UNIVERSITY OF ILLINOIS

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Susan Marie Shoemaker

ENTITLED: Elution and Purification of Anti-Uranyl(salophic)

Antibodies in Rabbits

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Chemistry

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HEAD OF DEPARTMENT OF Chemistry
ELICITATION AND PURIFICATION
OF
ANTI-URANYL (SALOPHIC) ANTIBODIES
IN RABBITS

BY

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THESIS

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I wish first to thank Dr. Russell Drake for giving me the opportunity to work on such a worthwhile, exciting project as "the rabbit project", and for his far-sighted wisdom in assigning me to work with James Stahlbush. Jim initiated me gently and kindly into the world of research. I must also thank the other members of the Drake research group for their help, advice, and (almost) encouragement.

I wish second to thank my adjunct research director and research group, Dr. Edward Voss and co-workers, for their tolerance of an inorganic chemist in an immunologists' world, and their help and advice.
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I. INTRODUCTION

Antigen-antibody interactions have long been the interest of immunologists. Since Porter first digested rabbit gamma globulin antibodies with crystalline papain (Porter, 1959), investigators have searched for methods which would give them information about the antibody molecule's structural, binding, and thermodynamic properties. It is now well established that the Fab portion of the molecule is responsible for binding the antigen. That this binding is so specific for the antigen, or, in many cases, structurally-related molecules, is why particular attention has been paid to the Fab active sites. Many studies have been conducted with haptens, molecules which alone do not stimulate the immune response but do react specifically with the appropriate antibodies. Haptens are generally coupled to carrier proteins to initiate the immune response. These studies have allowed the determination of such things as binding site dimensions (Dwek et al., 1975), hapten side group orientation effects (Voss and Watt, 1975), and degree of cross-reactivity (Voss et al., 1976; Dwek, Jones et al., 1975). Many haptens, however, even when coupled to a carrier, are not large enough to cause antibody formation to the hapten alone. As a result there have been problems separating the hapten effects from the carrier effects. These have been solved in part by designing haptens that will be large enough to approximate a complete antigenic group (Voss et al., 1976), and by changing carriers between the primary and secondary immunizations.

Repeated immunizations with the same antigen cause an increase in quantity and/or quality of antibody production, known as saturation. All antibodies, however, are not created equal. The affinity for the antigen increases with saturation. Because this is a continuous, in vivo process, antibody populations obtained are heterogeneous mixtures of affinity classes. The difficulty in physically separating affinity classes, necessary for present day thermodynamic techniques, has resulted in information on average
association constants for the entire population (Eisen and Siskind, 1961). It has also obscured the binding process enough so that the concept of what affinity is still being debated, as discussed in a chapter by Karush in Immunoglobulins (Gary Litman and Robert Good, editors, 1978).

The methods of immunology, however, have been improving by leaps and bounds, and will perhaps some day solve even the heterogeneity problem. A major advance was the adaptation of electrophoresis, first developed by Tiselius for analyzing and separating proteins, to study antigen-antibody reactions (Williams, 1960). This allowed identification of a single antibody protein in a mixture. Another advance was the use of fluorescent probes to study hapten-antibody binding. Voss and co-workers have demonstrated that the fluorescent probe fluorescein approximates a complete antigenic group, have obtained anti-fluorescein antibodies, and have used them in fluorescent quenching studies to determine binding constants (Lopatin and Voss, 1971; Voss et al., 1976). Immunologists are now turning to the techniques and instrumentation of the organic, inorganic, and physical chemist to obtain more precise information than immunological techniques can yield. Incorporation of nitroxide spin labels into hapten ligands (Humphries and McConnell, 1976) has allowed the use of electron spin resonance (ESR) spectroscopy to identify spin label resonances changed by antibody binding. The use of nuclear magnetic resonance (NMR) difference spectroscopy is also becoming widespread. In this technique a probe is attached to a ligand used as a hapten. Proton NMR spectra are taken of the free antibody and the hapten-antibody complex. All but the resonances shifted by the spin label are subtracted out of the spectra. Since the label was on the hapten against which the antibodies were formed, the shifted resonances will be those at or near the active binding site of the antibody. It cannot be known for certain, however, if the resonances are actually from the active site. The application of these probes in NMR studies of biological systems is extensively discussed in a review article by Morris and Dwak (1977). Possibly the most ambitious study undertaken to date using these techniques has been that by Dwak and co-workers of the structure on
the combining site of the myeloma protein MOPC 315 (Dwek et al., 1975; Dwek, Jones et al., 1975; Dwek, 1977). A myeloma produces large quantities of homogeneous immunoglobulins very similar to antibodies, so the problem of heterogeneity is bypassed, but it must be kept in mind that these proteins are the result of an artificial, abnormal system (cancer of immunoglobulin synthesizing cells). These structural data can be correlated with thermodynamic data. The vagueness of thermodynamic quantities obtained from equilibrium procedures has been replaced by the more exact microcalorimetry procedures, developed by Marisam and co-workers (see Skinner, editor, 1975) and discussed in a recent review article by Marisam and Jill (1978).

With sophisticated spectroscopic techniques, one thing is still lacking: the certain knowledge that changes in spectra caused by spin label binding to antibody occur at and in the active site of the molecule. To accomplish this one needs a paramagnetic label that is itself the hapten against which antibodies are produced. It is the purpose of this project to produce these antibodies. The paramagnetic hapten chosen was the ligand uranyl (salophic) (see Figure 1). There are two advantageous reasons why this particular ligand was chosen: 1) UO$_2$ (salophic) is structurally similar to fluorescein (see Figure 1), so should also approximate a complete antigenic group, thus minimizing carrier effects, and 2) a series of these ligands can be made using different transition metals (Pfeiffer et al., 1977). The differences in spectra and binding can thus be attributed to the change in the metal alone. This allows a fairly exact study of cross-reactivity. Another advantage of this system is that it will allow a study of affinity classes without physical separation of the classes by watching the growth, shifting, and disappearance of resonances by NMR difference spectroscopy during a titration of antibody with the hapten. We know these changes will be at the active site itself. We can then couple these data with ESR and microcalorimetry data to give a fairly complete picture of the active site of naturally produced antibody.
Fig. 1. Schematic molecular structures of uranyl (salophic) and fluorescein.
II. MATERIALS AND METHODS

A. Synthesis of Ligand Hanten

Uranyl (salophic) for use as the hanten was synthesized by James Stahlbush in the following manner (Reischwott, unpublished). Salophic was first synthesized by dissolving 1.52 g. (0.01 mole) 1,4-diaminobenzoic acid in 50 ml. boiling methanol and adding 1.22 g. (0.01 mole) salicylaldehyde (vacuum distilled and stored under argon in a dark bottle) dropwise. A second 1.22 g. of salicylaldehyde was then added dropwise to the resulting brown solution, with an orange microcrystalline precipitate forming. After cooling to room temperature, the precipitate was collected via suction filtration, washed with light petroleum ether and dried in vacuo. 1.00 g. (0.009 mole) of this salophic was then dissolved in 100 ml. hot methanol. 1.27 g. (0.01 mole) uranyl acetate in 20 ml. hot methanol was added dropwise, and the solution refluxed 0.5-2 hours, during which time an orange crystalline precipitate formed. The solution and precipitate were cooled in an ice bath for 15 minutes. The precipitate was collected via suction filtration, washed with cold methanol and diethyl ether, and dried in vacuo. The yield was 1.00 g. (57%).

 Elemental analysis | Calculated % | Found %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>40.12</td>
<td>41.75</td>
</tr>
<tr>
<td>H</td>
<td>4.06</td>
<td>4.14</td>
</tr>
<tr>
<td>N</td>
<td>5.47</td>
<td>5.64</td>
</tr>
</tbody>
</table>

\[
\begin{aligned}
\text{reaction:} & \\
\text{H}_2\text{N}-\text{C}-\text{OH} + 2 \text{C}_6\text{H}_4\text{OH} \rightarrow \text{C}_6\text{H}_4\text{N}-\text{N}=\text{C} \equiv \text{C}=\text{O} + \text{COOH} \\
\end{aligned}
\]

\[\text{+ UO}_4\text{(C}_6\text{H}_5\text{O}_4\text{)}\text{H}_2\text{O}\]
B. **Antigen Preparation**

Uranyl (salophic) was coupled to the protein carrier, bovine serum albumin (BSA), by James Stahlbusch. Coupling was through the lysine residues on the BSA and the carboxylic acid groups on the uranyl (salophic). A 10% solution of BSA was purchased from Fentex. A 1 ml. sample was precipitated in 25 ml. dimethylformamide (DMF), then washed once with ethanol, twice with 0.05 M potassium butoxide/ethanol, three times with ethanol, and once with DMF. 0.3215 g. (0.00051 mole) uranyl (salophic) and 0.2105 g. (0.00054 mole) NCDI, a coupling agent, were dissolved in 80 ml. DMF. To 40 ml. of this solution was added the prepared BSA, and the mixture stirred at room temperature for 18 hours. The BSA was then centrifuged and washed as before, put back into the reaction mixture, and stirred an additional 26 hours. It was then centrifuged, dialysed against 50/50 DMF/H₂O until the dialysate remained clear, and refrigerated. That coupling had occurred was indicated by a color change in the BSA from colorless to orange. Because of the insolubility of the complex, no attempt was made to determine the amount of substitution on the BSA.

C. **Polymer Immunoadsorbent Preparation**

1. Iodomethylation

A macroporous polystyrene bead substituted with uranyl (salophic) was chosen as the immunoadsorbent to give maximum separation of the anti-uranyl (salophic) IgG antibodies from the rest of the IgG fraction. Work was done as much as possible with plastic utensils because of the high electricity of the bead. Chloromethylated polystyrene was the starting material. The method of Gaul (Ph.D. thesis, 1978) was followed. 25 g. of chloromethylated polymer was refluxed for 2-4 days in 120 ml. of acetone/dioxane (9/1, v/v) containing 15-20 g. sodium iodide. The resin was cooled, filtered, and Soxlet extracted with acetone 24 hours, then dried at 80°C. This procedure was carried out twice on the same sample of polymer to give better substitution.
Elemental analysis:

<table>
<thead>
<tr>
<th></th>
<th>%C</th>
<th>%H</th>
<th>%Cl</th>
<th>%I</th>
</tr>
</thead>
<tbody>
<tr>
<td>chloromethylated</td>
<td>84.50</td>
<td>7.21</td>
<td>5.74</td>
<td>---</td>
</tr>
<tr>
<td>iodomethylated</td>
<td>79.67</td>
<td>6.83</td>
<td>1.19</td>
<td>9.44</td>
</tr>
</tbody>
</table>

Substitution calculation: Substitutions onto the polymer are not stoichiometric and therefore cannot be predicted. They, however, can be worked out afterward from the elemental analysis using the following method (Weichgott, unpublished). Calculation of chlorine substitution was not done because it was not known if the method was applicable. The elemental analysis was done to obtain approximate amounts of the elements involved in the subsequent substitutions.

In the calculations not every element is followed in all steps, because for some elements such as the halogens, what is unsubstituted in one step will remain unsubstituted and thus becomes unimportant for these purposes.

The polymer repeat units for 20% cross-linking (known for this sample as John Gaul worked it out) gives the formula: C_{17}H_{47}Cl_y

Iodination gives the formula (approximately):

C_{17}H_{47}I_{17}y

The C/I ratio using the results of the elemental analysis yields the value of y:

\[ \frac{79.67}{17.2} = \frac{128.6}{17.2} \]

Thus, \[ y = 0.424 \]

and \[ y = 0.424 \]

The formula, then, is C_{17.424}H_{17.424}I_{0.424}

In 2 polymer repeat units there are 9 styrene residues, so

9 \frac{1}{2} = \text{iodine residues}

9 \times 0.424 = 3.824

There are, therefore, approximately 1 iodine/11 styrene residues
Reaction:

\[
\text{CH}_2\text{Cl} + \text{NaI} \rightarrow \text{CH}_4\text{I}
\]

2. Ethylenediamine substitution

The iodomethylated beads were stirred with 25 ml. of DMF saturated with nitrogen. 25 ml. of ethylenediamine (distilled under argon and stored in a dark bottle) was injected, and the mixture stirred at room temperature for 2 days. The beads were next collected via suction filtration and washed in order with tetrahydrofuran (THF), H_2O, 1 M NaOH, H_2O, and THF. They were dried, and Soxlet extracted with 1/1 (v/v) dioxane/H_2O for 24 hours. Then they were washed with THF, and dried at 85°C.

Elemental analysis:  

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>86.61</td>
<td>8.01</td>
<td>2.28</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Substitution calculation: This was calculated in an analogous manner as for the iodine substitution, using the C/N ratio.

Formula: C_{76.10}H_{46}N_{0.46}O_{0.86}

Substitutional ethylenediamine/styrene residue

or 1 ethylenediamine/10 styrene residues

Reaction:

\[
\text{CH}_4\text{I} + H_2N(CH_2)_4NH_2 \rightarrow \text{CH}_3NHCH_2CH_2NH_2
\]

3. Uranyl (salophic) substitution

Uranyl (salophic) was synthesised as before and recrystallised in DMF.
Elemental analysis: | Calculated % | Found %
---|-------------|-------------
C | 41.11 | 15.51/15.77
H | 2.41 | 2.13/2.46
N | 4.79 | 4.54
U | 40.71 | 19.27/19.27

Next 90 mg. of the recrystallized uranyl (salophic) was dissolved in 90 ml. DMF, stirred for 1.5 hours, and filtered. 0.69 g. of a coupling agent (1-cyclohexyl-3-2-morpholinoethyl-carbodiimide) and 6.26 g. of the polymer bound ethylenediamine were added, and the mixture stirred for 2 days. During this time the solution went from orange to light yellow, and the beads went from yellow to orange. Then a fresh solution of 1 mg. uranyl (salophic)/ml. DMF was prepared as before. The beads were transferred into this solution, 0.68 g. coupling agent was added, and the mixture stirred an additional 2 days. The beads were then washed with THF, Soxlet extracted with THF for 24 hours, and dried in vacuo at 45°C. The beads remained orange after extraction. Calculations were done as outlined in IIIC1.

Elemental analysis: | C | H | N | U
---|---|---|---|---
Calc. | 39.14 | 40.09 | 0.93 | trace (0.12)
Found | 39.54 | 7.35 | 2.55 | ~

Formula: C_{39.14}H_{40.09}N_{0.93}U
Substitution (by C/N ratio): 1 UO_2 (salophic)/129 styrene residues

Reaction:

\[
\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NCH}_3\text{CH}_2\text{NH}_2 + \text{UO}_2\text{(salophic)} \rightarrow \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NCH}_3\text{CH}_2\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NCH}_3\text{CH}_2\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N} - \text{C} - \text{[UO}_2\text{(salophic)}]\]

D. Immunization and Bleedings

An adult albino rabbit was injected in the foot pads and intrascapularly with approximately 10 mg. UO_2 (salophic)--BSA in complete Freund's adjuvant. Bleedings were taken 17, 33, and 41 days after the primary immunization, with a booster immunisation identical to the primary given 17 days after the primary.
Approximately 50 ml. of blood was obtained through the marginal ear vein at each bleeding, yielding 18.9 ml., 20.4 ml., and 20.6 ml. of serum, respectively for the three bleedings.

E. Purification of Anti-Uranyl (salophic) IgG Antibodies

To obtain the IgG anti-uranyl (salophic) antibodies, the following procedure was followed for each serum fraction and a normal rabbit serum fraction. First the serum was treated with dextran and calcium chloride (CaCl₂) in an ice bath to remove interfering lipoprotein. Second, a 50\% saturated ammonium sulfate ((NH₄)₂SO₄) cut was done in an ice bath on the dextran-CaCl₂ supernate to precipitate the IgG fraction. Third, each (NH₄)₂SO₄ precipitate was dissolved in 5 ml. 0.1 M phosphate buffer pH 7 and dialysed against 0.1 M phosphate buffer pH 7 at 6°C for two days, with the dialysis buffer changed three times. During dialysis each fraction accumulated a small amount of white solid in the dialysis tubing. This was assumed to be denatured protein. Fourth, each dialysis fraction was incubated at 6°C with 2 ml. of the immunoadsorbent (described in IIIC) for 2 days. The fractions were then washed with 0.1 M phosphate buffer pH 7 until the A₂₇₈ was less than 0.100, centrifuging at 10,000 rpm between washings. Fifth, the anti-uranyl (salophic) antibodies were eluted off the immunoadsorbent by gradient elution with 4 M saline solution at room temperature. Two ml. fractions were collected. Protein was monitored by the A₂₇₈ read on a Beckman Model 25 spectrophotometer. The fractions with the highest A₂₇₈ were pooled and dialysed against 0.1 M phosphate buffer pH 7.95 at 6°C for 24 hours, changing the buffer twice. At this time it was thought that the total protein of the NBS, first, second, and third bleedings was 1.007 mg., 7.405 mg., 0.800 mg., and 1.082 mg., respectively. The fractions were concentrated to 1-2 ml. at 6°C with an Amicon ultrafiltration cell. It was later discovered that styrene contamination was present, so the fractions were run over a QAE-Sephadex (S100) column, using 0.1 M phosphate buffer pH 7 containing 0.02% sodium azide as the eluting buffer. Four ml. fractions were collected, and these with the highest A₂₇₈ pooled.
These were concentrated to 1-2 ml. as before. The total A$_{278}$ of the NRS, first, second, and third bleedings was 0.296, 0.447, 0.654, and 0.617, respectively.

F. **Immunoelectrophoresis**

Immunoelectrophoresis to determine purity of antibodies was done twice, once before and once after the QAE-Sephadex column was run. Both were done on glass slides coated with 1% agar in barbitol buffer pH 8.6. The first electrophoresis was run at 220 V and 15 mA for 50 minutes. Plates were developed with both mouse anti-rabbit plasma and goat anti-rabbit gamma chain plasma, placed in troughs following electrophoresis. The second electrophoresis was run at 220 V and 15 mA for 50 minutes, using fluorescein as a marker. Plates were developed with mouse anti-rabbit plasma, placed in troughs following electrophoresis. Both times a Buchler Instruments constant current constant voltage power pack was used.

G. **SDS Slab Gel Electrophoresis**

A 10% SDS slab gel electrophoresis was run in barbitol buffer pH 8.6 at 50 V and 25 mA until the samples were through the stacking gel, then at 95 V and 25 mA. The total run time was seven hours. Samples were prepared by boiling in a tris-SDS sample buffer for 10 minutes, then placed into wells in the slab gel. A Pharmacia low molecular weight calibration standard was run at the same time. This consists of six proteins (listed in order of decreasing molecular weight): phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and a-lactalbumin. An Arthur H. Thomas Company Electrophoresis Power Supply Model 21 was used. The gel was dyed in 0.25% Coomassie Blue in 25% isopropanol and 10% acetic acid, and destained in a 10% acetic acid, 7.5% methanol solution.
III. RESULTS

A. IMMUNELECTROPHORESIS

In both runs, the rabbit whole serum controls developed the usual precipitin bands when developed with mouse anti-rabbit plasma and goat anti-rabbit gamma chain plasma. None of the antibody fractions, however, formed any precipitin bands. This indicated that the technique was good, but that my antibody fractions contained no protein. A possibility existed that the assay was not sensitive enough to detect my low level of antibody. This is why SDS slab gel electrophoresis was run.

B. SDS Slab Gel Electrophoresis

The low molecular weight calibration standard formed its characteristic 6 band pattern. The tris-SDS digestion breaks antibodies into their heavy and light chains, with one band seen for each. My antibody fractions, however, showed no bands, thus confirming that I did not in reality have any antibodies.
IV. DISCUSSION

Although I did not obtain the desired antibodies, this project should not be abandoned. It has provided much useful information about the weaknesses of the system. By identifying and correcting them, the system should work, thus opening the way for NMR, ESR, and calorimetric studies. At this point four major problems can be identified: 1) synthesizing a water soluble ligand, 2) getting better substitution of the ligand on the carrier protein (along with developing a method to determine the amount of substitution), 3) developing a sensitive in serum assay, and 4) developing a new immunoadsorbent which can be substituted in the same manner as the old.

The first and second problems are related. The present ligand, UO$_2$ (salophic), and the carrier protein, BSA, are not soluble in the same type of solvent systems. As a protein, BSA is soluble in aqueous-type solvents. In contrast UO$_2$ (salophic), as a metal ligand, is soluble in solvents such as DMF. As little as 5% DMF is all that is necessary to precipitate BSA in a globular clump. This hides the lysine residues and makes them unavailable for substitution. Synthesis of a water soluble ligand would allow ligand and protein to be present in a solution together, thus increasing the chances of the two coupling. A water soluble system would also allow the use of the standard spectroscopic techniques to determine the amount of substitution, not possible with the present water insoluble system. Work is being carried out at the present by James Stahlbush to synthesize a water soluble ligand. A small possibility exists that good coupling was obtained, but because the ligand was hydrophobic, was hidden in a pocket in the BSA. Antibodies would then have been formed only to the BSA. The odds against this happening, however, are so great that this possibility can be ignored.

The third problem, that of developing a sensitive in serum assay, is basically a time saver. If it can be determined in the serum that the specific antibodies you wished to obtain were not there, the long purification process would not have to be done.
Immunelectrophoresis is a sensitive assay, but as with all precipitin-based assays, requires at least 0.5 mg/ml antibody. We cannot be certain we will have that great an immune response, almost certainly not in the primary response. Radioisotope techniques are even more sensitive than immunelectrophoresis. The BSA can be radiiodinated, the ligand attached to the BSA, and then the BSA-ligand put into the serum. By knowing the counts incorporated into the BSA, one can then check for counts in the serum and any precipitate that forms. Counts in the precipitate will indicate an antigen-antibody reaction has occurred, thus showing the presence of the antibodies in the serum. I am currently developing this technique with the help of Dr. Edward Voss.

The fourth problem, a change in immunoadsorbents, is connected with the problem of contamination. Styrene is one of the worst contaminants of immunoadsorbents, but is the only polymer for which the specific substitution needed is worked out. The problem can be partially alleviated by dialysis of the antibodies to remove small particle styrene and then running them over QAE-Sephadex to remove large particle styrene (both must be done), but to be solved a different immunoadsorbent such as substituted Sepharose or cellulose will need to be used.

In summary, the theory of the experiment is sound, but experimental flaws will need to be worked out before its full benefits can be realized.
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