up for Conjugate Analysis by TNBS

Volume of borate buffer (ml)	Volume of conjugate solution (μl)	Volume of protein solution (μl)
2*	—	—
2*	—	—
1.95	50 (500 μg)	—
1.90	100 (1 mg)	—
1.85	150 (1.5 mg)	—
1.95	—	50 (500 μg)
1.90	—	100 (1 mg)
1.85	—	150 (1.5 mg)

* Blanks.

7. Read blanks vs H₂O, then samples against the lowest blank.
8. Calculate the hapten substitution using the following formula:

$$9. \quad \% \text{ Substitution} = 100 - \frac{[100(\text{Absorbance conjugate})]}{(\text{Absorbance carrier protein})}$$

4. Notes

1. In general, conjugates of serum albumin are more soluble than those of globulins or ovalbumin. Because of its large size, KLH is more likely to precipitate during crosslinking conjugation. To preserve the solubility and stability of the conjugate, coupling reactions using KLH should be performed under conditions of high salt (at least 0.9 M NaCl) (4).
2. The haptenic content (*H/P*) of the final conjugate will vary with (1) the amount of hapten added, (2) pH of the solution with higher pH usually favoring greater substitution, (3) incubation temperature (higher temperatures favor higher substitution).
3. Conjugates having low hapten substitution will usually not precipitate at pH 4. Adjust the pH to 7 before starting dialysis.
4. Adding 5 mM *N*-hydroxysulfosuccinimide may enhance the efficiency (12).
5. To avoid amino acid modification in the hapten, you may block it with citraconic anhydride (CA). For this, dissolve the hapten in water to 10 mg/mL. Adjust the pH to 8.5 and stir at room temperature. Prepare a 10 mg/mL solution of CA in water and slowly add an equal volume to the hapten solution and incubate for 1 h. Following the reaction with ECD, the citraconylated amino groups are deprotected by dialysis against 5% acetic acid. Finally, dialyze the conjugate against PBS (1,12).
6. Adding *N*-methyl-imidazole to the coupling mixture may decrease the formation of acylurea adducts (12).

7. Larger haptens may not dialyze efficiently. Gel filtration can be used to separate the free hapten from the conjugate.
8. If the absorbance of the conjugate is equal to that of the carrier protein, *H/P* is very low. A more sensitive method of analysis is needed (such as electrospray mass spectrometry).

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