MODEL SYSTEMS FOR THE STUDY
OF DRUG HYPERSENSITIVITY

Thesis by
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In Partial Fulfillment of the Requirements

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Doctor of Philosophy

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(Submitted February 6, 1970)
Dedicated to
the loving memory of
MY MOTHER
ACKNOWLEDGMENTS

I would like to thank my advisor, Professor Dan H. Campbell, for his guidance and inspiration. His associate, Dr. Justine S. Garvey, rendered many services above and beyond the call of duty, including reading this manuscript during her vacation.

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An unrepayable debt of gratitude is owed to my Father, who has always supplied encouragement and helped with personal problems. And of course this acknowledgment would be incomplete without thanking Miss Barbara Adams.
ABSTRACT

I. It was not possible to produce anti-tetracycline antibody in laboratory animals by any of the methods tried. Tetracycline protein conjugates were prepared and characterized. It was shown that previous reports of the detection of anti-tetracycline antibody by in vitro methods were in error. Tetracycline precipitates non-specifically with serum proteins. The anaphylactic reaction reported was the result of misinterpretation, since the observations were inconsistent with the known mechanism of anaphylaxis and the supposed antibody would not sensitize guinea pig skin. The hemagglutination reaction was not reproducible and was extremely sensitive to minute amounts of microbial contamination. Both free tetracyclines and the conjugates were found to be poor antigens.

II. Anti-aspiryl antibodies were produced in rabbits using 3 protein carriers. The method of inhibition of precipitation was used to determine the specificity of the antibody produced. \( \varepsilon \)-Aminocaproate was found to be the most effective inhibitor of the haptens tested, indicating that the combining hapten of the protein is \( \varepsilon \)-aspiryl-lysyl. Free aspirin and salicylates were poor inhibitors and did not combine with the antibody to a significant extent. The ortho group was found to participate in the binding to antibody. The average binding
constants were measured.

Normal rabbit serum was acetylated by aspirin under in vitro conditions, which are similar to physiological conditions. The extent of acetylation was determined by immunochemical tests. The acetylated serum proteins were shown to be potent antigens in rabbits. It was also shown that aspiryl proteins were partially acetylated. The relation of these results to human aspirin intolerance is discussed.

III. Aspirin did not induce contact sensitivity in guinea pigs when they were immunized by techniques that induce sensitivity with other reactive compounds. The acetylation mechanism is not relevant to this type of hypersensitivity, since sensitivity is not produced by potent acetylation agents like acetyl chloride and acetic anhydride. Aspiryl chloride, a totally artificial system, is a good sensitizer. Its specificity was examined.

IV. Protein conjugates were prepared with p-aminosalicylic acid and various carriers using azo, carbodiimide and mixed anhydride coupling. These antigens were injected into rabbits and guinea pigs and no anti-hapten IgG or IgM response was obtained. Delayed hypersensitivity was produced in guinea pigs by immunization with the conjugates, and its specificity was determined. Guinea pigs were not sensitized by either injections or topical application of p-aminosalicylic acid or p-aminosalicylate.
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Chapter 1

IMMUNOCHEMICAL INVESTIGATIONS OF TETRACYCLINES

Introduction

The tetracyclines are one of the most important classes of broad spectrum antibiotics. Chlortetracycline (Aureomycin) was first isolated in 1947 from a mold, Streptomyces. Within a few years a large series of tetracycline compounds had been isolated from related molds and prepared by chemical modification of the natural compounds. In 1952 the structure of oxytetracycline (Terramycin) was determined (1,2), and in 1962 the structure was confirmed by synthesis (3,4). The structure of tetracycline and the ring numbering system are given in figure 1.

The rather complex chemistry of the tetracyclines has been reviewed recently (5).

Figure 1

Tetracycline
In the last fifteen years tetracyclines have come into widespread use as antibacterial drugs (6). The only antibiotics used more frequently are the penicillins. Usually tetracyclines cause only minor side effects, such as staining teeth and disturbing the digestive tract flora, causing gastrointestinal distress, nausea, etc. However, allergies to most of the commonly used tetracycline antibiotics have been reported. Only a few selected examples are given in the references. The earliest allergic reactions found were to Aureomycin (7,8,9). A short time after their introduction, allergy was also reported to tetracycline (10,11) and demethylchlortetracycline (Declomycin) (12,13). There are collections of case histories in several reviews (14,15) and an editorial warning about tetracycline allergy has appeared in The Journal of the American Medical Association (16).

Fellner and Baer (11) demonstrated that a reaction to tetracycline was the result of allergy by the use of direct skin tests, passive hemagglutination and the passive transfer (P-K) reaction. Shelly obtained a positive reaction using an indirect basophil test (17). Many individuals who developed tetracycline allergy were shown to be allergic to penicillin also, but clinical data indicate that the reactions are independent, and not due to a cross reaction or impurities. A photoreaction to demethylchlortetracycline has been investigated (18,19,20).
This reaction may or may not be caused by an immune mechanism. A similar reaction has also been observed with 6-deoxytetracycline (Doxycycline), but not with tetracycline. The high incidence of this reaction in patients who have had excessive solar exposure soon after taking the drug indicates that the reaction is probably phototoxic (20).

There have been several attempts to demonstrate anti-tetracycline antibodies either in vitro or in laboratory animals. In 1958 Muelling, Beven, Samson, Jenevein and Guillory (21) claimed to have found precipitating antibody against tetracycline in the sera of sensitive patients by using precipitation tests with free antibiotic as test antigen. In 1960 Spektorova (22) immunized rats with tetracycline and reported finding precipitation lines in gel diffusion tests with free tetracycline from 11 to 45 days after immunization. The most complete study to date was done by Queng, Dukes and McGovern (23). They immunized rabbits with free tetracyclines and used both the passive hemagglutination and anaphylactic methods to detect antibodies. The results and interpretations in each of these investigations in some way contradicted the general principles of immunochemistry. In the first two studies (21,22) a concentration of 1mg/ml of free tetracycline was used in the precipitation tests. It is very rare when a small sized, monovalent hapten can participate in immune precipitate formation. It has been clearly established
that precipitating antigens must be at least divalent (24). Tetracyclines are adsorbed to serum proteins (25) non-covalently, but this is an equilibrium with substantial amounts of free antibiotic in solution. This free antibiotic in solution should inhibit precipitate formation. Queng et al. (23) reported positive passive hemagglutination tests, Schultz-Dale reactions with guinea pig gut and a "reversed Schultz-Dale" reaction; however, they were unable to obtain positive passive cutaneous anaphylaxis (PCA) reactions in guinea pigs. It has been established that the PCA and Schultz-Dale techniques measure the same antibodies and that PCA is at least as sensitive (26).

Unlike the relatively rare tetracyline allergy, penicillin allergy has been extensively studied (27). Penicillin and several of its degradation products react in vivo with proteins to form immunogenic conjugates. The major determinant is the penicilloyl group, but in some cases the penicillenate group, other degradation products and impurities cause the allergy. A highly conjugated protein impurity has recently been found in many commercial preparations. Antibodies to these various determinants have been found in the IgE, IgG and IgM classes of immune globulins. IgE is responsible for the cases which exhibit urticarial reactions, and IgG and IgM have been detected in allergic sera by passive hemagglutination. Penicillin allergy has been estimated to occur in about 5% of the
American population and is the most common drug allergy known. Although at the early stages of investigation penicillin allergy seemed to exhibit anomalous immunochemical behavior, careful investigation showed that penicillin behaved as a collection of classical hapten systems when its chemistry was taken into account.

Because of the apparently anomalous behavior reported for tetracyclines, a two-fold investigation was undertaken. The first part involved the preparation of covalently bonded protein conjugates of tetracyclines and the study of the immunogenicity of these conjugates. The second part involved immunochemical and immunological studies of some of the previously reported investigations in order to clear up the inconsistencies mentioned above.
Results and Discussion

A. Conjugates

1. Bisdiazotized Benzidine Conjugates

In 1964 DeCarvalho, Rand and Lewis (28) coupled tetracycline and other drugs to gamma globulin using bis-diazotized benzidine. They did not report either experimental data or spectra for the tetracycline experiment. Their method was tried but it led to an intractable product. The method of coupling described in the materials and methods section of this chapter was then developed. Conjugates were prepared with tetracycline, demethylchlortetracycline, chlortetracycline and oxytetracycline (see figure 2 for structures), using bovine serum albumin (BSA), rabbit serum albumin (RSA), giant keyhole limpet hemocyanin (KLH) and bovine gamma globulin (BGG) as protein carriers. The conjugates were all brown or reddish brown in color and had broad absorption bands in the visible and ultraviolet. The spectral data for demethylchlortetracycline are given in table 1 and plotted in figure 3. The

<table>
<thead>
<tr>
<th>Substance</th>
<th>Color</th>
<th>Absorp. max. (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demethylchlortetracycline (DMC)</td>
<td>yellow</td>
<td>365,275,248,229</td>
</tr>
<tr>
<td>DiazobenzidineazoDMC</td>
<td>red</td>
<td>487,282,325-400</td>
</tr>
<tr>
<td>Bisdiazobenzidine</td>
<td>green</td>
<td>313</td>
</tr>
<tr>
<td>DMCazobenzidineazoBSA</td>
<td>red brown</td>
<td>492,282</td>
</tr>
</tbody>
</table>
Figure 2

Structures of Tetracyclines

Tetracycline

Chlortetracycline

Demethylchlortetracycline

Oxytetracycline

7-Amino-6-demethyl-6-deoxy tetracycline

9-Amino-6-demethyl-6-deoxy tetracycline
Figure 3
Spectra of Demethylchlortetracycline Derivatives

..... Demethylchlortetracycline
xxxxxx Demethylchlortetracyclineazodiazobenzidine
+++++ DemethylchlortetracyclineazobenzidineazoBSA

The concentrations are not equalized.
spectra of the other compounds are similar.

The spectral evidence indicates that the tetracyclines are not still intact in the conjugates, since most of the characteristic spectral features have disappeared. If the coupling proceeded without destruction of the tetracycline nucleus, the azo group would attach primarily at position 9 in the 7-chloro compounds and both positions 7 and 9 in the 7-unsubstituted compounds. The major directing effect on the electrophilic substitution is the o,p effect of the phenolic hydroxyl. This greatly outweighs the other directing effects.

Tetracycline, chlortetracycline and oxytetracycline are all subject to degradation by bases (29). The 6-de-methyl compounds are much more resistant to this degradation. The formation of iso-chlortetracycline from chlortetracycline is shown in figure 4. The iso-tetracyclines then degrade further. The half life of tetracycline in pH 8.85

Figure 4

Base Catalyzed Degradation of Chlortetracycline (29)
buffer is about 12 hours (30). Slightly basic conditions also favor epimerization at C-4. It is probable that much of the tetracycline in these antigen preparations is degraded. The conjugates are insolubilized by acidification or lyophilization, but remain soluble at pH 8.4 to 9.0. Storage under these basic conditions, however, favors further degradation. After extensive dialysis and storage for several months, very little color is removed by further dialysis. Under the same conditions other azoproteins release considerable amounts of colored hapten.

2. 7- and 9-Azotetracycline Conjugates

Small amounts of 7- and 9-amino-6-demethyl-6-deoxytetracyclines were obtained from Dr. J. H. Boothe of Lederle Laboratories. Both of these compounds can be easily diazotized, and the diazonium salts isolated and coupled directly to proteins. 6-Demethyl-6-deoxytetracyclines are also resistant to both acid and base catalyzed degradation (31). Spectral data for the 7- series of compounds are given in table 2 and figure 5.

These data indicate that the tetracycline is not degraded, since the chromophore is not destroyed. The conjugates do not have the deep red or brown color of the benzidine conjugates, but are either red or greenish. After extensive dialysis and prolonged storage, a material with a tetracycline-like spectrum is recovered by further dialysis.
Figure 5
Spectra of 7-Tetracycline Derivatives

..... 7-Amino-6-demethyl-6-deoxytetracycline
+++++ 7-azo-6-demethyl-6-deoxytetracycline\text{KLH}

The concentrations are not equalized.
Table 2
Spectral Data for 7-Amino-6-demethyl-6-deoxytetracycline
(7-AminoDDT)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Absorption Maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-AminoDDT</td>
<td>345, 274, 250, 200</td>
</tr>
<tr>
<td>7-DiazoDDT</td>
<td>345, 310, 255</td>
</tr>
<tr>
<td>7-AzoDDTKLH</td>
<td>346, 279</td>
</tr>
<tr>
<td>7-AzoDDTRSA</td>
<td>345, 272</td>
</tr>
<tr>
<td>7-AzoDDTBSA</td>
<td>346, 273</td>
</tr>
</tbody>
</table>

3. In Vivo Conjugates

Several studies have shown that tetracycline binds to serum proteins in vivo. Tetracycline is detectable in tissue and protein complexes by fluorescence microscopy. When sera from tetracycline treated individuals are separated by electrophoresis, the albumin region is labelled (25). There is no evidence for covalent bonding. The binding is probably ionic or adsorptive. Although much tetracycline is excreted in the urine unchanged (32), significant amounts are bound in various tissues (33). Table 3 gives the amounts of various tetracyclines that bind to mouse serum proteins under standard conditions, measured in vitro.
Table 3

Binding of Tetracyclines to Mouse Serum Proteins (34)

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Binding</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxyoxtetracycline</td>
<td>69.6</td>
<td>2.6</td>
</tr>
<tr>
<td>6-Methyleneoxytetracycline</td>
<td>69.7</td>
<td>2.1</td>
</tr>
<tr>
<td>6-Demethylchlortetracycline</td>
<td>73.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>54.8</td>
<td>2.2</td>
</tr>
</tbody>
</table>
B. Immunization

1. With Tetracycline-Protein Conjugates

Various immunization schedules and methods were tried. Intravenous injection, Freund's complete adjuvant intramuscularly and intradermally, and alumina adjuvant intramuscularly were all used. The species used were rabbits, guinea pigs and chickens. No animals produced significant amounts of antibody against tetracycline, although a few produced antibody against azo modified protein. All sera were tested by ring tests and passive cutaneous anaphylaxis (PCA) in guinea pigs. Precipitation tests with chicken sera were done in both 0.9% and 9% saline.

In order to further investigate the absence of antibody production against these haptens, a series of azophenol antigens were prepared and tested in rabbits. The phenols were selected because tetracycline is attached to protein carriers through the phenol ring. Results with these antigens were generally negative, as can be seen in table 4. This finding was at first attributed to the use of Freund's adjuvant (35), but later studies using intravenous immunization and glutaraldehyde crosslinked antigens (36) also gave negative results with most azoproteins, when rabbits of California origin were used. Recently some rabbits obtained from Illinois responded to several azo-proteins. A complete investigation of this problem has not been possible; the cause could be either genetic or
### Table 4
Assay of anti-hapten antibody in rabbit sera after immunization using Freund's adjuvant immunization

<table>
<thead>
<tr>
<th>Hapten</th>
<th>Carrier</th>
<th>Ring test†</th>
<th>PCA</th>
<th>Antibody*</th>
<th>Schedule‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 para-azophenol</td>
<td>KLH</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>2 para-azophenol</td>
<td>RSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>3 azobenzene</td>
<td>RSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>4 azobenzene</td>
<td>KLH</td>
<td>--,+,+,+</td>
<td>--</td>
<td>++,+,+,+</td>
<td>1</td>
</tr>
<tr>
<td>5 meta-azophenol</td>
<td>RSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>6 meta-azophenol</td>
<td>BSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>7 meta-azophenol</td>
<td>KLH</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>8 azonaphthyl</td>
<td>RSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>9 azonaphthyl</td>
<td>KLH</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>10 para-azophenylarsonic</td>
<td>KLH</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>11 para-azophenylarsonic</td>
<td>BSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>12 4-azosalicylic</td>
<td>BSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>13 4-azosalicylic</td>
<td>BGG</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>14 5-azosalicylic</td>
<td>BSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>15 5-azosalicylic</td>
<td>BGG</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>16 para-azobenzoic</td>
<td>BSA</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>17 para-azobenzoic</td>
<td>BGG</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>18 aspiryl</td>
<td>BSA</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>0·3</td>
<td>2</td>
</tr>
<tr>
<td>19 aspiryl</td>
<td>BGG</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>0·6</td>
<td>2</td>
</tr>
<tr>
<td>20 aspiryl</td>
<td>KLH</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1·4</td>
<td>2</td>
</tr>
<tr>
<td>21 benzoyl</td>
<td>BSA</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>0·3</td>
<td>2</td>
</tr>
<tr>
<td>22 benzoyl</td>
<td>BGG</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1·6</td>
<td>2</td>
</tr>
<tr>
<td>23 5-methyl aspiryl</td>
<td>BSA</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>24 5-methyl aspiryl</td>
<td>BGG</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>25 dinitrophenyl</td>
<td>KLH</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>26 dinitrophenyl</td>
<td>BSA</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>27 dinitrophenyl</td>
<td>BGG</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>28 trinitrophenyl</td>
<td>KLH</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>29 trinitrophenyl</td>
<td>BSA</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>30 trinitrophenyl</td>
<td>BGG</td>
<td>++++,+++++,+++++</td>
<td>++++,+++++,+++++</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Quantitative precipitin on pooled ammonium sulfate precipitated globulins. Maximum antibody precipitated in mg/ml.
†Individual rabbits.
‡1 - Intramuscularly 2 or 3 times weekly for 3 or 4 weeks. 2 - Intradermally weekly for 8 weeks.
environmental. It is not due to the antigen preparation. Antigens that had previously failed to produce an anti-hapten response have recently stimulated anti-azohapten antibody production in rabbits of midwestern origin. All of the animals immunized produced large amounts of antibody against the protein carrier.

Table 5 lists the animals, schedules and procedures used with the azobenzidine conjugates, and table 6 the data for the azotetracycline conjugates.

**Abbreviations for tables 5 and 6**

Tet -- tetracycline  
Terra -- oxytetracycline  
DMCT -- demethylchlortetacycline  
7-Tet -- 7-azo-6-demethyl-6-deoxytetracycline  
9-Tet -- 9-azo-6-demethyl-6-deoxytetracycline  
benz -- azobenzidineazo  
F -- Freund's complete adjuvant emulsion  
A -- alumina adjuvant  
iv -- intravenous  
im -- intramuscular  
sc -- subcutaneous  
B -- bleed  
da -- day  
wk -- week  
mo -- month  
All unspecified times are 7 days.
Table 5

Immunizations with Benzidine Conjugates

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Animals</th>
<th>Immunization Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 TetbenzKLH</td>
<td>10 guinea pigs</td>
<td>2 times F (4 weeks apart), wait 3 weeks, B</td>
</tr>
<tr>
<td>2 TetbenzKLH</td>
<td>6 guinea pigs</td>
<td>F, 3wk, B</td>
</tr>
<tr>
<td>3 TerrabenzBSA</td>
<td>2 guinea pigs</td>
<td>2xF (2wk), 5wk, B</td>
</tr>
<tr>
<td>4 TetbenzKLH</td>
<td>10 rabbits</td>
<td>6xiv(2xwkly), B, 2xiv, 3xiv (wk), 2wk, B, F, 3wk, B, 3wk, B</td>
</tr>
<tr>
<td>5 TerrabenzBSA</td>
<td>2 rabbits</td>
<td>2xF(10da), wk, B, wk, B, 2xiv, wk, B, 6xiv(3xwk), wk, B</td>
</tr>
<tr>
<td>6 TetbenzKLH</td>
<td>3 rabbits</td>
<td>A, 2mo, 2xiv, B, 3mo, 2xiv, 2wk, B, paint 8xTet (3wk), wk, B</td>
</tr>
<tr>
<td>7 TetbenzKLH</td>
<td>3 rabbits</td>
<td>F, 10da, 2xAiv (10da), 10da, B, 2xiv, 2wk, B</td>
</tr>
<tr>
<td>8 TetbenzRGG</td>
<td>4 rabbits</td>
<td>2xF (10da), 3wk, B, 6wk, 2xiv, 2da, B</td>
</tr>
<tr>
<td>DMCTbenzBSA</td>
<td>4 rabbits</td>
<td>2xF, 10da, B, 4xiv (2xwk), wk, B</td>
</tr>
<tr>
<td>9 TetbenzBSA</td>
<td>4 rabbits</td>
<td>2xim (10da), 3wk, B, 2xiv, 2wk, B</td>
</tr>
<tr>
<td>DMCTbenzRSA</td>
<td>3 rabbits</td>
<td>iv, 2wk, sc, 5da, B, 4xiv (2xwk), 7da, B</td>
</tr>
<tr>
<td>10 DMCTbenzRSA</td>
<td>3 rabbits</td>
<td>2xF (2wk), 3wk, B</td>
</tr>
</tbody>
</table>
Table 5, continued

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<th>Immune System</th>
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<tr>
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solid

Table 6

Immunizations with Tetracyclineazoproteins

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<td>6</td>
<td>7-TetKLH</td>
<td>3</td>
<td>chickens</td>
<td>iv, wk, B</td>
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</table>
2. With Free Tetracyclines

These investigations were undertaken to verify the results of Spektorova (22) and Queng, Dukes and McGovern (23). 10mg of antibiotic was dissolved in 0.5ml of water containing enough NaHCO₃ to dissolve the drug, and this solution was emulsified with 0.6ml of Freund's complete adjuvant and injected intradermally into 2 sites in each rabbit. This procedure was repeated 4 more times on alternate days. After resting 2 weeks each rabbit received on alternate days 3 intramuscular injections of 10mg of antibiotic in buffered saline pH 7.4. After 3 weeks' rest the first course of injections was repeated and the animals then rested for 5 weeks. Finally, 5 daily subcutaneous injections of 5mg each were administered. The rabbits were bled 7 days after the last injection in each series.

None of the sera obtained from these animals contained specific precipitating antibody. The results of hemagglutination and anaphylaxis experiments are discussed in later sections of this chapter. The antibiotics used in these experiments were tetracycline, demethylchlortetracycline, chlortetracycline, oxytetracycline, oxytetracycline hydrochloride and an old sample of oxytetracycline, which was light brown rather than yellow.

A group of rabbits was also immunized by painting a solution of tetracycline on a shaved area of their backs.
twice a week for 4 weeks. They showed no skin reaction and their sera tested negative by precipitation tests and PCA.

C. Precipitation Tests

1. Precipitation with Free Tetracycline

Two groups of investigators have reported the existence of antibody which precipitates with free tetracycline. Muelling et al. (21) reported this effect with human allergic sera and Spektorova (22) with immunized rat sera. Both groups used fairly high concentrations of antibiotic in their assays, about 10mg/ml, so their results are very questionable. In general, specific immune precipitation requires antigen which is at least divalent (24). Even if one postulates that the tetracycline reacts with or binds to the serum proteins of the test serum, one would still expect the high free hapten concentration to inhibit specific immune precipitation.

Spektorova's experiments with rats were repeated. Out of 6 rat sera tested, 5 showed precipitation lines in immunodiffusion. After incubating further for 72 hours the lines disappeared. 100 anti- and normal sera from rats, rabbits and guinea pigs were tested in immunodiffusion under the same conditions using 10mg/ml of tetracycline. 90% showed lines at 24 or 48 hours and in most cases the lines disappeared after further incubation. The percentage of positive reactions was almost identical in sera from
tetracycline treated and non-treated animals.

Four rabbits were immunized using a schedule similar to that used for the rats, and their sera were tested by the quantitative precipitation method with final dilutions of tetracycline from 5mg/ml to 0.1mg/ml. The data for rabbit 941 are given in figure 6. The amount of precipitate increased with increasing amounts of tetracycline up to the highest concentration used. There was free tetracycline left in all of the tubes. Both observations contradict the behavior of immune precipitates. In addition there was not inhibition by free hapten. Neither of the two articles referred to above mentioned control experiments with normal sera. The experiments reported here show that free tetracycline is able to non-specifically precipitate certain serum proteins when added at concentrations of 1-10mg/ml.

2. Immunoadsorbents

As a part of this study an evaluation was made of different methods of specifically purifying antibody. One reason was to find the best method for purifying antibody against tetracycline, when it was obtained, and another was to prepare highly purified antibody for developing and evaluating the various techniques used. Specifically purified antibody was of great value in developing the Schultz-Dale method and the equilibrium dialysis method.
Figure 6
Precipitation of Serum 941 (1/5/67) by Free Tetracycline

Abcissa- Concentration of tetracycline
Ordinate- Protein in absorbance units
used in these studies. The detailed methods for preparing and using the immunoadsorbents are given in the materials and methods part of this chapter.

Each adsorbent was prepared and tested with at least two antigen-antibody systems. Quantitative tests were performed to determine recoveries and purities from adsorbents which gave a good yield of pure antibody. The polyacrylamide gel method was the best system tried. It could be prepared from free proteins and amide coupled and azo coupled conjugates in less than 6 hours. It was easy to elute, and antibody was recovered in high yield. The adsorbent was reusable, but with loss of capacity. One special feature of this adsorbent is that the pore size of the gel can be enlarged or reduced for different sized antigens. It was also an efficient preparation, since it bound almost all of the antigen used in its preparation. Free proteins crosslinked by either glutaraldehyde or ethyl chloroformate were also excellent adsorbents, but many conjugates can not be crosslinked linked enough to become insoluble by these procedures. Azo and acid chloride coupling block the free amino groups necessary for crosslinking. The various immunoadsorbents tried are compared in table 7.
<table>
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<tr>
<th>Adsorbent</th>
<th>Ease of Preparation</th>
<th>Range of Use Proteins</th>
<th>Range of Use Azob-</th>
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<td>Yes</td>
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<td>M*</td>
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<tr>
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<td>Yes</td>
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<td>4 Bromoacetyl cellulose</td>
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<td>Yes</td>
<td>Hard</td>
<td>H</td>
<td>Med.</td>
<td>Exc.</td>
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<td>5 Glutaraldehyde crosslinking</td>
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<td>No</td>
<td>Easy</td>
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<td>No</td>
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<td>L</td>
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<td>7 Polyacrylamide gel</td>
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<td>Yes</td>
<td>Easy</td>
<td>L</td>
<td>High</td>
<td>Exc.</td>
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</table>

* M- medium, L- low, H- high

** Exc.- excellent
D. Passive Hemagglutination

Queng, Dukes and McGovern (23) tested sera from rabbits immunized with tetracycline, chlortetracycline, oxytetracycline and demethylchlortetracycline by passive hemagglutination and inhibition of passive agglutination. Two rabbits immunized with oxytetracycline showed titers of 640 and 5120 against oxytetracycline. All of the other sera tested negative or extremely low against the immunizing antigens. The positive sera exhibited little or no cross-reactivity with other tetracyclines. These results were unexpected since the incidence of allergy to oxytetracycline is significantly lower than that to any of the other compounds they used. The positive sera were chromatographed on DEAE-Sephadex and the activity was found after processing parts of the first two peaks. The first peak was IgG. The positive sera did not give PCA reactions in guinea pigs.

A group of rabbits was immunized with free tetracyclines according to the method of Queng et al. (23) and tested both by the tube hemagglutination technique (23) and a micro modification of it. It was found that the substitution of a buffered gelatin solution for normal serum in the final washing of cells and also as diluent greatly decreased the problem of non-specific agglutination and variation of the sensitivity of the assay. Three different samples of oxytetracycline were used; one was quite old and probably significantly degraded. The sera
from rabbits immunized with tetracycline, chlortetracycline and demethylchlortetracycline all tested negative or titered less than 20 each time they were tested. Each animal was bled before and 4 times during the immunization schedule and the sera were tested each time. At various times sera from rabbits immunized with oxytetracycline tested positive; however the variation in the titers was unsystematic. In the course of these experiments it was found that the oxytetracycline hemagglutination system was extremely sensitive to microbial contamination. The entire experiment was then repeated with new rabbits and the sera were kept sterile by membrane filtration and storage at -20°C. This time all of the sera were negative. 12 rabbits were used in this experiment. Since they all tested negative, it was not possible to evaluate the inhibition system. The micro method, using microtiter plates, was much more reproducible than the tube method for all four antigens. The cells were prepared by the literature method (23), since one could not maximize the sensitivity without a positive control serum for each drug.

E. In Vitro Anaphylaxis

It has been maintained by Queng et al. (23) that sera from rabbits immunized with oxytetracycline can passively sensitize guinea pig ileum in the Schultz-Dale technique. The same authors also state that the identical rabbit sera
could not sensitize guinea pig skin for passive cutaneous anaphylaxis. Both reactions are manifestations of sensitization by the same classes of antibodies. Rabbit IgG is known to sensitize guinea pig tissues, while rabbit IgM and IgA do not (37).

The sequence of events in anaphylactic reactions has been extensively studied (38) and more recent research has confirmed the proposed mechanism; a simplified version of the mechanism as it is presently understood follows.

The Fc end of certain globulins is able to couple to specific receptors on mast cells of various tissues. Globulins which can sensitize animals of the same species may be different from those that can sensitize other species. For example human IgG2 sensitizes guinea pig skin but not human or monkey skin, while human IgE sensitizes both human and monkey skin. Non-specific globulin competes with specific antibody on a concentration basis for the available mast cell combining sites. The globulins are bound firmly to the tissues and are not removed by extensive washing, but can be removed with non-specific globulin. The bound antibody can combine with antigen and initiate a series of events involving the cell, possibly by a steric effect. These events terminate in the release of vaso-active substances, particularly histamine and serotonin from the mast cells. Mast cell histamine is bound to heparin in the granules of the mast cells, and degranulation
is observed during an anaphylactic reaction. Figure 7 is a very schematized diagram of these events.

A second inconsistency in the interpretation of Schultz-Dale data by Queng et al. (23) is the claim that not only can guinea pig gut be passively sensitized to oxytetracycline with immune sera, but also that the gut can be passively sensitized with oxytetracycline and then react with immune sera. This interpretation is completely inconsistent with the mechanism presented above, which is based upon a large accumulation of empirical evidence.

The experiments of Queng et al. (23) were repeated and extensively studied to find the cause of this inconsistency. Figures 8 and 9 show experiments of Queng, Dukes and McGovern (23). Figure 10 shows the reactions of a piece of gut sensitized with oxytetracycline, challenged with immune globulin, washed again, and challenged with oxytetracycline. Figure 11 shows a piece of normal gut sensitized with immune serum, washed, challenged with tetracycline, washed, and challenged again with immune serum. Figure 12 shows the reaction of another piece of gut sensitized with anti-DNPBSA globulins and challenged with DNPBSA. This is a true Schultz-Dale reaction. Desensitization is observed with the anti-DNPBSA sensitized gut. The reaction is not visible after the second challenge, which is already quite weak. In all of the tetracycline and oxytetracycline experiments "desensitization" was
Figure 7

Schematic Diagram of Anaphylaxis

1. Mast cells and antibodies
2. Fixation of antibodies
3. Combination of antigen with antibody
4. Release of histamine and serotonin

Key
a  mast cell
b  cell
c  antibody
d  antigen
h  histamine
s  serotonin
.  mast cell granules
Schultz-Dale experiments from Queng, Dukes and McGovern (23)
Schultz-Dale experiment with normal guinea pig ileum

1 0.2ml serum A57 -- passive sensitization
2 0.02 micrograms histamine
3 0.4mg oxytetracycline

Discontinuities indicate time and washes.
Figure 11
Schultz-Dale experiment with oxytetracycline sensitized gut

1 0.05ml A61 total globulins
2 0.2mg tetracycline
3 0.02 microgram histamine
4 0.05ml A61 total globulins

Discontinuities are time and washes.
Schultz-Dale experiment with dinitrophenylBSA sensitized gut

1 0.02 microgram histamine
2 0.05mg dinitrophenylBSA
3 0.02 microgram histamine

Discontinuities are time and washes.
gradual and several challenges were required for the reaction to disappear. Tetracycline and demethylchlorotetracycline elicited only weak responses from sensitized gut. Queng et al. (23) claimed that 0.1mg of drug was non-irritating, but that amount of oxytetracycline was able to cause a contraction. They did not specify whether or not they used whole serum for testing. If they did, one would expect contractions from the effects of adding serum to the Tyrode and from the free histamine and histamine releasers in the Tyrode.

A recent study showed that tetracycline caused major reductions in the histamine and serotonin contents of mouse intestine (39). This effect could be the result of association of the tetracycline with serum proteins on the intestinal wall, causing the release of histamine and serotonin. This would also explain the results of the Schultz-Dale experiments.
Conclusions

It was not possible to produce a significant amount of antibody against tetracycline in laboratory animals by any of the methods tried. This was not unexpected, since tetracycline allergy is quite rare among patients given tetracycline therapy. However, it was found that the previous reports of the detection of antibody against tetracycline by in vitro experiments were in error.

The precipitation reactions reported in the literature were unequivocably shown to be non-specific, due to the association of tetracycline with serum proteins. The hemagglutination reaction was not reproducible and is highly suspect because of the interactions of some tetracyclines with proteins at cell surfaces. It was also extremely sensitive to microbial contamination. The anaphylactic reactions reported were the result of mis-interpreted data. None of the papers that reported in vitro detection of antibody against tetracyclines gave any evidence that proper control experiments were performed.

Tetracycline is a poor immunogen, and human hypersensitivity to it will probably have to be studied in allergic individuals and monkeys to elucidate the mechanism.
Materials and Methods

A. Materials

1. Antibiotics

The tetracycline antibiotics were all obtained from manufacturers and were all samples of batches intended for pharmaceutical use. The two aminotetracyclines were a gift from Dr. J. H. Boothe of Lederle Laboratories.

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<td>7Y536-51010</td>
</tr>
<tr>
<td>Oxytetracycline (old)</td>
<td>P</td>
<td>unknown</td>
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* L - Lederle Laboratories, Pearl River, N. Y.

P - Charles Pfizer and Co., Brooklyn, N. Y.
2. Freund's Adjuvant

Freund's adjuvant, complete, was obtained from Difco, Detroit, Michigan. This commercial product was superior to a preparation made with 9 parts of Marcol 52 (Humble Oil Co.), 1 part of Arlacel F (Atlas Powder Co.) and killed Mycobacteria. The latter antigen was more difficult to emulsify than the Difco product; 1.2 ml of adjuvant were mixed with 1 ml of antigen solution. The pH of antigen solutions should be either more than 7.4 or less than 5 to form stable emulsions.

3. Proteins

Bovine serum albumin (BSA) and bovine gamma globulin (BGG) were obtained from Armour Pharmaceuticals, Kankakee, Illinois. Rabbit serum albumin (RSA) was obtained from Pentex, Inc., Kankakee, Illinois.

Giant keyhole limpet hemocyanin (KLH) was prepared by a modification of the method of Campbell et al. (40). Fresh giant keyhole limpets, Megathura crenulata, obtained the same day from Marineland of the Pacific were slit in the foot and placed on 1/2 inch wire mesh that covered the top of enamelled pans in the cold room at 40°C. When all the body fluid had drained into the pan, the carcasses were discarded, and the fluid was centrifuged at 3000 rpm for 30 minutes, and the supernatant was then filtered through fluted filter paper. The hemocyanin was then sedimented in
the head of a Spinco Ultracentrifuge for 90 - 120 minutes at 30,000rpm. Samples were prepared and stored in two different ways. 5ml of saline was added to each of a group of tubes that were stored frozen at -20°. 5ml of saturated ammonium sulfate was added to each of a group of tubes that were stored at 4°. Before the use of any sample the liquid was poured from the tube and the pellet was dissolved in saline. The solution was centrifuged at 3000 rpm for 15 minutes; the supernatant was then filtered and the hemocyanin resedimented for 90 minutes at 30,000rpm in the ultracentrifuge. The pellets were redissolved in saline and again centrifuged at 3000rpm for 15 minutes. The supernatant was then dialyzed against saline. Recovery of hemocyanin from the material stored under ammonium sulfate solution was much greater than from frozen material. Ammonium sulfate appears to inhibit degradation. The concentration of hemocyanin after the above processing was generally from 25-40mg/ml.

4. Borate-saline Buffer

6.18g of boric acid, 9.54g of Na₂B₄O₇·10H₂O and 4.38g of sodium chloride were dissolved in one liter of distilled water. The pH was between 8.4 and 8.6.
B. Preparation of Antigens

1. Benzidine Coupling

Benzidine coupling was done by a modification of Kabat's method (41).

DemethylchlortetracyclineazobenzidineazoBSA

64mg of benzidine dihydrochloride was dissolved in 10 ml of water and 0.3ml of 6N HCl and cooled in ice. 0.85ml of sodium nitrite solution containing 35mg was added, and the colored solution was stirred for 25 minutes at 2-4°. Excess HNO₂ was destroyed by the addition of small quantities of solid sulfamic acid until tests with starch-iodide paper were negative. The tetra-azotized benzidine was poured into 50ml of cold 0.6% sodium acetate and the pH was adjusted to 8 with 2N NaOH. 125mg of demethylchlortetracycline hydrochloride was added in pH 8 buffer, and the solution was stirred in the cold for 10-15 minutes at pH 8. The demethylchlortetracyclineazodiacobenzidine was then slowly added to a cold solution of 250mg of BSA in saline, while the pH was kept between 8.2 and 8.6. After the addition was completed, the solution was allowed to stand for 20 hours in the cold, and then it was dialyzed against 1:20 borate-saline buffer in saline pH 8.4 until there was no ultraviolet absorption in the dialysate. The solution was then dialyzed against borate-saline buffer, which solubilized almost all of the conjugate. Conjugates can also be purified on Bio-Gel or Sephadex columns.
2. Diazo-6-demethyl-6-deoxytetracyclines (42)

28 microliters of n-butyl nitrite were added to 25mg of 7 or 9-amino-6-demethyl-6-deoxytetracycline hydrochloride in 0.5ml of 0.1M HCl in methanol at 0°. The solution was allowed to stand for 30 minutes; then 10ml of ether was added, the mixture centrifuged and the liquid discarded. 5ml of cold saline was added, and the remaining ether was removed with a capillary pipet. The resulting solution was used directly for protein coupling. The ultraviolet absorption spectra of both diazo compounds matched those reported in the literature.

3. Protein Conjugation

The diazotetracycline solution from 2 was added to 225mg of RSA in 25ml of 0.05M sodium phosphate-0.1M saline, pH 7, at 4°. The pH was then adjusted to 7.8 and the coupling was allowed to proceed overnight in the cold. The solution was then dialyzed against borate-saline buffer pH 8.4 diluted 1:20 in saline, until there was no absorbance in the dialysate at 315nm.

4. Diazophenols

109mg of meta-aminophenol in 10ml of water and 0.4ml of 6N HCl was cooled to 2°. 1.7ml of cold 4% sodium nitrite was added and the solution was stirred for 15 minutes. The excess nitrous acid was destroyed with sulfamic acid until
the starch-iodide test was negative. This solution was then added to a cold solution of 300mg BSA in 25ml of borate-saline pH 8.4. After 18 hours in the cold, the solution was extensively dialyzed. The same procedure was used with various haptens and carriers.

5. Alumina Adjuvant (43)

4ml of water was added to 1.6ml of 10% AlKSO₄. The mixture was brought to pH 7.2 by adding about 1ml of 1N NaOH and then diluted to 25ml. It was then centrifuged for 5 minutes and the supernatant was discarded. The alumina was suspended in 4ml of saline, and 0.2ml of 5mg/ml antigen in 0.1M TrisHCl pH7.9 was added. The solution was diluted to 9.3g with saline with the pH adjusted to 6.4 with 1N HCl. After standing 30 minutes 1.5ml of the suspension was injected intramuscularly into each rabbit.

C. Immunochemical Methods

1. Ring Test

Interfacial tests were used for qualitative detection of precipitating antibody. 3-5mm of each component was layered in a 6x50mm test tube so that an interface formed. If both of the components were dilute solutions, sucrose was added to one in order to make the interface more stable. Tubes were coated on the outside with mineral oil to reduce light scattering by scratches on the glass, so
that reactions could be more clearly observed. All observations were made in a shadow box.

2. Immunodiffusion

1g of Noble agar (Difco) or 0.9g of Ionagar was dissolved at 95° in 95ml of 0.9% saline and 5ml of borate-saline buffer pH 8.4. The hot medium was pipetted into 35x10mm plastic Petri dishes and was allowed to solidify. Wells were cut with LKB punches, and the plugs removed using a disposable pipet connected to vacuum. 2-3 micro-liter samples of both antigen and antibody were used. Diffusion took 24-48 hours at room temperature or at 37°. The plates were then washed for 36-48 hours in saline and air dried under filter paper. The dried plates were stained with 0.1% amido black in 5% sulfosalicylic acid and 5% trichloroacetic acid. The backgrounds were cleared with 5% acetic acid.

3. Immunelectrophoresis

Immunelectrophoresis was performed using the Millipore Immuno-PhoroSlide system. Strips of either pattern were pre-buffered by standing on edge in a slide box containing 3mm of half strength Millipore barbital buffer (original ionic strength 0.075, pH 8.6) until the liquid reached the top of the strip. The strip was then blotted and placed in the Millipore chamber containing 5.5ml of half strength
buffer in each buffer compartment. 0.8 microliter of sample was deposited in each well, and the chamber was covered. It was then connected to a modified Spinco power supply and operated at 100 volts for 18 minutes. The slide was then removed and fastened to a glass slide with double sided tape. 25-40 microliters of Hyland goat anti-rabbit or anti-human serum was placed along each trough, and the slide was placed in a water saturated diffusion chamber for 48 hours. The PhoroSlide was then removed from the glass slide and the top plastic layer was peeled off. The cellulose acetate strip was then washed in normal saline for 2-12 hours at 40°. The slide was then stained in 0.05% nigrosin in 5% sulfosalicylic acid, 5% trichloroacetic acid. The background was cleared in 5% acetic acid and the slide washed in water before drying in air.

Regular electrophoresis was performed by a similar procedure using Millipore PhoroSlides. Full strength Millipore buffer was used and samples were applied with Millipore applicators. The diffusion and washing steps were not necessary and the stain already contains a protein fixative. Either amido black, Ponceau S or nigrosin can be used for staining electrophoresis slides.

4. Precipitation Tests

Equal amounts of antigen and antibody solution (0.25 or 0.5 ml each) were mixed in 13x100 mm test tubes. Serial
dilutions were made in borate-saline buffer pH 8.4 and were either a twofold series or a 1,2,3,4,6,8,10 series. Each dilution was prepared in duplicate. The tubes were incubated 1-2 hours at 37° followed by 48 hours at 4°. The precipitates were resuspended with a vortex mixer at 24 hours. After the incubation was completed, the tubes were centrifuged at 3000rpm for 35 minutes at 4° and the supernatants were decanted. The equivalence zone was located by ring testing the supernatants. Each precipitate was washed twice with 5ml of borate-saline by centrifuging at 3000rpm for 35 minutes at 4°. After decanting, the tubes were inverted to drain. Protein was assayed by either the Nessler reaction or ultraviolet absorption. Other methods of protein determination were usually not applicable to the systems being studied. Precipitation curves were plotted, and the amount of antibody was calculated from the curve by taking the highest value after subtracting the antigen added. This value should occur in either the equivalence or antibody excess zones. Antibody solutions were decomplemented either by adsorption with a preformed heterologous precipitate or by the use of serum globulin fraction or specifically purified antibody.

5. Nessler Reaction

Protein nitrogen was determined by the Nessler reaction (44). Standards were prepared that contained ammonium
sulfate in the range of 10-200 micrograms of nitrogen. 0.2 ml of concentrated sulfuric acid was added to each tube: sample, standard and blank. The tubes were placed in a metal rack and heated over a low flame from a Fisher burner for 15-20 minutes until all the water was removed. The flame was turned up to digest the samples. The rack was periodically rotated to heat the tubes evenly, and the sides of the tubes were flamed with another burner to prevent condensation. The tubes were digested for 5-10 minutes after the sulfuric acid refluxed. The total heating time was about 20-25 minutes. The tubes were cooled for 1-2 minutes and 2 drops of 30% hydrogen peroxide were carefully added to each tube. The tubes were then heated over a low flame; hydrogen peroxide was added again to all tubes which were not colorless. They were heated for 10-15 minutes until the acid refluxed. The tubes were removed from the flame and cooled to room temperature. 7ml of water was added to each tube, and then the contents of each tube was poured into a separate 19x150mm tube. Each digestion tube was then rinsed with another 7ml of water, which was also transferred to the large tube. A button bottom stirring rod was placed in each tube and 6ml of Koch and McMeekin formula Nessler reagent (45), freshly filtered through a sintered glass funnel, was added, and the mixtures stirred. After standing for 30 minutes, the OD readings were made at 440nm.
6. Protein Determination by Ultraviolet Absorption

Free protein or protein in precipitates was determined by solution in 0.1N NaOH and measuring absorbance at 280nm. Either 3 or 5ml of NaOH was used for precipitation tests. The method was standardized for each protein by Nessler determination. The values are given in table 8.

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<th>Protein</th>
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<tbody>
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</tr>
<tr>
<td>Rabbit antibody (IgG)</td>
<td>1.50-1.61</td>
</tr>
</tbody>
</table>

7. Separation of the Serum Globulin Fraction

0.5 volume of saturated reagent grade ammonium sulfate was dropped into 1 volume of serum with stirring at room temperature. After stirring for 30 minutes, the suspension was centrifuged for 10 minutes at 3000rpm and reconstituted to the original volume of serum with saline. The precipitation was repeated twice more and the precipitated globulins were dissolved in \( \frac{1}{2} \) to \( \frac{3}{4} \) volume of saline. The solution was then dialyzed against 6-8 changes of normal saline, buffered at pH 7 to 8.4 and was then centrifuged to remove the small amount of insoluble material. These preparations were albumin free by immunoelectrophoresis.
8. Ultrafiltration

Purified protein solutions were concentrated by ultrafiltration. The membrane that gave the best results was an Amicon Corporation (Cambridge, Mass.) UM20E, which passed molecules smaller than molecular weight 20,000. 50 psi of prepurified nitrogen was used to force the liquid through the membrane in the 65ml cell. Other membranes were much slower or did not retain all of the antibody.

9. Animals and the Preparation of Sera

Rabbits were obtained from Adams Caviary, Horton's Laboratory Animals, and California Caviary; all were New Zealand albino males. No animals were used that weighed less than 5lbs. Bleeding was from the ear vein using a vacuum bleeder, except when the animals were sacrificed by cardiac exsanguination. The blood was allowed to clot at room temperature for 3 hours and then was centrifuged twice for 15 minutes at 1700rpm. The sera were clarified by ultracentrifugation at 30,000rpm for 45 minutes, which made the lipids float so that they could be easily removed. Any solids present were removed by pressure filtration through an 0.45 micron membrane filter. When possible, sera were stored frozen, otherwise they were kept at 4°C. Preservatives were not used.

Guinea pigs were all from the Caltech colony. They are an albino, American type called Campbell-Trapani strain.
The animals have been randomly bred in a completely closed colony. The strain is distinguished by large ears and very constant immune reactivity; passive cutaneous anaphylaxis reactivity differs among various individuals by less than a factor of 5 (46). The animals were bled by heart puncture, and the serum was processed the same way as rabbit serum.

Rats were obtained from various sources and were bled by heart puncture.

Chickens were bantams of both sexes, obtained locally. They were injected in the wing vein or intramuscularly in the leg. Bleeding was usually done by cardiac puncture. Sera were processed in the usual manner.

All animals were fed proper laboratory diets. Guinea pigs received either fresh kale or carrots and vitamin C in their water in addition to pellets. At various times guinea pigs and rabbits received either sulfa or peptide antibiotics in their feed to control infections. At one time there was a pneumonia outbreak in the guinea pig colony. Snuffles (Pasteurella leptiseptica) was usually present in the rabbit colony and caused the loss of many experimental animals. No animals received either tetracycline or penicillin antibiotics as either therapeutic or control measures. The only exposure to tetracyclines was the immunization.
10. Passive Cutaneous Anaphylaxis (26)

250-500g guinea pigs were sensitized by injecting 0.1 ml of varying dilutions of antisera intradermally using a 26 or 27 gauge intradermal bevel needle into an area of their backs shaved with a number 000 electric clipper. Sensitization times were 3-4 hours for rabbit antibody and either 3-4 or 18 hours for guinea pig antibody. Passive Arthus reactions were challenged immediately after intradermal injection with a 30 gauge needle. The guinea pigs were challenged by intracardiac injection of 0.5ml of antigen in saline mixed with 0.5ml of 1% Evans blue in saline. Reactions were observed and measured at 15, 30 and 120 minutes after challenge. The guinea pigs were then sacrificed and skinned. The skins were stretched and covered with Boraxo to dry. The diameters of the reactions were then measured on the reverse side of the skin. Saline controls were performed on each animal, and normal serum and carrier controls were always done to verify positive anti-hapten reactions.

11. Passive Hemagglutination (23,47,48)

Sheep blood was obtained from Davis Laboratories, Davis, California, and was always from the same animal. Red cells were always centrifuged at 1000-1500rpm for 7-10 minutes. The red cells were washed 3 times with 0.9% saline and suspended to a concentration of 2.5% (v/v) in 1:1
saline-0.7M sodium phosphate buffer pH 7.2. An equal volume of freshly diluted 1:20,000 tannic acid in phosphate buffered saline pH 7.2 was added, and the cells were incubated at 37° for 10 minutes. They were then washed with phosphate buffered saline pH 7.2 and with saline. The cells were suspended in phosphate buffered saline pH 6.4 to a concentration of 2.5% and a solution of tetracycline in phosphate buffered saline was added to give a final concentration of 2.5mg of drug per milliliter of packed cells. After incubation at 37° for 30 minutes, the cells were washed with saline and then twice with freshly prepared 0.2% Difco Bacto gelatin in 0.11M phosphate pH 7.3. The cells were resuspended in gelatin phosphate to a final concentration of 0.5%. Complement was inactivated by heating the sera at 60° for 30 minutes, and the sera were adsorbed with 50% tanned red cells to remove non-specific agglutinating substances. The adsorbing cells were prepared by the above procedure leaving out the tetracycline. Dilutions were made using gelatin phosphate as the diluent. The tests were carried out with 0.025ml volumes using a Cooke Engineering Microtiter set. The titer was expressed as the reciprocal of the highest dilution which agglutinated the cells, when read at 12-18 hours. A tube method that gave similar results was used earlier, but it was experimentally more difficult and required much more material.
12. **In Vitro Anaphylaxis** (49)

**Tyrode Solution**

- 8.000g Sodium Chloride, Biological Grade
- 0.195g Potassium Chloride
- 0.146g Calcium Chloride, Anhydrous
- 0.213g Magnesium Chloride·6H₂O
- 1.000g Sodium Bicarbonate
- 1.000g Glucose, recrystallized from ethanol-H₂O

The above chemicals were dissolved in order in 1 liter of glass distilled water. The solution was then saturated with O₂, not O₂-CO₂ as is given in the reference. The final pH was adjusted to 7.8 at 37°. Lower pH inhibits the reaction. The solution was prepared fresh each day.

**Isotonic Buffered Glucose**

55.1g of recrystallized glucose and 43mg of sodium bicarbonate were dissolved in 1 liter of glass distilled water. The final pH was 7.3. Isotonic buffered glucose was sometimes used for passive sensitization.

**Guinea Pig Gut**

Guinea pigs were sacrificed by exsanguination or by a blow to the base of the skull. The abdomen was opened and 10ml of warm Tyrode solution was poured into the cavity. The small intestine was cut 7-10cm from the cecum (junction with the large intestine). The gut was extended and it was pulled free from the mesentery. 20-30cm was removed and
the remaining mesentery and fat was trimmed off. The intestine was washed by allowing Tyrode solution to flow through it from a 10ml volumetric pipet by gravity. The intestinal contents were completely removed by 2 rinses. The gut was then placed into fresh Tyrode solution and refrigerated for 2-3 hours; this decreased spontaneous contractions. 1.5-2cm pieces were used for experiments. A length of silk thread with a pretied loop was tied through each end of the gut using a curved suture needle. The ends of the gut should not be tied off, if they are, gas collects in the lumen. Each piece of gut was always equilibrated for at least 20 minutes before being tested. Uterus horns were tried in several experiments, but the background contractions were much stronger than those from gut. Gut was used on the same day it was obtained. Samples stored at 4°C overnight were significantly less sensitive and required much longer equilibration times, 2 to 3 times those of fresh gut.

**Apparatus**

Contractions were recorded on either a Grass Instrument (Quincy, Mass.) model 5A polygraph using an FT.03c strain gauge (50g/mm) or a Harvard Apparatus Co. (Millis, Mass.) model 356 transducer and model 350 recording module. The Grass Instruments apparatus measures the contractions isometrically, since the distance the strain gauge arm moves is less than 0.02mm. The Harvard apparatus measures
the contraction isotonically; the load was set at 2g. The kymograph speed for the Harvard apparatus was 2.06 cm/minute.

The vessel is diagrammed in figure 13. The inside of

![Schultz-Dale Tissue Bath Vessel](image)

Figure 13
Schultz-Dale Tissue Bath Vessel

the vessel is made from a 13 mm o.d., 10 mm i.d. Pyrex fine porosity (F) sintered glass disc. The height above the disc is 115 mm giving a working volume of 6-7 ml. The bottom is tapered to attach to 5 mm i.d. Tygon tubing, which connects to the oxygen supply. Oxygen is regulated by a two-stage regulator and accessory fine taper needle valve. The entire vessel is water jacketed and is kept at 37 ± 0.05 °C by a circulating bath. The inside 3 mm rod is held in position by a rubber band attached to the water outlet. The vessel is filled by an all glass automatic syringe or pipet and emptied with a 16 gauge needle connected to an
aspirator line. Test substances are added with a microliter syringe and are stirred by the oxygen flow.

All glassware and surgical instruments used in Schultz-Dale experiments were rinsed at least 3 times in glass distilled water and air dried before use. Most apparatus was used only for Schultz-Dale to prevent contamination by tissue toxic substances. Guinea pig gut is very sensitive to many impurities, much more so than many cell cultures.

**Procedures**

The gut was attached to the rod and to the transducer with silk thread and then rinsed twice with Tyrode solution. It was then equilibrated for 20-30 minutes at 37° and tested for viability with histamine. 0.01 microgram of histamine should give a pen deflection of at least 5mm with either apparatus. The normal contractions should be less than 50% of the histamine caused deflection. The piece was challenged 3 consecutive times with histamine and then washed and re-equilibrated for 10-15 minutes. Passive sensitizations were done in either Tyrode solution or isotonic buffered glucose for varying times from 10-60 minutes. 30 minutes was the most common sensitization time. The gut was tested with histamine and washed 3 times with Tyrode solution and equilibrated for 20 minutes. The gut was then challenged with antigen; after 4 washings and equilibration, it was rechallenged to test for desensitization and then retested with histamine. Results
were compared as histamine equivalents. The variation among different pieces of gut was usually less than a factor of 2 on this basis.

D. Immunoadsorbents

1. Para-Aminobenzyl cellulose (50)

Machery Nagel 300 or Whatman CF11 cellulose powder was washed in 3% NaOH, 2N HCl, water and acetone. It was then dried under vacuum. 2g of washed cellulose powder, 6g of p-nitrobenzyl chloride and 6g of NaOH in 30ml of water were mixed in a three neck flask equipped with stirrer, condenser and thermometer. The reaction was stirred at 93° for 4 hours using a water bath to regulate the temperature. The mixture was then poured into 400ml of ice water, filtered and washed with water, alcohol and then acetone. The solid was then extracted with acetone for 4 days in a Soxhlet extractor. The yield was 1.8g.

The dried nitrobenzyl cellulose from the previous step was suspended in 20ml of ethanol in a three neck flask equipped as above. The alcohol was refluxed, and 1.8g of sodium dithionite dissolved in the minimum amount of water was slowly added with vigorous stirring. The reaction was refluxed for 30 minutes after the addition was completed, and the light yellow product was filtered, washed with water and vacuum dried. The yield was 1.7g.

The p-aminobenzyl cellulose was coupled by
diazotization. 1g of p-aminobenzyl cellulose was suspended in 2ml of 2N HCl and 4ml of water and cooled in ice. 0.5-1 ml of 0.5M NaN02 was added until there was an excess of nitrite for 15 minutes when tested with starch-iodide paper. After stirring an additional 15 minutes, the cellulose was filtered and washed with 200ml of 0.3% sulfamic acid. The diazotized cellulose was added to 10ml of 2mg/ml BSA in borate-saline pH 8.4 and stirred at 4° for 5 days. The adsorbent was filtered and washed with 300ml of borate-saline. Free diazonium groups were blocked by stirring the adsorbent in 50ml of saturated β-naphthol in borate buffer pH 8.7 for 30 minutes at 4°. It was then filtered and thoroughly washed with buffer and water.

Adsorption and elution procedures were similar to those described below. The best procedure for this system was glycine-HCl buffer pH 2.0.

2. Half benzidine amide of carboxymethylcellulose (51)

1g of Machery Nagel CMC 300 was stirred in 200ml of 2N HCl for an hour, filtered, stirred with 400ml of water, filtered and washed again with water. The CMC was slowly added to 0.2g of benzidine in 2ml of water in a small flask until 0.8g of the CMC had been added together with enough water to make the mixture semi-solid. 0.4g of dicyclohexyl-carbodiimide in 1ml of tetrahydrofuran and 1ml of water were added along with the rest of the CMC. The creamy
mixture was stirred for 2 days. The mixture should not be dry after this. 20ml of water was added and the reaction mixture was poured into 400ml of dilute NaHCO₃. The final pH should be 8-9. After stirring overnight the mixture was filtered and water washed. The derivative was suspended in 200ml of acetone for an hour and then filtered; this was repeated twice. The dry CMC was washed 3 times with dilute NaHCO₃, 3 times with dilute HCl and 3 times with water. It was then acetone washed and vacuum dried.

Three methods of coupling were used. Diazotization was performed as described for p-aminobenzyl cellulose. Acid chlorides were coupled by Schotten-Baumann reaction. The benzidine CMC was suspended in water and a solution of acid chloride in ether was dropped in, while the pH was kept at 7-9 by the addition of 1N NaOH. The derivative was washed thoroughly with water and acetone. This reaction was also used with aminoethyl cellulose (Bio-Rad). The adsorbent could also be prepared and coupled to haptens by the use of Woodward's reagent (52), N-ethyl-5-phenylisoxazolium-3'-sulfonate in acetonitrile.

3. Cyanogen bromide treated agarose

1g of cyanogen bromide in 40ml of water at pH 11 was added to 1g of 4% agarose (Sepharose 4B, about 56ml). The pH was immediately adjusted to 11 and held at 11 for 6 minutes with 2N NaOH. The solid was then immediately
washed with cold water and then 0.1M NaHCO₃. This product was immediately suspended in 30ml of 0.1M NaHCO₃ and placed in a stoppered tube with 500mg of KLH in 10ml of saline. The tube was slowly rotated end over end for 24 hours at 50°. The coupled adsorbent was then poured into a column and washed at 20ml/hour for 48 hours with borate-saline pH 8.4, 24 hours with 0.1M acetate pH 4.1 in 0.1M NaCl, and 24 hours with 0.1M acetate pH 4.1. The gel was then washed with saline and used in 0.1M Tris-HCl pH 7.4. Tetracycline did not couple under these conditions. Antibody was eluted using a column method with 0.1M acetic acid or free hapten.

4. Bromoacetylcellulose (54,55)

Whatman CF11 cellulose powder was washed with large volumes of acetone and dioxane and vacuum dried over P₂O₅. 57g of bromoacetic acid was dissolved in 18ml of dioxane freshly distilled from sodium. 6g of cellulose powder was added, and the mixture was stirred for 22 hours in a stoppered flask. 43ml (100g) of bromoacetyl bromide (Aldrich, others are usually of an inferior quality) was added and the flask was connected to a NaOH trap and stirred for 9½ hours. The mixture was poured into 4 liters of iced distilled water and stirred for 15 minutes. It was then centrifuged, and the solid washed several times with water. The BAC was then stirred overnight in 1200ml of 0.1M NaHCO₃, filtered and washed 4 times with 0.1M
bicarbonate by centrifuging. It was then washed 5 times with water and suspended in 100ml of water. 2ml was vacuum dried. The yield was 3.7g.

1g of BAC in 46ml was added to 400mg of RSA in 35ml of 0.1M phosphate-0.05M citrate pH 3.8 (for KLH, pH 4.6, for BSA, pH 3.8). A drop of toluene was added and the mixture was vigorously stirred for 30 hours at room temperature. The mixture was then centrifuged, and the BAC was suspended in 30ml of 0.1M bicarbonate pH 9 and stirred in the cold for 24 hours. The RSABAC was centrifuged and then suspended in 0.05Maminoethanol-0.1M bicarbonate pH 8.8 for 36 hours in the cold. It was then centrifuged and washed 3 times with normal saline and then stirred with 30 ml of 8M urea for 24 hours at room temperature. The adsorbent was then washed with saline until the absorbance of the wash at 280nm was less than 0.03. The adsorbent was stored in 0.15M phosphate pH 7.0 at 4°.

10ml of antiserum or total globulins was diluted with 10ml of borate-saline pH 8.4 and stirred with the adsorbent prepared from 0.5g of BAC, washed twice just before use. The suspension was stirred for 45-120 minutes at 4°. The adsorbent was then centrifuged and washed 3 times with borate-saline pH 8.4 or until the absorbance of the wash was less than 0.04 at 280nm. The antibody was eluted by stirring with 25ml of 0.1M acetic acid for 1 hour at 37° or with glycine-HCl pH 2.2. The adsorbent was extracted twice.
After centrifugation the eluate was neutralized, then
dialyzed against dilute borate-saline buffer in saline.
The antibody was then filtered through a 0.45 micron
membrane filter and concentrated by ultrafiltration.

5. Glutaraldehyde crosslinking (56)

250mg of BGG was dissolved in 5ml of 0.1M phosphate pH
7. 1ml of 2.5% glutaraldehyde was dropped in with stirring
at room temperature. The resulting gel was then allowed to
stand for 3 hours at room temperature. It was then
homogenized in 0.2M phosphate pH 7.3 in a blender and
centrifuged; this was repeated 3 times and the insoluble
polymer was then washed twice in 0.1M glycine-HCl pH 2.2
and twice again in phosphate. Antibody was purified as in
4.

6. Ethyl chloroformate crosslinking (57)

5g of BGG was dissolved in 100ml of 0.11M phosphate pH
7.3 and 4ml of ethyl chloroformate was added with stirring
while the pH was kept between 7.1 and 7.3. The solid
formed in 30 minutes and was allowed to stand for an
additional hour. It was filtered and homogenized in 2
liters of phosphate-saline, centrifuged and washed with 400 ml of 0.3% Na₂CO₃, then twice with phosphate-saline, 4
times with glycine-HCl pH 1.8, and 3 times with phosphate-
saline. BSA was polymerized in 0.1M acetate pH 4.5-5.0
and KLH at pH 6.1-6.4. Antibody was purified using the method given for bromoacetylcellulose.

7. Polyacrylamide gel (58)

7.5g of acrylamide and 2.5g of N,N'-methylene-bis-acrylamide were dissolved in 100ml of 0.025M Tris-HCl pH 7.4. 700mg of BGG in 10ml of water and 1ml of a suspension containing 0.3mg of riboflavin were added. Nitrogen was slowly bubbled through the solution for 15 minutes and the flask was stoppered and then irradiated with a 100 watt incandescent frosted bulb. The gel polymerized in about 20 minutes and irradiation was continued for another hour. The gel was cut up and dispersed into 0.025M Tris-HCl in saline by forcing it through a syringe and then through a 15 gauge needle. The gel was washed with the following in order: 4 times with Tris-saline, once with saline, twice with 0.1M glycine-HCl pH 2.2, once with saline and twice with Tris-saline. It was stored in Tris-HCl 0.01M pH 7.4. This method works well with azo and amide coupled proteins.

8. Sephadex

Methods 1, 3 and 4 were tried with Sephadex instead of cellulose and method 2 with CMSephadex, but all of the reactions destroyed the crosslinked dextran.
9. Elution

Several elution methods were used. They included 0.1N HCl, 0.1M acetic acid, glycine-HCl buffer pH 2.2, 5M iodide and 0.1M hapten. 0.1M glycine-HCl pH 2.2 at 37° was found to be the best method for most systems. Free hapten is the best eluant for antibody against dinitrophenyl determinants.
REFERENCES


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Introduction

The existence of hypersensitivity type reactions to aspirin has been known since 1902 (1). However, further studies have accomplished little in elucidating whether or not the adverse clinical reactions to aspirin are due to antigen-antibody reactions or to some other mechanism. No one has yet found either a precipitating or reaginic type antibody in the serum of individuals who react adversely to aspirin.

Weiner, Rosenblatt and Howes (2) were able to detect a factor in sera from aspirin intolerant individuals, which was able to agglutinate red cells treated with aspiryl proteins; this factor was destroyed by heating to 56°. Daniel, Perloff, Major and Baum (3) investigated patients treated with para-aminosalicylic acid and found an equal percentage of positive hemagglutination reactions among both individuals who demonstrated clinical sensitivity and normal individuals. Yurchak, Wicher and Arbesman (4) were unable to detect hemagglutinating antibody in sera from aspirin sensitive individuals using aspiryl proteins.
Many other attempts to detect antibodies against aspirin in sensitive individuals have all given negative results. The methods used have included direct and passive skin tests with aspirin (5,6,7) and with aspirol proteins (4,7). Results from lymphocyte stimulation tests, immunofluorescent studies of nasal polyps (4) and leukocyte histamine release (8) were also negative.

Two immunological mechanisms have been proposed for aspirin sensitivity. The first is reaction against the aspirol or salicylyl moiety (2,7,9,10), and the second is a reaction to serum proteins acetylated by aspirin (11). One approach to the resolution of this problem is to select and study clearly defined model systems and then to apply this knowledge to the study of the clinical problem. One such system is the aspirol hapten in the rabbit. The specificity and combining properties of rabbit antibodies against aspirol proteins are reported and discussed in this section.
Materials and Methods

5-Methylaspirin (12). 14ml of acetic anhydride and 5 drops of concentrated sulfuric acid were added to 9 grams of 5-methylsalicylic acid (Aldrich). The mixture was kept at 50-60° for 15 minutes, cooled and 150ml of water was added. The 5-methylaspirin was filtered off, vacuum dried over KOH and then crystallized from benzene and vacuum dried. The yield was 7.5g; the melting point was 146-148°.

Aspiryl Chloride (13). 36g of aspirin, 100ml of benzene and 40ml of thionyl chloride were refluxed for 5 hours. The solvent was stripped off under vacuum and the residue was vacuum distilled. The boiling point was 93-94° at 1mm. 5-Methylaspiryl chloride was prepared similarly; its boiling point was 107-108° at 1mm.

Protein Carriers. Bovine serum albumin (BSA) and bovine gamma globulin (BGG) were purchased from Armour Pharmaceuticals, Kankakee, Illinois. Keyhole limpet hemocyanin (KLH) was prepared by a modification of the method of Campbell, Garvey, Cremer and Sussdorf (14) and was resedimented by centrifugation at 30,000rpm and redissolved before use.
**Antigens.** Conjugates were prepared by Schotten-Baumann reaction of acid chlorides with protein carriers. The procedure of Wichor, Schwartz, Arbesman and Milgrom (10) was followed with only minor modifications. The conjugates were purified by precipitation with acetic acid at pH 4 and were then redissolved in sodium carbonate; this was repeated 4 or 5 times (15). The conjugates were dialyzed against 0.01N Na₂CO₃ 4 times, and finally 6 times against borate-saline pH 8.4. KLH proteins are irreversibly denatured by acid precipitation and become insoluble. Protein concentrations were determined by the Nessler reaction (14) and the hapten was estimated by UV absorption at 350nm (the numbers are transposed to 305 in reference 10). AspirylBSA had about 16 hapten residues per protein molecule, and aspirylBGG had about 31. The absorption of KLH interferes with the determination of aspiryl groups by UV.

**Preparation of Antisera.** New Zealand albino male rabbits weighing at least 5 pounds were injected intradermally in two sites with 1.25-2.5mg of antigen emulsified with an equal volume of Freund's complete adjuvant (Difco). This procedure was repeated weekly for a total of 8 weeks and the rabbits were bled 10 days after the last injection. All sera were stored frozen. All of the precipitation and most of the other experiments were performed with total globulin fractions, prepared by precipitation 3 times with
33% saturated ammonium sulfate (14).

**Preparation of Haptens.** Acid chlorides were coupled to ε-aminocaproic acid (ACA) and α-carbobenzoxy-L-lysine (CBZ-Lys) by Schotten-Baumann reaction. Yields of purified materials were generally about 30-50% after recrystallization from benzene or benzene-pentane mixtures. All chemicals were either reagent grade or were the highest purity commercially available and were used without further purification. The identity of the compounds synthesized was verified by NMR spectroscopy and the purity was checked by melting point. The melting points are listed in table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Melting Points of Haptens</th>
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<tbody>
<tr>
<td>ε-Aspirylaminocaproic acid</td>
</tr>
<tr>
<td>ε-5-Methylaspirylaminocaproic acid</td>
</tr>
<tr>
<td>ε-Benzoylaminocaproic acid</td>
</tr>
<tr>
<td>ε-p-Chlorobenzoylaminocaproic acid</td>
</tr>
<tr>
<td>ε-Anisoylaminocaproic acid</td>
</tr>
<tr>
<td>ε-m-Bromobenzoylaminocaproic acid</td>
</tr>
<tr>
<td>5-Methylaspirin</td>
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</table>

Aspirin was Merck USP grade, the same grade used clinically. Aspirin solutions were prepared immediately before use, since aspirin decomposes in solution. Most
other solutions were also prepared immediately before use.

**Purification of Anti-Aspiryl Antibody.** An immunoabsorbent consisting of a protein antigen trapped in a polyacrylamide gel (16) was used. A mixture of 15ml of 12.5% acrylamide and 4.2% N,N'-methylene-bis-acrylamide, 20ml of 0.8% AspBSA and 1ml of 0.3mg/ml riboflavin under a nitrogen atmosphere was irradiated with a 100 watt incandescent bulb for 1 hour. The gel was dispersed twice through a 15 gauge needle and was washed 4 times with 0.1M Tris-HCl buffer pH 7.4, twice with 0.2M glycine-HCl pH 2.2 and finally twice with Tris pH 7.4 after a saline wash. 23ml of anti-aspirylBGG total globulins containing 25mg of anti-aspiryl was added to the gel and the suspension was stirred for 30 minutes at room temperature. The mixture was centrifuged for 5 minutes at 2000rpm. The supernatant gave a negative ring test with aspirylKLH. The solid was washed once with Tris-HCl pH 7.4 and 4 times with saline. The absorbance of the last wash was less than 0.03 at 280nm. The antibody was then eluted with 25ml of 0.02M glycine-HCl pH 2.2 for 30 minutes and again with another portion for 15 minutes. The eluant was filtered through a 0.45 micron membrane filter after neutralization and then dialyzed 3 times against 1 liter volumes of borate-saline pH 8.4 and filtered again. The antibody was concentrated by ultrafiltration with a UM20E membrane. Over 75% of the protein was precipitable antibody.
Aspiryl-carboxyl-\textsuperscript{C\textsubscript{14}}-ACA. Aspirin-carboxyl-C\textsuperscript{14} was purchased from Amersham-Searle. 50 microcuries (0.55mg) of aspirin-C\textsuperscript{14}, 1.5ml of benzene and 5 drops of thionyl chloride were refluxed for 5 hours. The solvent was then removed by vacuum distillation. The residue was dissolved in ether and added to 100mg of \(\epsilon\)-aminocaproic acid in 2.5ml of methanol. 30mg of aspiryl chloride was added, and the solution was stirred for 70 minutes. It was then diluted with water, acidified with acetic acid and extracted 3 times with chloroform. The CHCl\textsubscript{3} was extracted once with 0.5N Na\textsubscript{2}CO\textsubscript{3} and twice with 0.1N Na\textsubscript{2}CO\textsubscript{3}. The carbonate solution was acidified with acetic acid and extracted 4 times with CHCl\textsubscript{3}. The CHCl\textsubscript{3} was dried with sodium sulfate and evaporated. The residue was crystallized from benzene-pentane. 8mg of aspiryl-C\textsuperscript{14}-aminocaproic acid was dissolved in 7ml of dilute NaHCO\textsubscript{3} and diluted to 25ml with borate-saline pH 8.4. Dilutions of \(2 \times 10^{-5}\) to \(10^{-7}\) molar were prepared from this stock solution using borate-saline buffer as diluent. The specific activity was determined by scintillation counting, and the actual concentrations were determined by radioactivity.

Quantitative Precipitation and Inhibition of Precipitation Analyses. Hapten specific antibody was determined by mixing equal volumes (0.5 or 0.25ml) of serum globulin fraction with dilutions of heterologous non-cross reacting carrier
antigen. After standing 2 hours at 37°, the tubes were stored for 48 hours at 4°, centrifuged 30 minutes at 3000 rpm, and the precipitates were washed twice with borate-saline pH 8.4 and assayed by Nessler reaction on a 2/5 scale (14). Inhibition tests were performed by mixing 0.25 ml of antibody, 0.5ml of hapten dilution in borate-saline and 0.25ml of the equivalence dilution of antigen. The same pool of sera was used for all inhibition tests. Each hapten was measured in a duplicate series of 4 to 6 dilutions.

Equilibrium Dialysis. Equilibrium dialyses were carried out in a water bath at 4°. The cells were made by cutting off 18/9 Pyrex "O" ring joints to a capacity of 1.5ml. The two halves of the cell were sealed with a teflon "O" ring and Saran Wrap covered rubber stoppers and were held together by two ball joint clamps, one of which was drilled to fit on an immersible rotator driven by a flexible cable. The cell is illustrated in figure 1. The membrane was cut from number 36 Visking tubing that had been boiled twice in dilute sodium bicarbonate and 3 times in distilled water. 0.9-1.0ml of either specifically purified antibody or of total serum globulins was pipetted into one side of the cell and the same amount of hapten solution was pipetted into the other side. After the cells were rotated at 29rpm for 48 hours, 0.5ml samples were taken from each half of
the cell with volumetric pipets. Each sample was added to a scintillation vial containing 15ml of a fluor consisting of 7g of 2,5-diphenyloxazole (PPO), 0.7g of 1,4-bis-2-(4-methyl-5-phenyloxazoyl)benzene (dimethyl-POPOP), 100g of naphthalene and 40g of Cab-O-Sil (Cabot Corporation) in 1000ml of reagent dioxane (17). The samples were counted for several 10 minute cycles in a scintillation counter. The counting efficiency was 83% for C\textsuperscript{14}.

**Serological Procedures.** All sera were tested by ring tests with heterologous non-cross reacting carrier antigen, passive cutaneous anaphylaxis (PCA) (18), and immunodiffusion in 1% Noble agar (Difco) in 5% borate-saline pH 8.4 -- 95% saline.

![Equilibrium Dialysis Cell](image)

**Figure 1**

Equilibrium Dialysis Cell

a - membrane
Results

All rabbits immunized with aspiryl, 5-methaspiryl and benzoyl proteins produced specific anti-hapten antibody in medium to high titer (0.2 - 3.0 mg/ml). Animals immunized with antigens made with KLH as the carrier protein produced the most anti-hapten antibody in each case. BGG was not as effective a carrier, and BSA was significantly less effective than either of the other two. There was no cross reactivity between KLH and BSA and none between KLH and BGG. The cross reactivity between BSA and BGG was less than 0.1% as measured by PCA reaction in guinea pigs. Because of their higher titers and lack of carrier cross reactivity, sera produced by rabbits immunized with KLH conjugates were used in most experiments.

Neither free aspirin nor aspiryl-ε-aminocaproate (aspiryl-ACA) gave a positive PCA reaction or precipitation reaction as did the protein conjugates. Both haptens could inhibit precipitation and PCA, but aspirin was only effective at relatively high concentrations, i.e. greater than 0.01M in the inhibition system described.

The data from quantitative inhibition precipitation reactions were analyzed by the method of Pauling et al. (19). This method assumes an error function for the distribution of the free energies of combination, and then the data are fitted to calculated curves by the use of an empirically determined heterogeneity index,6. The method is given in
detail by Pressman and Grossberg (20). The relative combining constant, $K_{rel}$, is the ratio of the combining constants of test to reference haptens or the ratio of the fiducial concentration of the reference and test haptens. The relative free energies of combination, $\Delta F_{rel}$, are calculated at 37°. The results are given in table 2 for the inhibition of the reaction between anti-aspirylKLH and aspirylBSA at pH 8.4.

Average binding constants, $K_H'$, were determined by equilibrium dialysis. The binding constants, $K_H$, were assumed to be a Sips distribution about the average, $K_H'$. The method of treating the data was that of Nisonoff and Pressman (21). The reciprocal of the concentration of antibody sites, $1/A_0$, was determined by taking the intercept of a plot of the reciprocal of the bound hapten concentration, $1/b$, against the reciprocal of the free hapten concentration, $1/c$. In each experiment the extrapolated value of $A_0$ agreed with the value determined by quantitative precipitation tests within the experimental errors.

The average binding constant, $K_H'$, was obtained from a plot of $\log \left( \frac{A_0}{b} - 1 \right)$ against $\log \left( \frac{1}{c} \right)$. $K_H'$ is the value of $1/c$ when $(A_0/b) - 1$ equals 1, the point at which half of the antibody sites are occupied. The data for a sample experiment are in table 3 and these data are plotted in
### Table 2

Inhibition of Precipitation of Anti-AspirylKLH with AspirylBSA

<table>
<thead>
<tr>
<th>Haptena</th>
<th>$\varepsilon$</th>
<th>$K_{rel}$</th>
<th>$\Delta F_{rel}$b</th>
<th>log $C_f$c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspiryl-ACA</td>
<td>3.5</td>
<td>1.00</td>
<td>0</td>
<td>-4.98</td>
</tr>
<tr>
<td>5-Methylaspiryl-ACA</td>
<td>3</td>
<td>.37</td>
<td>610</td>
<td>-4.52</td>
</tr>
<tr>
<td>Benzoyl-ACA</td>
<td>4</td>
<td>.0058</td>
<td>3170</td>
<td>-2.73</td>
</tr>
<tr>
<td>$\varepsilon$-Aspiryl-$\alpha$-CBZ-L-lysine</td>
<td>2.5</td>
<td>.73</td>
<td>200</td>
<td>-4.83</td>
</tr>
<tr>
<td>$\varepsilon$-CBZ-L-lysine</td>
<td>0</td>
<td>.044</td>
<td>1920</td>
<td>-3.60</td>
</tr>
<tr>
<td>DNP-L-lysined</td>
<td>0</td>
<td>&lt;.005</td>
<td>&gt;3250</td>
<td>&gt;-2.65</td>
</tr>
<tr>
<td>p-Chlorobenzoyl-ACA</td>
<td>5</td>
<td>.0025</td>
<td>3670</td>
<td>-2.36</td>
</tr>
<tr>
<td>Anisoyl-ACA</td>
<td>4</td>
<td>.0013</td>
<td>4080</td>
<td>-2.06</td>
</tr>
<tr>
<td>m-Bromobenzoyl-ACA</td>
<td>5</td>
<td>.0014</td>
<td>4030</td>
<td>-2.10</td>
</tr>
<tr>
<td>Aspirin</td>
<td>2</td>
<td>.00010</td>
<td>5650</td>
<td>-0.96</td>
</tr>
<tr>
<td>Benzoate</td>
<td>1</td>
<td>.000055</td>
<td>6020</td>
<td>-0.70</td>
</tr>
<tr>
<td>Salicylate</td>
<td>1</td>
<td>.00018</td>
<td>5280</td>
<td>-1.22</td>
</tr>
<tr>
<td>4-Aminosalicylate</td>
<td>0</td>
<td>.00017</td>
<td>5260</td>
<td>-1.18</td>
</tr>
<tr>
<td>5-Methylsalicylate</td>
<td>1</td>
<td>.00020</td>
<td>5230</td>
<td>-1.27</td>
</tr>
<tr>
<td>5-Methylaspirin</td>
<td>1</td>
<td>.00018</td>
<td>5280</td>
<td>-1.21</td>
</tr>
<tr>
<td>$\varepsilon$-CBZ-L-lysine</td>
<td>-</td>
<td>&lt;.0001</td>
<td>&gt;6000</td>
<td>&gt;-1.0</td>
</tr>
<tr>
<td>ACA</td>
<td>-</td>
<td>&lt;.0001</td>
<td>&gt;6000</td>
<td>&gt;-1.0</td>
</tr>
</tbody>
</table>

---

a See figure 2 for structures.
b at 37° in calories/mole
c log fiducial concentration
d $\varepsilon$-2,4-dinitrophenyl-L-lysine
Figure 2
Structures of Haptens
Table 3

Equilibrium Dialysis of Anti-aspirylKLH Globulins against Aspiryl-\(^{14}\)amino-caproate. \(A_o=1.66 \times 10^{-5}\) molar, temperature 4°.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Counts bound side</th>
<th>Counts free side</th>
<th>(1/b \times 10^{-5})</th>
<th>(1/c \times 10^{-5})</th>
<th>((A_o/b)-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23473</td>
<td>6489</td>
<td>0.89</td>
<td>3.13</td>
<td>0.13</td>
</tr>
<tr>
<td>2</td>
<td>18663</td>
<td>4253</td>
<td>1.02</td>
<td>5.3</td>
<td>0.70</td>
</tr>
<tr>
<td>3</td>
<td>14087</td>
<td>3352</td>
<td>1.40</td>
<td>6.7</td>
<td>1.32</td>
</tr>
<tr>
<td>4</td>
<td>13928</td>
<td>2196</td>
<td>1.28</td>
<td>7.8</td>
<td>1.13</td>
</tr>
<tr>
<td>5</td>
<td>12954</td>
<td>2588</td>
<td>1.43</td>
<td>9.6</td>
<td>1.40</td>
</tr>
<tr>
<td>6</td>
<td>9190</td>
<td>2359</td>
<td>2.16</td>
<td>10.0</td>
<td>2.6</td>
</tr>
<tr>
<td>7</td>
<td>7473</td>
<td>1525</td>
<td>2.50</td>
<td>18.2</td>
<td>3.15</td>
</tr>
<tr>
<td>8</td>
<td>5424</td>
<td>1277</td>
<td>3.56</td>
<td>21.7</td>
<td>4.95</td>
</tr>
<tr>
<td>9</td>
<td>3044</td>
<td>824</td>
<td>6.67</td>
<td>38.5</td>
<td>10.0</td>
</tr>
<tr>
<td>10</td>
<td>1593</td>
<td>505</td>
<td>13.6</td>
<td>100</td>
<td>21.6</td>
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</tbody>
</table>

\[ a=1.0, \quad K_H' = 5.6 \pm 0.6 \times 10^5 \text{ liters/mole} \]
Figure 3

Equilibrium dialysis experiment. Anti-aspirylKLH globulins dialyzed against aspiryl-$C^{14}$-aminocaproate at pH 8.4 for 48 hours at $4^\circ$. The data are in table 2. $A_0=1.66 \times 10^{-5}$ molar, $a=1.0$, $K'_H=5.6\pm0.6 \times 10^5$ liters/mole.
Dialysis experiments with aspirin-C\textsuperscript{14} were unsuccessful since the binding constant was too low to measure. Threefold concentrated anti-aspirylKLH serum globulins bound less than 1% of the hapten at free hapten concentrations of $5 \times 10^{-4}$ to $10^{-7}$ molar. Both sides of the cell contained the same concentration of hapten within experimental error, as is shown in table 4.

In experiments with aspiryl-C\textsuperscript{14}-ACA good results were obtained. The average binding constants for anti-aspiryl KLH serum globulins were found to be between 4 and $8 \times 10^5$ liters/mole. In a typical experiment, reproduced in table 3 and figure 3, the average binding constant was found to be $5.6 \pm 0.6 \times 10^5$ l/m. The exponent of the Sips equation, $a$, (the slope of the log-log plot) fitted by least squares was 1.0, which corresponds to a relatively small amount of heterogeneity. An experiment with specifically purified anti-aspiryl antibody from anti-aspirylBGG sera gave an average binding constant of $2.2 \pm 0.6 \times 10^6$ l/m with an exponent of 0.6.

Discussion

Rabbits formed specific antibody against the aspiryl hapten, when various protein conjugates were used for immunization. Immunodiffusion analysis yielded results similar to those previously reported (9,10).
Table 4

Binding of Aspirin-C\textsuperscript{14} to Anti-aspirylKLH by Equilibrium Dialysis. \( A_0 = 0.40 \text{mg/ml} = 5.4 \times 10^{-6} \) molar, temperature 4\(^{\circ}\).  

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Free Side</th>
<th>Antibody Side</th>
<th>&quot;Binding&quot; \textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>730000</td>
<td>731000</td>
<td>+</td>
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<td></td>
<td>731000</td>
<td>711000</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>395000</td>
<td>400000</td>
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<tr>
<td></td>
<td>388000</td>
<td>396000</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>161000</td>
<td>156000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>150000</td>
<td>155000</td>
<td>+</td>
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<tr>
<td>10</td>
<td>80000</td>
<td>79000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>75700</td>
<td>77300</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>39900</td>
<td>39300</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>38500</td>
<td>38200</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>15800</td>
<td>16100</td>
<td>+</td>
</tr>
</tbody>
</table>

\textsuperscript{a} sign of antibody side cpm minus free side cpm
At the pH used in these studies, 8.4, it is very probable that most of the compounds referred to in the text as aspiryl are partially hydrolyzed and are actually mixtures of salicylyl and aspiryl compounds. The haptens are not soluble in neutral or acidic aqueous solutions. However, blood is slightly basic, so the experimental situation probably reflects the physiological situation.

The inhibition of precipitation experiments showed that aspiryl-ACA was the most effective inhibitor of the haptens tried. It was even more effective than ε-aspiryl-α-CBZ-L-lysine, the hapten which in some ways most closely approximates the combining part of the protein antigen; although the relative free energy difference was only about 0.2 kcal/mole. This order of reactivity has also been observed with 2,4-dinitrophenyl haptens (23). The aspiryl-aminocaproate is smaller at the carboxyl end and can probably fit in the antibody site in more conformations than the CBZ-lysine haptens. The ε-aspiryl-α-CBZ-lysine has significantly fewer stable conformations in solution because of the large CBZ group and the presence of an additional functional group which can be involved in hydrogen bonding. Lysine haptens can also form internal hydrogen bonds with the free amino group. The most stable conformations of the lysine haptens in solution may be significantly different from their conformations in protein antigens. It is known that hydrophilic groups like the
ε-amino group of lysine or the aspiryl-amide generally stick out from globular proteins (22), so the actual determinant can be more closely approximated by aspiryl-ACA than by aspiryl-lysine.

The fit of the antibody combining site around the aspiryl part of the hapten does not seem to be particularly close. Addition of a 5-methyl (meta to carboxyl) group to aspiryl-ACA causes only a 3 fold decrease in inhibitory power. However, the acetyl or hydroxyl group is very important; benzoyl-ACA is about 160 times less effective than aspiryl-ACA in inhibiting precipitation. If one adds a para-chlorine, the effectiveness is reduced again by a factor of 2; para-methoxy and meta-bromo groups each cause a 4 fold decrease in effectiveness beyond that of benzoyl-ACA. ε-CBZ-L-lysine is 1/25 as effective as aspiryl-ACA and 7 times more effective than benzoyl-ACA. The structural formulas of the aspiryl, benzoyl and CBZ groups show that the spatial arrangement of the CBZ group is in many ways between those of the aspiryl and benzoyl groups. The $K_{rel}$ for 6-CBZ-lysine is more than 10,000 times less than that of aspiryl-ACA and more than 440 times less than that of ε-CBZ-lysine. This indicates that the carbon chain is an extremely important part of the determinant.

Aspirin, 5-methaspirin, salicylate, 4-aminosalicylate and 5-methylsalicylate were all 5000 to 10,000 times less effective in inhibiting precipitation than aspiryl-ACA.
This is consistent with the observations of Butler et al. (9). The difference between salicylate and benzoate still remains, although its magnitude is reduced to only a factor of 2. Feinberg and Malkiel (7), Storm van Leeuwen and Drzimal (24) and Lester, Lolli and Greenberg (25) all measured salicylate binding to sera and all obtained variable results indicating an easily reversible, weak binding of small amounts. In this study threefold concentrated serum globulins containing specific antibody bound only small amounts of aspirin-C^14; the amounts bound were of the order of the experimental uncertainty.

The average binding constant of pooled anti-aspiryl KLH globulins to aspiryl-ACA was found to be $5.6 \pm 0.6 \times 10^5$ liters/mole. The constant for specifically purified anti-aspirylBGG was $2.2 \pm 0.6 \times 10^6$ liters/mole. Both of these are within the normally observed range of antibody-hapten binding constants. For purposes of comparison the values of the average binding constant for the binding of DNP-lysine to anti-DNP range from $0.4$ to $8.7 \times 10^5$ liters/mole according to Eisen and Siskind (23). No high affinity antibody with average binding constants of $10^7$ to $10^8$ as is found for DNP (23) was observed, although immunizations were carried out with small amounts of protein, 1.25 to 2.5 mg per injection.

In the rabbit anti-aspiryl system the antigenic determinant is closely approximated by $\epsilon$-aspiryl-amino-
caproate and is probably in large part $\epsilon$-aspiryl-lysyl. Aspirin and salicylates bind very poorly to the specific antibody and must be present in high concentration to inhibit precipitation. The ortho-acetyl, or at least the ortho-hydroxyl group, is important in the combination of antigen and antibody. Average binding constants for aspiryl ACA were between 4 and $22 \times 10^5$ liters/mole.

Conclusions

Anti-aspiryl antibodies were produced in rabbits using three protein carriers. The method of inhibition of precipitation was used to determine the specificity of the antibody produced. $\epsilon$-Aspirylaminocaproate was found to be the most effective inhibitor of the haptens tested, indicating that the combining hapten of the protein is $\epsilon$-aspiryl-lysyl. All aspiryl haptens are actually mixtures of aspiryl and salicyl derivatives. Free aspirin and salicylates were poor inhibitors and did not combine with the antibody to a significant extent in equilibrium dialysis experiments. The ortho group in aspiryl and salicyl amides was found to participate in the binding to antibody. The average binding constants of antibody globulins and specifically purified globulins were found to be in the usual range of hapten-antibody binding constants, when measured with aspirylaminocaproate.
B. Model Studies of the Acetylation Mechanism for Aspirin Intolerance

Introduction

It has recently been shown that aspirin (acetylsalicylic acid) can acetylate human serum proteins under \textit{in vitro} (11) and \textit{in vivo} (26) conditions. Both \textit{in vitro} (27) and \textit{in vivo} (4) studies have shown that the aspiryl/salicylyl model is not relevant to the clinically observed syndrome, although salicylyl proteins are known to form under \textit{in vivo} conditions (28). The determinant in the aspiryl/salicylyl conjugates is aspiryl/salicylyl-lysine, and free aspirin and salicylates are bound very weakly by antibodies made against this determinant; $K_H$ is less than 100 liters/mole (27).

Aspirin has been found to acetylate human serum albumin in a reproducible manner at specific locations (26). Antibody against acetylated proteins has been found in both control and aspirin intolerant individuals after they were given aspirin orally (29). The sera from rheumatic patients who had received extensive aspirin therapy almost all contained anti-acetylated serum albumin antibodies.

In these studies rabbits were used as a model to study the ease of protein acetylation by aspirin and the immunogenicity of these acetylated proteins.
Materials and Methods

**Acetylated Antigens.** 25ml of 1M NaHCO₃ or 1M K₂HPO₄ was added to 25ml of normal rabbit serum and cooled to 4°C. 2.5ml of acetic anhydride was dropped in, keeping the pH between 7 and 8 with 2N NaOH. After stirring for an hour, the acetylated rabbit serum antigen was dialyzed.

1g of rabbit serum albumin (Pentex) was dissolved in 30ml of 1M K₂HPO₄ and reacted as above with 1.5ml of acetic anhydride (30).

**Aspirin Treated Sera.** 25ml of 1M NaHCO₃ containing 1g of aspirin was added to 25ml of each normal rabbit serum. The pH was 7.5-7.7. Each sample was filtered through a sterile 0.45 micron membrane filter and then incubated for 5 days at 37°C. Each serum antigen was extensively dialyzed against normal saline, pH 8.4. A second series of antigens was prepared using saturated aspirin in a saturated sodium bicarbonate solution.

**Immunization (31).** Aspirin treated or acetylated serum antigens were prepared from pre-immunization sera from each of the rabbits used. Each rabbit received antigen made from its own serum, except those immunized with acetylated rabbit serum albumin, which was made from a purified product. 0.5ml samples of each antigen at the concentrations resulting
after dialysis (approximately 40-50% of normal serum concentration) were emulsified with 0.6ml volumes of Freund's complete adjuvant and injected intradermally in 2 sites in each animal. This was repeated in 14 days. The animals were bled 14 days later.

**Animals.** The rabbits were all New Zealand albino males, obtained in California. They weighed at least 2kg at the time of immunization. Guinea pigs were albino, Campbell-Trapani strain, bred in the Caltech closed colony. PCA animals weighed about 250g.

**Precipitation Tests.** Antigen and antibody were measured by the quantitative precipitin test, set up as previously described. Precipitates were quantitated by measuring the absorbance at 280nm after dissolving them in 3ml of 0.1N NaOH. The absorbance of 1mg/ml of RSA was found to be 0.67, and the absorbance of 1mg/ml of rabbit antibody was assumed to be 1.55.

**Passive Cutaneous Anaphylaxis.** PCA was performed as previously described. Sera were diluted with 4 parts of sterile normal saline. Saline and normal serum controls always gave negative tests.
Results and Discussion

All of the aspirin treated sera precipitated with anti-acetylated rabbit serum, anti-acetylated rabbit serum albumin and anti-acetylated bovine gamma globulin. The sera treated with saturated aspirin in sodium bicarbonate were largely irreversibly insolubilized, so quantitative determinations of the acetylated protein in them was not possible. When injected into rabbits in Freund's complete adjuvant, each serum antigen stimulated the production of anti-acetylated protein antibody, which reacted with acetylated rabbit serum albumin and acetylated bovine gamma globulin. The amount of immunologically precipitable acetylated protein in the aspirin treated sera was quite small and was near the limit of detection of the precipitin test, so the results were verified by comparing ring tests of them with a series of rings prepared with known concentrations of acetylated rabbit serum albumin. Antibody was determined by quantitative precipitation tests. The anti-acetylated protein antibody was shown to be IgG by immunoelectrophoresis of a concentrated sample. The results of the acetylated protein and the anti-acetylated protein antibody determinations are given in table 5. In all cases controls with pre-immune, untreated sera were negative. The amount of acetylated protein measured by this procedure is a lower limit, since precipitation tests only detect multivalent antigen.
Table 5

Acetylation of normal rabbit serum by aspirin and the production of anti-acetylated protein antibody by rabbits immunized with these antigens.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Precipitable acetylated protein precipitated by anti-acetylated rabbit serum</th>
<th>Anti-acetylated protein antibody stimulated by immunization with aspirin treated serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitin Test</td>
<td>Ring Test</td>
</tr>
<tr>
<td>253</td>
<td>3 microgram/ml</td>
<td>2</td>
</tr>
<tr>
<td>254</td>
<td>0</td>
<td>2</td>
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<tr>
<td>255</td>
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<td>256</td>
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</tr>
<tr>
<td>257</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>258</td>
<td>immunized with acetylated rabbit serum</td>
<td></td>
</tr>
</tbody>
</table>
The cross reactivity of the anti-acetylated protein antibody with aspirylKLH (keyhole limpet hemocyanin) was measured by PCA reaction. The strongest reaction was given by an antiserum prepared against acetylated rabbit serum albumin; anti-aspirin treated serum antisera gave weaker, although still positive reactions. These results indicate that aspirylKLH acetylates itself upon standing. Very little anti-aspiryl/salicylyl antibody is produced by immunization with aspirin treated sera; since aspiryl/salicylyl proteins are potent antigens, this would indicate that there is significantly less aspiryl/salicylyl protein than acetylated protein in the aspirin treated sera.

Aspirin easily acetylates serum proteins under in vitro and in vivo conditions. Acetylated proteins are extremely potent antigens in rabbits; as little as 2 micrograms of precipitable acetylated proteins can stimulate the production of 0.1mg/ml of precipitating antibody, when divided into 2 injections in Freund's adjuvant.

Samter and Beers (32) have studied the possibility that aspirin intolerance is not an immune reaction. The ease of protein acetylation by aspirin and the immunogenicity of acetylated proteins would lead one to expect that aspirin intolerance would be a common result of aspirin ingestion, if acetylation were the mechanism of aspirin intolerance. The binding properties of the aspiryl/salicylyl system indicate that this is not the
mechanism of aspirin intolerance, since salicylates combine as well as aspirin and the specificity of anti-aspiryl/salicylyl-lysine is quite broad. The symptoms of aspirin intolerance are not produced in sensitive individuals by propionylsalicylic acid, salicylic acid, or by diacetyl-fluorescein, an easily detectable acetylating agent. In some intolerant patients ingestion of other analgesics can cause the same symptoms as ingestion of aspirin (32). Further studies to elucidate the mechanism of aspirin intolerance require further investigation with aspirin intolerant human subjects.
REFERENCES


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Chapter III

IMMUNOCHEMICAL STUDIES OF CONTACT DELAYED TYPE HYPERSENSITIVITY TO ASPIRIN IN GUINEA PIGS

Introduction

The clinical syndrome of aspirin intolerance does not include the classical symptoms of delayed hypersensitivity, such as fever and a latent period of more than six hours after exposure. However, the facile formation of aspiryl/salicylyl (1) and acetyl (2) proteins in vivo from aspirin fulfills one of the conditions necessary for the production of contact hypersensitivity. In order to study these systems guinea pigs were injected with aspiryl chloride in oil for the aspiryl/salicylyl system and with both acetic anhydride and acetyl chloride in oil for the acetylation system. In addition, guinea pigs were injected intradermally with various amounts of aspirin to find out if contact sensitivity could be induced.

Contact sensitivity has been extensively investigated with both the dinitrophenyl (DNP) and trinitrophenyl (TNP) systems in guinea pigs (3). Several efficient methods of sensitization have been developed (4, 5) which require only a few micrograms of sensitizer. The hypersensitivity is caused by conjugates, which
are formed from the in vivo reaction of the sensitizer with body proteins to form an antigenic foreign protein. The specificity of contact sensitivity is similar to that of delayed hypersensitivity induced by the injection of hapten protein conjugates (6) and in most cases involves carrier as well as hapten specificity.

Materials and Methods

Materials for Immunization and Skin Testing.

Freund's adjuvant, complete, and also dried Mycobacterium butyricum were obtained from Difco. Marcol 52 was purchased from Humble Oil Company and olive oil was purchased in a local supermarket. Other solvents were reagent grade.

It was found that when Marcol or Freund's adjuvant was used for immunization, skin tests with Marcol produced positive reactions. In these cases olive oil was used as the test vehicle. It was also necessary to clean apparatus used for preparing Freund's emulsions meticulously, since traces of protein as small as 0.001 microgram can induce delayed hypersensitivity.

Immunization.

1. Each animal was injected with 0.05ml of Marcol or olive oil containing 5 micrograms of acid chloride intradermally on 10 consecutive days. After resting for 14 days the animals were tested.
This method was used in early experiments.

2. 5 skin sites on the flank of each guinea pig were injected with 0.05ml of olive oil containing 2.5 micrograms of sensitizer (a total of 12.5 micrograms per animal). After 90 minutes the same sites were injected with another 0.05ml of oil containing 2.5 micrograms of heat killed dried *Mycobacteria.* The animals were tested at days 13, 20 and 27. The injection sites usually become partially healed in the first few days, and then open sores form coincident with the appearance of reactivity to tuberculin (5). This is a split adjuvant technique.

3. Freshly prepared aspirin solutions in pH 8 buffer were emulsified with Freund's complete adjuvant. Each animal received intradermal injections of 0.1ml each in 5 sites and was tested on days 14 and 21.

**Skin Tests.**

Oil soluble sensitizers were applied in olive oil with a disposable Pasteur pipet, generally at a concentration of 1%. The guinea pigs were carefully shaved with a number 000 electric clipper and one drop of test solution was spread on the skin. The skin was only touched with the smooth area of the pipet and rubbing was only with the grain of the hair. 0.1ml of water soluble sensitizers were injected intradermally for challenge. All challenge solutions were tested on unsensitized guinea pigs to determine if
they caused non-specific irritation. Some animals were tuberculin tested to provide a positive control. A few experiments were tried using dimethyl sulfoxide, methyl ethyl ketone and propylene glycol as contact testing solvents.

The reactions were read at 6, 24 and 48 hours. They were graded as follows; +, faint pink; ++, pale pink; +++, pink; ++++, pink and thickened. Readings were verified by several observers, who were not informed of the substances used, but only of the number and approximate location on each animal. Most of the reactions were evaluated equally by several observers; at most there was a variation of one degree among the observers.

**Passive Cutaneous Anaphylaxis.**

PCA was performed as previously described, except that the latent period was 18 hours, which is required for maximal sensitization with guinea pig antibody (4).

**Arthus Reactions.**

Arthus reactivity was measured by intracardiac injection of 0.5ml of 1% Evans blue followed by intradermal injection of 0.1ml of antigen using a 30 gauge special intradermal bevel needle. Only actively sensitized animals were used. Positive reactions were maximal within less than 4 hours. Arthus reactions were
distinguished from anaphylactic reactions by performing PCA tests.

Sensitizers.

The preparation of aspiryl chloride and 5-methylaspiryl chloride are described in chapter II. The other acid chlorides were all the best available commercial grade. In some cases the acid chlorides were vacuum distilled.

Acetyl Guinea Pig Serum.

3 ml of normal guinea pig serum was added to 3 ml of 1N NaHCO₃ and cooled in ice. 0.3 ml of acetic anhydride was dropped in with stirring over a period of 30 minutes. After standing an additional hour, the antigen was dialyzed against saline containing 5% borate-saline buffer pH 8.4. The concentration of the stock solution was 13.8 mg/ml.

Guinea Pigs.

Guinea pigs were all Campbell-Trapani strain albinos, bred in the Caltech closed colony. Animals from 250-500 grams were used for both PCA and contact tests. Adult and older animals gave less consistent results in contact tests.
Results and Discussion

Every guinea pig immunized with aromatic acid chlorides by either method developed contact sensitivity. The first experiments were done using 10 daily injections without Mycobacteria (4) and later experiments were done using 5 simultaneous injections followed by Mycobacteria (5). Both methods gave similar results, but the split adjuvant technique was easier experimentally and allowed results to be obtained in less time. Testing with the immunizing hapten acted as a booster injection. This effect is shown in table 1 along with the effect of Mycobacteria. Animals immunized with aspiryl chloride, using either method, gave negative PCA and Arthus reactions at day 14. After tests on day 14 some animals gave positive PCA tests at day 21 when tested with aspiryl KLH. Both PCA and Arthus reactivity are caused by IgG antibodies, although only electrophoretically fast moving guinea pig IgG can cause homologous anaphylactic sensitization. Mycobacteria and oil stimulate the production of homocytotropic antibody in guinea pigs (8). Passive sensitization with guinea pig homocytotropic antibody is maximal at 17-18 hours rather than at 3-4, as with rabbit antibody (4).

Contact tests were performed with 1% solutions of acid chloride in olive oil. A 1% solution of aspiryl chloride gave an average ++++ reaction. The effect of concentration is shown in
Table 1

Booster Effects of Contact Testing

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Test at Day 14</th>
<th>2nd Test at Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyl Chloride</td>
<td>+\textsuperscript{a}</td>
<td>++</td>
</tr>
<tr>
<td>o-Chlorobenzoyl Chloride</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>p-Chlorobenzoyl Chloride</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>5-Methylaspiryl Chloride</td>
<td>++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Aspiryl Chloride (Mycobacteria) \( +++++, +++\textsuperscript{b} \)

Aspiryl Chloride (10x) \( ++++, + \)

\( \text{PCA -} \)

\( \text{a. average of three animals} \)

\( \text{b. 2 experiments, 4 animals each} \)

Table 2

Effect of Concentration of Test Antigen on Contact Tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Average of 4 Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Aspiryl Chloride</td>
<td>++++</td>
</tr>
<tr>
<td>0.1% Aspiryl Chloride</td>
<td>+++</td>
</tr>
<tr>
<td>0.01% Aspiryl Chloride</td>
<td>++</td>
</tr>
</tbody>
</table>
104

table 2. 1% solutions of each test hapten were non-irritating to normal guinea pig skin. Olive oil was used as a test vehicle because both Freund's complete adjuvant and Marcol contained an impurity which sensitized guinea pigs, so that contact tests gave an average ++ reaction.

A series of experiments summarized in table 3 was undertaken to determine the cross reactivity of guinea pigs immunized with aspiryl chloride against other aromatic acid chlorides, and also the specificity of delayed reactions in guinea pigs sensitized with these other haptens. Guinea pigs sensitized with aspiryl chloride gave weaker reactions to 5-methylaspiryl chloride, para-chlorobenzoyl chloride and ortho-chlorobenzoyl chloride, the reaction was much weaker to benzoyl chloride, but it was still clearly positive. The specificity is quite broad; but apparently there are some limitations due to steric factors as well as to chemical factors. The binding in the site is apparently quite loose since a 5- (meta) methyl can be accommodated in addition to the ortho-hydroxyl. The reactions with both the para and ortho-chloro compounds were approximately equal. The significantly smaller benzoyl chloride reacted only weakly.

Benzoyl chloride was a significantly less potent sensitizer
Table 3

Cross Reactivity of Aromatic Acid Chlorides in Contact Sensitivity

<table>
<thead>
<tr>
<th>Immunized with</th>
<th>Tested with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AspCl</td>
</tr>
<tr>
<td>24 hour observation</td>
<td></td>
</tr>
<tr>
<td>AspCl</td>
<td>+++</td>
</tr>
<tr>
<td>BenCl</td>
<td>1/2+</td>
</tr>
<tr>
<td>o-ClBenCl</td>
<td>1/2+</td>
</tr>
<tr>
<td>p-ClBenCl</td>
<td>-</td>
</tr>
<tr>
<td>5-MeAspCl</td>
<td>+++</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
</tr>
</tbody>
</table>

48 hour observation

| AspCl         | +++   | +     | ++         | ++        | ++        | -  |
| BenCl         | ++    | +++   | +++        | ++++      | +         | -  |
| o-ClBenCl     | ++    | ++    | +++        | ++++      | 1/2+      | -  |
| p-ClBenCl     | +     | ++++  | +++        | ++++      | +         | -  |
| 5-MeAspCl     | +++   | ++++  | +++        | ++        | ++++      | -  |
| Control       | -     | -     | -          | -         | -         | -  |

Each reading is the average of 3 animals. The animals were sensitized by injection with Mycobacteria at day 0, contact tested with the immunizing antigen on day 14 and tested on day 21.
than the other compounds tested. Guinea pigs immunized with it do not react as strongly with aspiryl and 5-methylaspiryl chlorides as with the substituted benzoyl chlorides. Ortho-chlorobenzoyl chloride reacts more strongly than para-chlorobenzoyl chloride, indicating a close fit around the para position.

Para-chlorobenzoyl chloride is a very potent sensitizer. The test haptens reacted in the following order: p-chlorobenzoyl > o-chlorobenzoyl > benzoyl > 5-methylaspiryl > aspiryl. Ortho-chlorobenzoyl chloride sensitized guinea pigs reacted in the following order: o-chlorobenzoyl > p-chlorobenzoyl > benzoyl > aspiryl > 5-methylaspiryl. In this case both chemical and steric factors are involved. The ortho-hydroxyl group interferes with the combination of the aspiryl haptens. Para-chlorobenzoyl chloride has a significantly different shape than the sensitizer, o-chlorobenzoyl chloride, but it still combines more strongly than the ortho substituted aspiryl chloride. The 5-methyl group in the 5-methylaspiryl sterically interferes with the combination in o-chlorobenzoyl sensitized animals.

Aspiryl conjugates of KLH, BGG and BSA gave either negative or very weak reactions in aspiryl chloride sensitized guinea pigs when observed at 15 minutes and 2, 6, 24 and 48 hours after challenge. The existence of carrier specificity and
the relatively low degree of hapten specificity are similar to the specificity observed in delayed hypersensitivity to pre-formed hapten-protein conjugates (6, 7). The relative amounts of hapten and carrier specificity differ in various systems studied by other investigators (6, 7, 9, 10).

If this type of sensitivity were present in human subjects, it would be easy to detect because of its broad specificity. Human beings are significantly more susceptible to contact sensitization than guinea pigs, because of the greater vascularization of human skin. Human skin has been estimated to be about 1000 times more sensitive to DNP than guinea pig skin. Aspiryl chloride would probably be a relatively potent sensitizer of people exposed to it. Fortunately, it is a rare chemical. Attempts to sensitize guinea pigs with aspirin are discussed below along with the possibility of a contact reaction due to acetylation.

Two groups of four guinea pigs were immunized with 12.5 to 25 micrograms of acetyl chloride and acetic anhydride by the split adjuvant technique. Marcol was used as a vehicle because it is relatively anhydrous. Another group was immunized with aspiryl chloride for comparison. The animals were contact tested on days 14 and 20 and tested by injections of acetylated proteins on day 20. The results are given in table 4. On day 14 the tests were performed
Table 4

Attempted Sensitization to Acetyl Proteins

Immunization 5 x 2.5 micrograms in 0.05ml Marcol and 2.5 micrograms of Mycobacteria in 0.05ml Marcol. Each result is the average of 5 guinea pigs.

Day 14

<table>
<thead>
<tr>
<th>Test</th>
<th>Acetic Anhydride</th>
<th>Acetyl Chloride</th>
<th>AspCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Anhydride 1%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetyl Chloride 1%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p-Chlorobenzoyl Chloride 1%</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Aspiryl Chloride 1%</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

Day 20

<table>
<thead>
<tr>
<th>Test</th>
<th>Acetic Anhydride</th>
<th>Acetyl Chloride</th>
<th>AspCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Anhydride 5%, 10%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetyl Chloride 5%, 10%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aspiryl Chloride 1%</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Acetyl Guinea Pig Serum 0.1%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
with 1% solutions in olive oil. All 12 animals as well as a control animal tested negative with both acetic anhydride and acetyl chloride at concentrations of 1%, and on day 20 with 5 and 10% solutions. None of these solutions were non-specifically irritating. The guinea pigs immunized with aspiryl chloride tested positive with 1% aspiryl chloride and p-chlorobenzoyl chloride, but were negative with acetyl chloride and acetic anhydride. All tests with acetylated proteins were negative. Both 0.1% acetylated RSA and 0.1% acetylated guinea pig serum were tried. A couple of animals gave positive results with acetylated RSA, but also tested positive with a similar concentration of RSA. Both acetic anhydride and acetyl chloride are more easily hydrolyzed than the aromatic acid chlorides used, but they react more rapidly with free amino groups of proteins than with water. Acetic anhydride should be able to react with guinea pig skin proteins under the conditions used for immunization and testing. So the problem is the absence of induction of delayed hypersensitivity, not the lack of production of protein conjugates in vivo.

Aspirin was dissolved in water by adding the minimum amount of NaHCO₃ necessary to dissolve it just before it was emulsified with Freund's complete adjuvant. Each animal received 5 simultaneous injections, each of 0.05ml containing either 5 or 25 micrograms of aspirin. The experiments are summarized in table 5. The tests
Table 5

Attempted Sensitizations With Aspirin

1. day 0  immunize 5 x 5 micrograms aspirin in 0.05ml
day 17  test with 20% aspirin in DMSO  irritant
   20% in propylene glycol  -
   20% in methyl ethyl ketone  -
   inject 1% in water  -
day 23  test by i.d. injection
   2% aspirin in water  -
   1% aspirin in water  -
   0.1% aspirin in water  -
   1% acetylated RSA  +
   1% aspiryl KLH  -
   1% RSA  +
day 31  1% acetylated BGG  -

2. day 0  immunize as in 1.
day 14  test by i.d. injection
   0.1% aspirin  -
   1% RSA  -
   0.05% aspiryl KLH  -
   0.1% acetyl RSA  -

continued
Table 5, Continued

0.01% acetylated RSA -

0.1% acetylated BGG -

day 21 contact test with 1% aspiryl Cl -

inject 0.1% aspirin -

3. day 0 immunize 5 x 25 micrograms aspirin in 0.05 ml

day 14 test by i.d. injection

0.1% aspirin -

0.1% acetylated guinea pig serum -

0.01% acetylated guinea pig serum -

with aqueous solutions were all performed by intradermal injection of 0.1 ml. Some of the solutions produced non-specific reddening of the skin so a control animal was used with each set of tests. Only reactions significantly larger or redder than those on the control animal were graded as positive. There is necessarily a subjective element in these evaluations.

A series of contact tests with aspirin in dimethylsulfoxide (DMSO), propylene glycol and methyl ethyl ketone were attempted. Dimethylsulfoxide caused non-specific reddening and increased vascular permeability, which was observed in guinea pigs that had
received Evans blue intravenously. Dimethylsulfoxide was selected because of its ability to carry small molecules through the skin. Tests with the other two solvents were uniformly negative. Both propylene glycol and methyl ethyl ketone have been used successfully for contact testing with human patients.

Tests with aspirin solutions, aspiryl KLH, acetylated RSA, acetylated BGG and acetylated guinea pig serum were all negative in all animals in which the carrier proteins tested negative. Aspirin solutions from 0.1% to 2% were used. Higher concentrations caused significant irritation to the skin of normal animals. One group of animals was tested with 1% aspiryl chloride in olive oil. All of these reactions were also negative.

Aspirin did not induce either contact or delayed sensitivity when guinea pigs were immunized using conditions that induce sensitivity with other reactive compounds. The acetylation mechanism is not relevant to this type of hypersensitivity, since contact sensitivity is not produced in guinea pigs by strong acetylating agents like acetyl chloride and acetic anhydride. Aspiryl chloride, a totally artificial system, is a good sensitizer and behaves in the manner previously reported for other delayed and contact hapten systems.
REFERENCES


Chapter IV

IMMUNOCHEMICAL INVESTIGATIONS OF IMMEDIATE AND DELAYED HYPERSENSITIVITY TO Para-AMINOSALICYLIC ACID IN ANIMALS

Introduction

Para-aminosalicylic acid is commonly used in the treatment of tuberculosis and leprosy. Lehmann (1) found that p-aminosalicylic acid inhibited the growth of *Mycobacterium tuberculosis* in vitro, but later it was found that p-aminosalicylic acid was not particularly effective in vivo when compared to other anti-Mycobacterial drugs like streptomycin (2). Although streptomycin and Isoniazid (isonicotinic hydrazide) are usually used for primary treatment of tuberculosis, p-aminosalicylic acid is often used as an adjunct to therapy, because of its action in greatly reducing the rate of appearance of streptomycin resistant strains in both laboratory animals (3, 4) and human patients (5).

Normally a dose of about 4 grams is administered orally. The free acid is found at a concentration of 3 - 12mg% in the blood 1-1/2 to 2 hours after administration. The sodium salt is absorbed even more rapidly. Within 10 hours over 85% of the drug is excreted in the urine. In man more than half of the drug
that is excreted is acetylated. The retained drug is found in the kidneys, lungs and liver and a significant amount is bound by serum proteins (6, 7).

Para-aminosalicylic acid is slightly soluble in water, and the free acid readily decarboxylates in aqueous solution to form meta-aminophenol. The sodium salt is very soluble and is more stable, although it decarboxylates upon heating (8).

An allergic reaction to p-aminosalicylic acid was first reported in 1949 (9). A large number of cases have since been reported. A 1960 review (10) tabulates 229 well documented cases, most of which were verified by the administration of a second dose after the reaction once occurred. The clinical doses used have ranged from 1 to 20 grams per day. Incidence of all forms of allergy to this drug has been estimated to be from 0.3 to 5 per cent of the patients treated with the drug. At least three distinct types of hypersensitivity to p-aminosalicylic acid have been observed. Each of them is discussed separately below, since they appear to have different mechanisms.

The most common form of hypersensitivity to p-aminosalicylic acid is completely described by Simpson and Walker (10). The usual symptoms are fever and a non-urticarial skin rash; abnormal lymphocytes, disturbed hepatic function, leukocytosis
and eosinophilia can also be observed in many cases. Many other symptoms have been associated with this syndrome in individual cases. Many patients also demonstrate sensitivity to streptomycin and Isoniazid simultaneously. In more than half of the reported cases symptoms appeared between 15 and 35 days after the start of p-aminosalicylic acid therapy. A second challenge dose, administered after recovery from the symptoms caused by the original drug treatment, caused a recurrence of the symptoms. Desensitization has been reported in many cases. This form of hypersensitivity is considered to be of the delayed type, although there is no direct evidence such as observations of the lack of transferability by serum. This classification is made on the basis of the symptoms and also the presence of Mycobacteria, which act as an adjuvant to stimulate delayed hypersensitivity.

A second type of hypersensitivity observed with p-aminosalicylic acid is allergic contact dermatitis (11). This reaction is probably related to the fairly common "p-amino group" sensitization, which is elicited in many individuals by a variety of p-amino compounds. The reaction elicited by p-aminosalicylic acid may actually be a cross-reaction caused by sensitization to p-aminobenzoic acid, p-phenylenediamine, procaine or some
other similar compound. The cross-reactivity of patients sensitized to various p-amino compounds is quite wide and unpredictable. After extensive studies, Mayer (12) has proposed a mechanism for this type of hypersensitivity. His scheme for p-aminobenzoic acid is shown in figure 1. Mayer considers the reactive substances to be quinonediimines and quinoneimines. In a series of guinea pigs sensitized with p-phenylenediamine, tests with the sensitizer produced a ++++ reaction, and tests with quinonediimine produced a ++++ reaction. Other quinone forming compounds usually gave positive reactions, and non-quinone formers gave negative reactions. Meta-aminophenol was considered a non-quinone former.

![Figure 1. Scheme for the formation of reactive intermediates from p-aminobenzoic acid (12).](image-url)
The third type of hypersensitivity reaction to p-aminosalicylic acid is hemolytic anemia (13, 14). Most of the cases reported have been from France and North Africa. Several authors have reported that the solutions used in these areas were many times discolored and contained from 2.6 to 17% m-aminophenol and up to 58% of a black tarry compound (15, 16). Both of these substances have been accused of being the cause of the hemolysis. In a few cases glucose-6-phosphate dehydrogenase deficiency has been demonstrated in the red cells of the hemolytic anemia patients (17). Deficiency of this enzyme many times causes drug induced hemolytic anemias (14). The enzyme deficiency disease is found in two forms, both of which are hereditary and most commonly found in areas of Africa and Mediterranean Europe. However, a few cases have been found in which the hemolytic anemia is definitely caused by p-aminosalicylic acid. Dausset and Bergerot-Blondel (18) extensively investigated a case in which hemagglutination, but not hemolysis, took place. They found a serum antibody which could agglutinate erythrocytes, leukocytes and platelets, but which did not fix complement. It appeared to be in the \( \gamma G \) fraction of the serum. The specificity of this antibody was determined by passive hemagglutination. Their results are given in table 1. Dausset and Thierfelder
Table 1. Specificity of Antibody against p-aminosalicylate (19)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Titer</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-aminosalicylate</td>
<td>1/64</td>
<td>++++</td>
</tr>
<tr>
<td>3-aminosalicylate</td>
<td>1/2</td>
<td>++</td>
</tr>
<tr>
<td>5-aminosalicylate</td>
<td>1/8</td>
<td>++</td>
</tr>
<tr>
<td>p-aminobenzoate</td>
<td>1/64</td>
<td>++++</td>
</tr>
<tr>
<td>salicylate</td>
<td>1/16</td>
<td>+++</td>
</tr>
<tr>
<td>gentisate</td>
<td>1/32</td>
<td>+++</td>
</tr>
<tr>
<td>3-nitrosalicylate</td>
<td>1/2</td>
<td>+</td>
</tr>
<tr>
<td>5-nitrosalicylate</td>
<td>1/2</td>
<td>+</td>
</tr>
<tr>
<td>benzoate</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>acetylsalicylate</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>p-phenylenediamine</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>p-aminosulfanilate</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>m-aminophenol</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>p-quinone</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>salicylamide</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>phenacetin</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
(19) described another case where hemolytic anemia was present. A similar level of cross-reactivity was observed, but the relative reactivities were slightly different from the other case. These symptoms are probably caused by antibodies of the IgG and IgM classes.

A few cases have been observed with urticarial reactions (10). These have not been studied in detail and could be caused by IgE antibodies.

In the investigations reported here, various conjugates of p-aminosalicylic acid were prepared and injected into rabbits and guinea pigs in order to observe the immune reactions elicited. Guinea pigs were also sensitized with free p-aminosalicylic acid and its sodium salt and their response studied.
Results and Discussion

A. Azo Coupled Antigens

Aromatic amines were diazotized and coupled to proteins by the methods described in Chapter I. Conjugates of para-azosalicylate, 5-azosalicylate and p-azobenzoate were prepared using several carriers, including KLH (keyhole limpet hemocyanin), BGG (bovine gamma globulin), BSA (bovine serum albumin) and GPS (normal guinea pig serum). After extensive dialysis the conjugates were passed through a column of either Bio-Gel P2 or P10 to remove unreacted hapten and side products. After chromatography there was no dialyzable color in any of the preparations. The absorption spectra of 4-azosalicylate GPS and 5-azosalicylate GPS are shown in figures 2 and 3.

A group of small azohaptens were prepared to help in characterizing the conjugates and to be used as inhibitors in inhibition of precipitation experiments. The azosalicylates were coupled directly to phenol (20) and to protected amino acids (21). Aniline derivatives were prepared by coupling the azo compound to aniline-ω-methylsulfonate, followed by basic hydrolysis (20). The yields of all the compounds were lower than expected after base-acid cycling for purification. The absorption spectra of six of these derivatives are shown in figures 4 and 5. The N-acetyl
Figure 2. Absorption spectrum of 4-azosalicylate guinea pig serum. Protein concentration 0.18mg/ml.
Figure 3. Absorption spectrum of 5-azosalicylate guinea pig serum. Protein concentration 0.09mg/ml.
Figure 4. Absorption spectra of azosalicylate derivatives.

- 4-azosalicylate-para-phenol
- 4-azosalicylate-para-aniline
- 5-azosalicylate-para-aniline
Figure 5. Absorption spectra of azosalicylate amino acid derivatives.

- 4-azosalicylate-ortho-N-acetyltirosine
- 4-azosalicylate-N-acetylhystidine
..... di-(4-azosalicylate)-ε-aminocaproate
tyrosine, N-acetyl histidine and ε-aminocaproate derivatives all had similar spectra. The purity of all of these compounds is questionable and could not be readily determined. Attempts to prepare di-(4-azosalicylate)-N-acetyl tyrosine gave the same compound as the preparation of the mono derivative. ε-Aminocaproate forms only a di-(4-azosalicylate) derivative. Both the preparation and characterization of the azosalicylate derivatives were more difficult than for the corresponding p-azobenzene-arsonate, p-azobenzenesulfonate and p-azobenzoate derivatives (22).

The antigens used for immunization were partially cross-linked by glutaraldehyde (23). Rabbits and guinea pigs were injected intradermally every two weeks with antigen emulsified in Freund's complete adjuvant. They were tested two weeks after the third injection. All of the animals immunized with azosalicylate conjugates tested negative by both precipitation and passive cutaneous anaphylaxis (PCA) tests. The rabbits were then injected intravenously with 0.5 - 1mg three times a week for three weeks. They were bled and tested seven days after the last injection. All of the 4-azosalicylate rabbits were negative; however, a group of three rabbits immunized with a p-azobenzoate BGG antigen prepared the same way as the 4-azosalicylate conjugates all produced medium to low titers of anti-hapten
antibody, when tested against p-azobenzoate KLH by ring test and PCA. The results did not change after further immunization. Several preparations of 4-azosalicylate antigens were tried in both rabbits and guinea pigs, but no antibody was produced. These conjugates, however, did elicit delayed skin reactions in guinea pigs. These results are discussed in section IV, D.

Daniel, Perloff, Major and Baum (24) used 4-azosalicylate human serum albumin sensitized erythrocytes to study serum from human patients in a passive hemagglutination system. 52 out of 114 samples tested positive. The incidence of positive reactions did not show statistically significant differences among normal controls, p-aminosalicylic acid treated patients, p-aminosalicylic acid sensitive patients and patients treated with large amounts of aspirin for rheumatoid arthritis. Therapy with p-aminosalicylic acid caused a small increase in the serum hemagglutination titer of those patients whose sera tested positive the first time. These observations give very little information about the mechanism of the hypersensitivity and, moreover, could be the result of some non-specific or non-pathological reaction. There are no reports in the literature of 4-azosalicylate antibody from laboratory animals.
B. Carbodiimide Coupled Antigens

Para-aminosalicylate was coupled to proteins and polypeptide carriers using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, CH$_3$CH$_2$N=C=NCH$_2$CH$_2$CHN(CH$_3$)$_2$, abbreviated as ECDI (25). 100mg of p-aminosalicylic acid was dissolved by neutralization with 1N NaOH in a total volume of 5ml; 100mg of protein or polypeptide was then dissolved in the solution. 1g of ECDI in a volume of 2ml was added and the mixture was gently stirred for 18 hours. The solution was then dialyzed against dilute sodium bicarbonate and four times against saline. Any precipitate that formed was redissolved by adjusting the pH with NaOH or HCl.

The reactions involved in carbodiimide coupling and the side reactions that occur have been extensively investigated (26). The coupling takes place by the reaction of free amino groups with either the intermediate compound resulting from the reaction of carbodiimide with free carboxyl groups and/or the reaction of acid anhydrides formed from the intermediate compound. The intermediate is an O-acyl urea whose reactivity is not known; however, acids that have symmetrical anhydrides that are unreactive toward amines do not form amide bonds with carbodiimides and amines (26). The most common reactions in
Figure 6. from reference 27.
carbodiimide coupling are diagrammed in figure 6 (27). The by-product of the coupling is a disubstituted urea, which can be removed by dialysis. The major side reaction is the formation of N-acylureas. Although this side reaction is controllable in the coupling of small peptides, it is a serious problem in the coupling of macromolecules (28). The conjugates prepared in this study were highly immunogenic as were those prepared by Goodfriend et al. (28). However, precipitation of antigen was not inhibited by p-aminosalicylate, and sera prepared against p-aminosalicylic KLH (coupled by ECDI) cross reacted with ECDI treated BGG. Passive cutaneous anaphylaxis experiments with rabbit immune sera in guinea pigs gave similar results.

A second problem of ECDI coupled conjugates is the presence of reactive groups, which can cross-link the antigen to other serum proteins, forming a non-specific precipitate. This was discovered by observing that precipitation reactions still occurred after immune serum was absorbed three successive times with huge excesses of antigen trapped in a polyacrylamide gel matrix (29). The antigen also gave a precipitate with normal serum. These reactive groups were not blocked by treatment of the antigen with a large excess of free p-aminosalicylate, or large excesses of glycine or lysine. Coupling was also tried
over a pH range of 5 to 8, but the same problems persisted in each preparation.

P-aminosalicylate was coupled to BGG by ECDI. Spectral evidence obtained from a thoroughly dialyzed sample showed a significant amount of coupling, approximately 20 hapten residues coupled to each BGG molecule. The spectrum of p-aminosalicylate in base is shown in figure 7 and those of p-aminosalicylic BGG and ECDI treated BGG are shown in figure 8. The concentrations of BGG in both samples in figure 8 differ by less than 5%. The changes are quite obvious.

Two experiments were done to determine the direction of coupling of p-aminosalicylate. In the first experiment p-aminosalicylate was coupled to polyglutamic acid. The spectrum of the dialyzed product is shown in figure 9 and it shows that coupling did take place to form polyglutamylaminosalicylate. The results of the second experiment are shown in figure 10; p-aminosalicylate also coupled to polylysine to form p-aminosalicylylpolylysine. This product was used to demonstrate that the p-aminosalicylate is covalently bound. A thoroughly dialyzed sample was diluted 10 times with water and then pressure diafiltered through an Amicon UM20e (cutoff 20,000 molecular weight) membrane. This was repeated three times for a total
Figure 7. Absorption spectrum of para-aminosalicylate in base.

Extinction coefficients 14,000, 9000.
Figure 8. Absorption spectra of carbodiimide coupled antigens.

--- p-aminosalicylic BGG, ECDI coupled, approximately 20 moles of hapten coupled per mole of BGG.

----- BGG treated with ECDI, about the same concentration of protein.
Figure 9. Absorption spectrum of polyglutamylsalicylate amide.
Figure 10. Absorption spectra of p-aminosalicylylpolylysine.

----- Dialyzed

----- Dialyzed and then diafiltered 3 times for a total dilution of 1 to 1000. Final dilution is 1.3 times that of the original sample.
dilution of 1 to 1000. After correction for volume change, the spectra agreed within 2%, which is less than the experimental error. The polyglutamate conjugates caused only a faint non-specific precipitation, while the polylysine conjugates caused a strong non-specific precipitation.

There are several other side reactions of carbodiimides which may be important in the systems investigated. Aromatic amines can react with carbodiimides under mild conditions to form trisubstituted guanidines (30) as is shown in figure 11.

Only strongly acidic phenols such as picric acid react with carbodiimides, and this reaction only occurs at elevated temperatures (31). Non-aromatic hydroxy groups are not reactive with carbodiimide under mild conditions (32). Formation of cyclic compounds such as β-lactones from amino acids by carbodiimide treatment is not unusual (33). Figure 12 shows the reaction of protected histidine. Similar reactions may take place with the free functional groups on macromolecules.

\[
\begin{align*}
R'\text{NH}_2 + ArN=C=NAr & \rightarrow ArN=CNHAr \\
& \quad | \\
& \quad NHR
\end{align*}
\]

Figure 11. Reaction of carbodiimide with aromatic amines.
The reaction of carbodiimide with gelatin has been studied to some extent (34). Sheehan and Hlavka performed the reaction of gelatin with carbodiimide in aqueous solution and measured the cross-linking by the decrease in gelling time and eventual liquefaction. After extensive treatment with an equal weight of water soluble carbodiimide only 10% of the free amino and carboxyl groups remained. Benzoylated gelatin did not gel or cross-link with carbodiimide, showing the necessity of free amino groups for intermolecular coupling.

$\text{P-aminosalicylate may also react with carbodiimide without coupling. The phenol is not particularly acidic so it does not participate directly (31). Zetzsche and Voigt (35) have found that ortho, meta and para-aminobenzoic acids react with carbodiimides to form a urea at the amine and an amide at the carboxyl.}$
Figure 13. Reaction of p-aminobenzoic acid with diaryl carbodiimides.

P-aminobenzoic acid reacts with diaryl carbodiimides under mild conditions to give the product shown in figure 13. The final product is not reactive with either amino or carboxyl groups.
C. Mixed Anhydride Coupling by Ethyl Chloroformate

Ethyl chloroformate (EtChf) has been used for crosslinking proteins to prepare immunoadsorbents and insoluble enzymes (36). The two primary reactions that take place are mixed anhydride coupling to form peptide bonds and the formation of ureas by the coupling of two amino groups. The presence of ureas was established by the carbamido diacetyl test (36). Insolubilization takes place most rapidly around the isoelectric point, and with many proteins does not occur when the reaction is carried out at a pH 2 or 3 units away from the isoelectric point.

In the first experiment p-aminosalicylate was coupled to normal guinea pig serum by ethyl chloroformate without buffer. The pH was in the range of 5 to 7. After extensive dialysis and diafiltration, the absorption spectrum measured in acid showed coupling had occurred. The spectrum lost the peaks characteristic of p-aminosalicylate (see figure 7), when it was measured in base. The spectra are reproduced in figure 14.

When this experiment was repeated, no coupling took place. A series of reactions were set up using several amino haptens and proteins at various pH's. Some of them are tabulated in table 2. No coupling was observed in any case. Where precipitation occurred, the supernatant was examined, and if the hapten was colored, the
Figure 14. Absorption spectra of p-aminosalicylic guinea pig serum coupled by ethyl chloroformate.

--- in acid

----- in base
precipitate was thoroughly washed and visually examined. The amino and carboxyl groups of the protein appear to react more rapidly with the ethyl chloroformate than with the haptens. Outside the range of 2 pH units around the isoelectric point the functional groups of the protein are unreactive.

The coupled p-aminosalicylic guinea pig serum from the first experiment was injected in Freund's adjuvant into guinea pigs and rabbits. None of the sera obtained from these animals gave positive reactions in PCA against ECDI coupled p-aminosalicylic KLH.
D. Delayed Skin Reactions to para-Aminosalicylic Acid Conjugates in Guinea Pigs

Normal guinea pigs were immunized with five 0.1ml intradermal injections of antigen emulsified in Freund's complete adjuvant on day 0. Each animal received a total of 7 micrograms of antigen. Each animal was tested by intradermal injection of 0.1ml of each test antigen on day 10 and the reaction was measured after 4, 24 and 48 hours. A tuberculin test was performed on each animal to provide a positive control. The Mycobacteria in the adjuvant cause tuberculin reactivity to develop in the animals. The polylysine conjugate was an irritant and caused a reaction of about 9mm diameter to develop in both sensitized and control animals. The other conjugates were relatively non-irritating. The results of the tests are tabulated in table 3. The orders of reactivities are consistent with other systems that have been studied (37, 38, 39, 40).

The animals that had been sensitized with 4-azosalicylate guinea pig serum (GPS) reacted most strongly with the sensitizing antigen. The reaction with PABGPS was very weak; only one out of three animals tested positive. 4-azosalicylate KLH gave a strong reaction, probably due to the high hapten coupling ratio of the preparation; there was about 5 to 7 times the amount of
Table 3. Delayed Reactions to Conjugates in Guinea Pigs

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>Sensitized with</th>
<th>mg/ml</th>
<th>Control</th>
<th>PABGPS*</th>
<th>4-SalGPS</th>
<th>5-SalGPS</th>
<th>PASGPS (EtChf)</th>
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<tr>
<td>4-SalGPS</td>
<td></td>
<td>.35</td>
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<td>11</td>
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<td>-</td>
<td>8</td>
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<td>-</td>
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<tr>
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<td>-</td>
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</tr>
<tr>
<td>5-SalGPS</td>
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<td>.36</td>
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<td>9</td>
<td>5</td>
<td>13</td>
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continued
Table 3. continued

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<tr>
<td>Saline</td>
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</table>

Notes

* PAB is p-azobenzoate, 4-Sal is p-azosalicylate, PAS is p-aminosalicylic acid or salicylate, PPD is purified protein derivative from tuberculin, TU are tuberculin units, the methods of antigen preparation are in parentheses.

** - is no reaction, numbers in the last five columns are the average diameters of the reactions from at least three animals, the units are mm. Blanks were not done.
hapten coupled per mg protein more than the other conjugates used. 4-azosalicylate BSA gave a very weak reaction. 5-azo­salicylate GPS gave a reaction half the size of the homologous reaction; p-aminosalicylate GPS (ethyl chloroformate coupled) gave a negative reaction.

Guinea pigs immunized with 5-azosalicylate GPS reacted very strongly with the sensitizing antigen and somewhat more weakly with 4-azosalicylate GPS and p-azobenzoate GPS. The reaction with p-aminosalicylic GPS was very weak.

Para-aminosalicylic GPS coupled by ethyl chloroformate was as potent an antigen as p-azobenzoate GPS. The carbodiimide coupled preparation reacted almost as strongly as the immunizing preparation. 4-azosalicylate GPS and the p-aminosalicylic acid polyglutamic acid conjugate each gave reactions about half the diameter of those caused by the immunizing conjugate.

Experiments were done with p-azobenzoate GPS for comparison. The immunizing conjugates reacted quite strongly and 4-azosalicylate GPS, 5-azosalicylate GPS and p-azobenzoate BSA all caused reactions about half the diameter of those caused by p-azobenzoate GPS.

The 4-azosalicylate GPS guinea pigs were bled and their sera were tested against 4-azosalicylate KLH and 4-azosalicylate
GPS by PCA in normal guinea pigs, after an 18 hour sensitizing period. These tests were all negative, showing the absence of skin sensitizing antibodies. Precipitation tests were also negative.

The experiments described above indicate a significant degree of hapten specificity in the salicylate systems studied in delayed hypersensitivity. 4-azosalsalicylate cross reacts less with 5-azosalicylate than the reverse. P-azobenzoate does not react significantly with 4-azosalsalicylate sensitized animals, but cross reacts strongly with the 5-azosalsalicylate sensitized animals. The two p-aminosalicylic acid preparations each gave similar size reactions in the same animals. This indicates that they probably share many determinants; however these animals did exhibit a broad range of cross-reactivity. The variable amounts of carrier specificity were expected. The reactivity of the polyglutamic acid conjugate showed that some of the coupling was through the amino group of the p-aminosalicylate.
E. Contact Sensitivity to p-Aminosalicylate in Guinea Pigs

Guinea pigs were injected with p-aminosalicylate in Freund's complete adjuvant emulsions using the amounts and procedures described for aspirin in chapter III. The animals were tested by topical application of p-aminosalicylic acid in propylene glycol and methyl ethyl ketone, and by both application and injection of p-aminosalicylate in water. Concentrations of up to 2mg/ml were non-irritating. All of these tests were negative as were the tests with free hapten on the animals sensitized with conjugates.

A method was tried with one group of guinea pigs that had given excellent results with p-phenylenediamine (12). 1 gram of p-aminosalicylic acid was mixed with 9g of Vaseline brand petroleum jelly. The mixture was topically applied to a shaved area on the flank of the guinea pigs daily for 7 consecutive days. On day 21 the guinea pigs were tested with 10% p-aminosalicylic acid, p-phenylenediamine, 5-aminosalicylic acid and p-aminobenzoic acid in Vaseline. All of the reactions were either negative or weak enough to be masked by the color of the test substance. At day 14 the area where the sensitizer was applied seemed thickened. If any sensitivity was produced, it was very weak. These animals did not react to p-aminosalicylic acid conjugates.
F. The Farr Technique for Determining Binding Constants

Stupp, Yoshida and Paul (41) found that a modification of the Farr technique could be used for determining the binding of dinitrophenyl-€-aminocaproate to antibody. The method consists of incubating dilutions of hapten with immune serum, immune serum globulins, or purified antibody, then adding 2 volumes of 75% saturated ammonium sulfate and centrifuging. Aliquots of the supernatant were counted to determine free hapten concentrations.

This technique was tested with the model system, p-azo-benzoate. Anti-p-azobenzoate BGG serum was incubated with dilutions of p-aminobenzoate-14C (specific activity 13.5 mc/mM). The experiment was repeated several times and each time the log-log plot had a slope of 1. Binding constants were from $10^5$ to $1.6 \times 10^5$; this is about an order of magnitude higher than expected from previous results.

Six series of tubes were set up and buffer was added to one set. Five substituted benzoates were added at three concentrations each to the other five series of tubes. The measured binding was the same within experimental error for each series including the buffer control. This showed that inhibition did not occur. The results were nothing like calculations predicted for competitive binding (42). The binding observed was probably a result of non-specific absorption of the
hapten to precipitated protein and precipitation of hapten caused by the decrease in pH when the ammonium sulfate was added. This method does not appear to be applicable to substituted benzoic acids and may only work for special cases like dinitrophenyl haptens.
Materials and Methods

Most of the materials and methods used in this investigation are described in the previous chapters.

Reagents

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl was lot number SN1895 from Ott Chemical Company, Muskegon, Michigan.

Poly-l-lysine had an average molecular weight of 62,000 as the hydrobromide, and poly-l-glutamic acid had an average molecular weight of 59,000. Both of these polypeptides were products of Yeda, Ltd., Rehovoth, Israel.

Both 4-aminosalicylic acid and 5-aminosalicylic acid were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. There were no significant impurities in the 4-aminosalicylic acid. Both crystals and solutions of the 5-aminosalicylic acid were colored; this color was not removable by either recrystallization or acid precipitation.

Methods

Aniline-ω-methylsulfonate (43).

1.1 equivalents (1.14g) of sodium bisulfite in a saturated
solution was added to 1 equivalent (8ml) of formalin. After standing 30 minutes, 1 equivalent (9.3g) of aniline was added and the mixture was heated to 75 - 80° with stirring until it became clear. It was then cooled and a mass of crystals formed. The crystals were filtered off, washed in ice water and ether and finally vacuum dried.

Glutaraldehyde Crosslinking (23)

8ml of 1M dibasic potassium phosphate was added to 50ml of p-azobenzoate BGG containing 350mg, and the pH was adjusted to 6.4 with 1M acetic acid. 2ml of 2.5% glutaraldehyde was dropped in over 10 minutes. The pH was reduced to 5.9 and then to 5.5 until slight cloudiness appeared. 7ml of 1M lysine HCl was then added. After stirring for 15 minutes, the crosslinked antigen was extensively dialyzed.
Conclusions

Protein conjugates were prepared with p-aminosalicylic acid and various carriers using azo coupling, carbodiimide coupling and mixed anhydride coupling by ethyl chloroformate. These antigens were injected into rabbits and guinea pigs and no anti-hapten IgG or IgM response was obtained. Delayed hypersensitivity was produced in guinea pigs by immunization with the conjugates, and its specificity was determined. Guinea pigs were not sensitized by injections or topical applications of p-aminosalicylic acid or p-aminosalicylate.
REFERENCES


