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Synthesis of 6-Aminopenicillanic Acid-Protein Conjugates for Development of Enzyme Immunoassay for B-Lactam Antibiotics

Alice Ann Heth
Utah State University

SYNTHESIS OF 6-AMINOPENICILLANIC ACID-PROTEIN CONJUGATES FOR DEVELOPMENT OF ENZYME IMMUNOASSAY FOR β-LACTAM ANTIBIOTICS

by

Alice Ann Heth

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Alice A. Heth

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LIST OF ABBREVIATIONS

AMPC	ampicillin
APA	6-aminopenicillanic acid
BGG	bovine gamma globulin
DNB	dinitrobenzene
DTT	dithiothreitol
EIA	enzyme immunoassays
EDC	1-ethyl 3(3-dimethylaminopropyl) carbodiimide
FDNB	fluorodinitrobenzene
HRP	horseradish peroxidase
OVA	ovalbumin
PBS	phosphate buffered saline
Pen	penicillin-G
RSA	rabbit serum albumin
TBS	tris barbituate buffer
TNFB	trinitroflurobenzene
UV	ultraviolet

ABSTRACT

Synthesis of 6-Aminopenicillanic Acid-Protein Conjugates for Development of Enzyme Immunoassay for β-Lactam Antibiotics

by

Alice Ann Heth, Doctor of Philosophy

Utah State University, 1984

Major Professor: Dr. Rodney J. Brown
Department: Nutrition and Food Sciences

An enzyme immunoassay specific for several β-lactam antibiotics rather than individual antibiotics was investigated. The goal to develop an enzyme immunoassay for analysis of a whole class of compounds at one time is different than the goal of most enzyme immunoassays which desire specificity for drugs or hormone levels. Detection of the presence of all β-lactam antibiotics is wanted and identification of specific antibiotics is not needed.

6-Aminopenicillanic acid, the common structural moiety of β-lactam antibiotics was used tin this investigation. Methods of preparation of 6-aminopencillanic acid conjugates and antibodies needed for enzyme immunoassays have been developed. 6-Aminopenicillanic acid was conjugated to ovalbumin and bovine gamma globulin for production of antibodies with specificity to 6-aminopenicillanic acid. 6-Aminopenicillanic acid was also linked to the enzyme horseradish peroxidase for future use in enzyme immunoassays.

Antibodies produced against 6-aminopenicillanic acid are antigenic towards the thiazolidine ring of penicillins as shown by their affinity to ampicillin and penicillin. Anti-6-aminopenicillanic acid antibodies should therefore be antigenic towards other semisynthetic penicillins because 6-aminopenicillanic acid is usually used in their synthesis.

(79 pages)

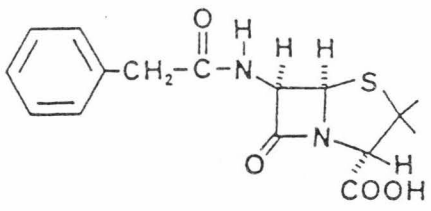
INTRODUCTION

Antibiotics are used for treatment of bacterial mastitis in dairy cows. Residual antibiotics in milk may induce allergic response in susceptible individuals causing a public health hazard. Antibiotics also cause financial losses for both farmers and milk processors by inhibiting the growth of lactic microorganisms in dairy products. An estimated 20 million dollars per year is spent by the U.S. dairy industry to test for and discard contaminated milk (Ryan, 1983). In addition, contaminated market milk exposes consumers and the environment to low levels of antibiotics and enhances the development of antibiotic-resistant pathogenic bacterial strains.

The most frequently used β-lactam antibiotics in mastitis preparations (Figure 1) are penicillin-G, ampicillin, cloxacillin and cephapirin (Herbst, 1982). Penicillin-G is the most commonly used of all antibiotics (Macaulay and Packard, 1981). The Food and Drug Administration standard for antibiotics in milk is zero-tolerance (Kornfeld, 1977). Presence of any penicillin in milk constitutes adulteration.

Several microbial and nonmicrobial assays exist for detection of antibiotics in milk, but it is desired that a rapid and affordable method for an on-the-farm use be developed. Enzyme immunoassay for β-lactam antibiotics could possibly be used to achieve this goal. Preliminary aspects for development of such an assay have been studied. An immunoassay specific for several β-lactam antibiotics rather than individual antibiotics was investigated. 6-Aminopenicillanic acid (APA), the common structural moiety of all

β-lactam antibiotics (Figure 1), was linked to a protein carrier for production of antibodies. Crossreactivity of anti-APA antibodies with β-lactam antibiotics was shown. APA is used for the synthesis of many semisynthetic β-lactam antibiotics, therefore anti-APA antibodies should be antigenic to those antibiotics.



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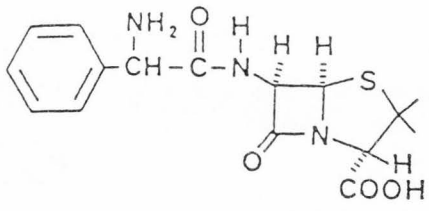

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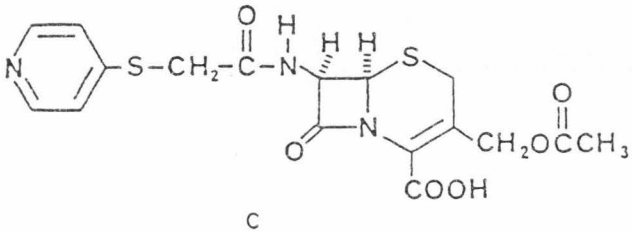

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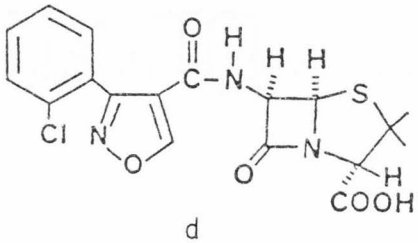

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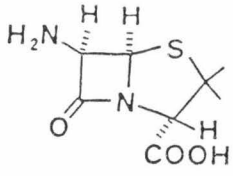

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Figure 1. Chemical structures of a) penicillin-G, b) ampicillin, c) cephapirin, d) cloxacillin and e) 6-aminopenicillanic acid.

REVIEW OF LITERATURE

Occurrence of Antibiotic Contaminated Milk

Antibiotics may be present in milk after treatment of dairy cows. Undetected antibiotics in milk present problems for dairies that attempt to utilize the milk for cultured dairy products. This is due to inhibition of the culture microorganisms. Mastitis is a costly disease for the dairy industry, and has led to the use of antibiotics for treating affected animals. Mastitic milk and milk following antibiotic treatment must be disposed of, thus decreasing production. Occurrence of mastitis is high during the first stages of lactation then decreases until the drying off period when the incidence of mastitis increases dramatically (Natzke, 1981). This has led to the practice of dry therapy where all cows are given antibiotic treatments to reduce the incidence of mastitis within the herd.

Penicillin is the most commonly used antibiotic for treatment of mastitis. Approximately ninety percent of antibiotic products contain penicillin alone or in combination with other antimicrobial agents (Macaulay and Packard, 1981). Manufacturers of antibiotic preparations recommend withholding milk from treated cows after the last injections of antibiotics. Withholding times vary between manufacturers, antibiotic preparations and types of treatment, i.e., intramammary and intramuscular injections (Freke and Bates, 1981; Johnson et al., 1977). The occurrence of antibiotic contaminated milk and laboratory studies indicate that statutory withholding times should be a minimum standard and not the only criteria for forwarding

milk. Secretion of antibiotics varies between animals and is affected by the bases and preservatives added to the antibiotic preparations.

Detection of Antibiotics

New methods for detecting of antibiotics and improving old methods are continually being investigated. The ultimate goal is to develop a rapid and sensitive method to detect residual antibiotics at minimal cost. There are three major classes of methods for antibiotic detection: microbial, non-microbial, and dye marking. The microbial methods are most commonly used. Table 1 is a summary of characteristics of available penicillin assays.

Micobial Assays

Standard Methods for the Examination of Dairy Products (Marth, 1978) describes three methods for detecting of antibiotic residues in milk: (1) Bacillus subtilis disc assay; (2) Modified Sarcina lutea cylinder plate method; and (3) specific antimicrobial cylinder methods (Marth, 1978). Since publication of Standard Methods for the Examination of Dairy Products several procedures with improved sensitivities have been defined.

The Bacillus subtilus disc assay is the assay specified by the Association of Official Analytical Chemists (AOAC) (Kaufman, 1977). Spores of B. subtilus are incorporated into the seed agar. Sterilized paper discs which have been dipped in a standard solution of antibiotics and discs saturated with milk are placed on the agar surface. The plates are incubated at 32°C for 14-24 h. Presence

Table 1. Comparison of characteristics of available antibiotic tests.

Assay	Penicillin Sensitivity (IU/ml)	Time
Disc Assay (*Bacillus subtilus*)	0.05[a]	14-24 h
Cylinder Method (*Sarcina lutea*)	0.01[b]	14-24 h
Disc Assay (*Sarcina lutea* w/lysozyme)	0.0075[c]	14-24 h
Delvotest P Kits (*B. stearothermophilus* var. *calidolactics*)	0.0084[d]	2.5 h
Difco Disc Assay	.005[d]	14-24 h
Charm Test	0.001[e] 0.01-.005[f]	7-30 min
Penzyme Assay	0.008-0.009[g]	20 min
Spot Test	0.008-0.009[h]	6 min

[a]Kaufman, 1977

[b]Johnson et al., 1977; Kornfeld, 1977

[c]Johnson et al., 1977; Macaulay and Packard, 1981; Van O's and Beukers, 1980.

[d]Ginn et al., 1978; Macaulay and Packard, 1981.

[e]Charm, 1980a

[f]Macaulay and Packard, 1981

[g]Smithkline Beckman, 1984

[h]Angenics, 1984

of an inhibitory substance causes a zone of clearing around the discs. The sensitivity of the assay for penicillin is 0.05 I.U./mL.

Sarcina lutea is another indicator organism commonly used for microbial plate assays (Marth, 1978). Agar is seeded with a supension of S. lutea. The cylinder method is usually used with this organism, but the disc method also may be employed. Stainless steel cylinders (8.0 mm O.D. X 10 mm) are placed onto the agar, and are filled with the sample to be tested. The plates are incubated at 32°C overnight. Presence of antibiotic in the sample inhibits the growth of S. lutea, causing a zone of clearing around the cylinder. The literature reports various values for the penicillin sensitivity of the assay, but is generally sensitive down to 0.01 I.U./ml (Johnson et al., 1977; Kornfeld, 1977).

Lysozyme incorporated into the seed agar of S. lutea enhances the sensitivity of the test to 0.0075 I.U. pencillin/ml. (Kornfeld, 1977). Addition of lysozyme increases the zone of inhibition and also increases its transparency. Dilution of standards with milk decreases the size of the zone areas. This is believed to result from binding of some penicillin to milk proteins which decreases the amount of free penicillin in the sample. Both factors affected by addition of lysozyme enhance the detectability of antibiotics in milk. It is proposed that lysozyme enhances lysis after the action of penicillin on the cell wall. At low concentration of penicillin a distinct zone of clearing is not noted, but addition of a glycosidase causes increased lysis due to weakening of the cell wall. The S. lutea

cylinder method is more sensitive than the disc method, but requires more equipment and time to perform.

Bacillus stearothermophilus var. calidolactis is very sensitive to a variety of antibiotics. New methods to store and propogate spores have allowed incorporation of this organism into antibiotic assays. The Delvotest P and Delvotest P multiassays use B. stearothermophilis var. calidolactis as the indicator organism (Huhtanen et al., 1977; Johnson et al., 1977; Macaulay and Packard, 1981; Van Os and Beukers, 1980). This test has the highest sensitivity to penicillin of all the microbiological assays and can also detect fourteen other antibiotics: cloxacillin, nafcillin, ampicillin, tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, dihydrostreptomycin, neomycin, kanamycin, gentamycin, oleandomycin, rifamycin, and erythromycin (Van Os and Beukers, 1980).

Delvotest P kits are commercially available. A 0.1 ml milk sample and a nutrient tablet are placed in an ampul containing 0.3 ml plain agar inoculated with 10^6 stabilized spores of the indicator organism. The agar also contains a pH indicator, bromcresol purple. The ampul is incubated in a water bath at 64°C for 2.5 h. A sample containing an inhibitory substance prevents growth of the spores and the agar remains purple. Growth of the spores causes a decrease in pH and the media turns yellow. An incomplete color change indicates a false-positive result and occurs at low antibiotic concentrations.

The Delvotest is more sensitive than previously mentioned microbial tests and requires only a 2.5 h incubation period. Penicillin concentrations greater than 0.0084 I.U./ml are detected

with 100% accuracy. Lower concentrations increase the occurrence of false-positive tests. In this range (0.002 - 0.004 I.U./ml), penicillin does not inhibit lactic culture growth, but false-positive tests should be treated as positive because the lower limit of human hypersensitivity is not known.

The Difco disc assay is another commercially available antibiotic test which uses B. stearothermophilus var. calidolactis as the indicator organism (Ginn et al., 1978; Macaulay and Packard, 1981). The Difco disc assay is more sensitive than the S. lutea and B. subtilis disc assays. Bromocresol purple is incorporated in the seed agar. A positive test results in a color change and a zone of inhibition. The sensitivity to penicillin has been reported as 0.005 I.U./ml with 100% accuracy (Macaulay and Packard, 1981). Values for penicillin sensitivity as low as 0.002 - 0.004 I.U./ml have been recorded (Ginn et al., 1978). The sensitivity of the test decreases with the age of the agar plate used.

All the microbiological tests can be quantitated by use of standards. A standard curve for each test can be determined by a series of assays of samples with known concentrations of antibiotics. All the methods are more sensitive to penicillin than to other antibiotics, but the assays will detect other antibiotics which inhibit their indicator organisms. Microbial assays use penicillinase to identify penicillin as the inhibitory substance.

Non-Microbial Tests

Direct assays for residual antibiotics using non-microbial methods are less common than microbial assays, but they may prove to be more sensitive. Three such assays are now commercially available: the Charm Test (Penicillin, Inc., Boston, MA), Penzyme (Smithkline Beckman Co., West Chester, PA) and the Spot test (Angenics, Cambridge, MA). The Spot test and Penzyme assay have become available within the past year so comparative data for these tests are not available.

The Charm test is a non-microbial assay specific for detection of penicillin (Charm, 1980a; Charm, 1980b). Penicillin-sensitive cells (lyophilized B. stearothermophilus) and C^{14} labelled penicillin are added to samples. Labelled and free pencillin compete for receptor sites on the cell walls. Cells are removed by centrifugation. The precipitate is washed and the amount of C^{14} labelled penicillin present is determined by counting radioactive emissions. The amount of labelled penicillin detected is inversely proportional to the amount of free penicillin in the sample. This test is rapid, requiring less than 30 min. The initial investment for the centrifuge and Geiger counter required is substantial, and expense of each assay is greater than for microbial tests. Proper procedure for disposal of radioactive wastes is required. In comparison with microbial assay standards, 15.6% of samples tested by the Charm test were classified incorrectly (Thorogood et al., 1983). Precision of the test also decreased with age of the reagents. Daily standard curves would increase precision, but also increase time and expense.

The Penzyme assay is based on inhibition of D-ala-carboxypeptidase by binding of β-lactam antibiotics at its active site (Laloux et al., 1982; Smithkline Beckman, 1984). Carboxypeptidase is a penicillin-binding protein isolated from some species of bacteria. In the initial step of the assay, enzyme is incubated with milk for 5 min. Penicillin in the sample partially or completely inhibits carboxypeptidase. Substrate (Ac-L-lys-D-ala-D-ala) and another enzyme system (D-amino oxidase, flavin adenine dinucleotide, peroxidase, and o-dianisidine) are added and incubated at 47°C for 15 min. Carboxypeptidase releases D-ala from the substrate. D-ala is converted to pyruvate and hydrogen peroxide. In the presence of peroxidase and hydrogen peroxide, reduced o-dianisidine is oxidized. Addition of sulfuric acid results in a colored product. Fifty microliters of milk containing greater than 0.017 I.U. antibiotics per ml will totally inhibit the reaction. A semiquantitative estimate of β-lactams in the range of 0.008-0.009 I.U./ml can also be determined.

Enzyme linked immunoassays (ELISA) have been developed for detection of penicillin in milk, and one version of the assay is available commercially from Angenics, Inc. (Brown and Swaisgood, 1982; Miura et al., 1981; Ryan, 1983). ELISA provides a rapid method to detect penicillin, but like the Charm test, this assay is specific only for penicillin. Other antibiotics are not detected.

The Spot test utilizes monoclonal antibodies to detect penicillin-G and cephapirin, a synthetic cepholosporin. Antibiotics are bound to latex particles. Reagents and milk are mixed on a glass

slide. If no antibiotics (penicillin or cephapirin) are present in the test sample, interaction of the antibodies with bound antibiotics causes the latex particles to cluster. Within 4 min uncontaminated samples develop a grainy appearance and samples with antibiotics appear smooth. Free antibiotics bind antibodies and thereby prevent agglutination of the latex particles. The total time for the Spot Test is 6 min.

A sandwich ELISA for detection of ampicillin in milk has been reported (Miura et al., 1981). Ampicillin concentrations as low as 0.01 μg/ml can be detected. β-D-galactosidase is used as the enzyme label with 7-β-D galactopyransyloxy-4-methylcoumarin as its substrate. The activity of the enzyme label was measured by fluorescence intensities.

An ELISA for penicillin has been reported by Brown and Swaisgood (1982). Penicillin concentrations of 0.01-0.05 I.U./ml were detected. Horseradish peroxidase was used as the enzyme label. Using m-phenylenediamine and hydrogen peroxide as co-substrates the enzymatic reaction can be followed spectrophotometrically or visually. This assay is a competitive immunoassay and activity of the enzyme is inversely proportional to penicillin concentration.

Dye-Infusion Methods

Addition of dye to antibiotic preparations used for mastitis treatments is the only indirect method used for detecting antibiotics (MacIntosh and Vilim, 1977; Van Os, 1978). Brilliant Blue F.C.F. is added to antibiotic preparations. The rate of excretion of dye parallels the excretion rate of penicillin. It is only applicable if

intramammary injections are used. The main advantage is that the dye is visible in the milk of individual cows. This method is commonly used in Australia and New Zealand. Main disadvantages are that the rate of excretion of antibiotics can vary and direct controls may be ignored. Thus, contamination from non-dye preparations may go undetected.

Enzyme Immunoassays

The technique of enzyme immunoassays (EIA) has been extensively reviewed (Borrebaeck and Mattiasson, 1979; Carlier et al., 1981; Halbert, 1981; Maggio, 1980; O'Beirne and Cooper, 1979; Pesce et al., 1981; Schuurs and vanWeeman, 1977; Schuurs and vanWeeman, 1980; and Trivers et al., 1983). Development of EIA began in the early 1970's as a method to replace radio-isotopes used as labels for radio immunoassays (RIA). The basic principles of EIA and RIA are the same, but some enzyme labels have an advantage over RIA in that separation of bound and free antigens is not required, which allows for automated systems. EIA reduce equipment cost and also eliminate problems associated with use of radio-isotopes. Common acronyms in the literature for different types of EIA are: ELISA, enzyme-linked immunosorbent assay or enzyme-linked immunospecific assay and EMIT, enzyme-multiplied immunoassay technique. EIA are currently used as analytical tools for determination of many different substances including: drugs, hormones, proteins, and antibodies. Specificity and sensitivity of immunoassays are determined by the binding avidity of the antibody for a specific antigen.

Heterogeneous EIA

Heterogeneous EIA require bound antigen to be separated from free antigen prior to addition of enzyme substrate. The major advantage of separation of bound and free antigens is increased sensitivity by eliminating substances in the sample which may alter enzymic activity (Bastinani and Wilcox-Thole, 1982; Carlier et al., 1981). Enzyme activity is inversely proportional to antigen concentration in heterogeous EIA. Criteria for enzymes chosen for EIA include high specific activity, high rate constants, stability during conjugation, pH optimum compatibility with ligand-antibody binding, easily detectable product, absence of endogenous activity in sample and low cost availability (Guesdon and Avrameas, 1981; Maggio, 1980). Enzymes used for heterogeneous EIA include oxidoreductases (horseradish peroxidase and glucose oxidase), and hydrolytic enzymes (alkaline phosphatase and β-galactosidase).

Competitive EIA (Figure 2) parallel the principles of RIA. Free antigens and labeled antigens compete for antibody binding sites. Sample containing antigen is added to antibodies bound to a solid phase. The bound antibodies are thoroughly rinsed between each step to prevent adhesion of any endogenous proteins to the solid support. Following incubation with enzyme-labeled hapten, enzyme substrate is added. Reactions can be qualitative by visually noting color change and quantitative with spectrophotometric methods.

Sandwich assays are noncompetitive immunoassays. The most common types employ labeled and immobilized antibodies. Figure 3 is a schematic representation of a typical sandwich assay. It is necessary

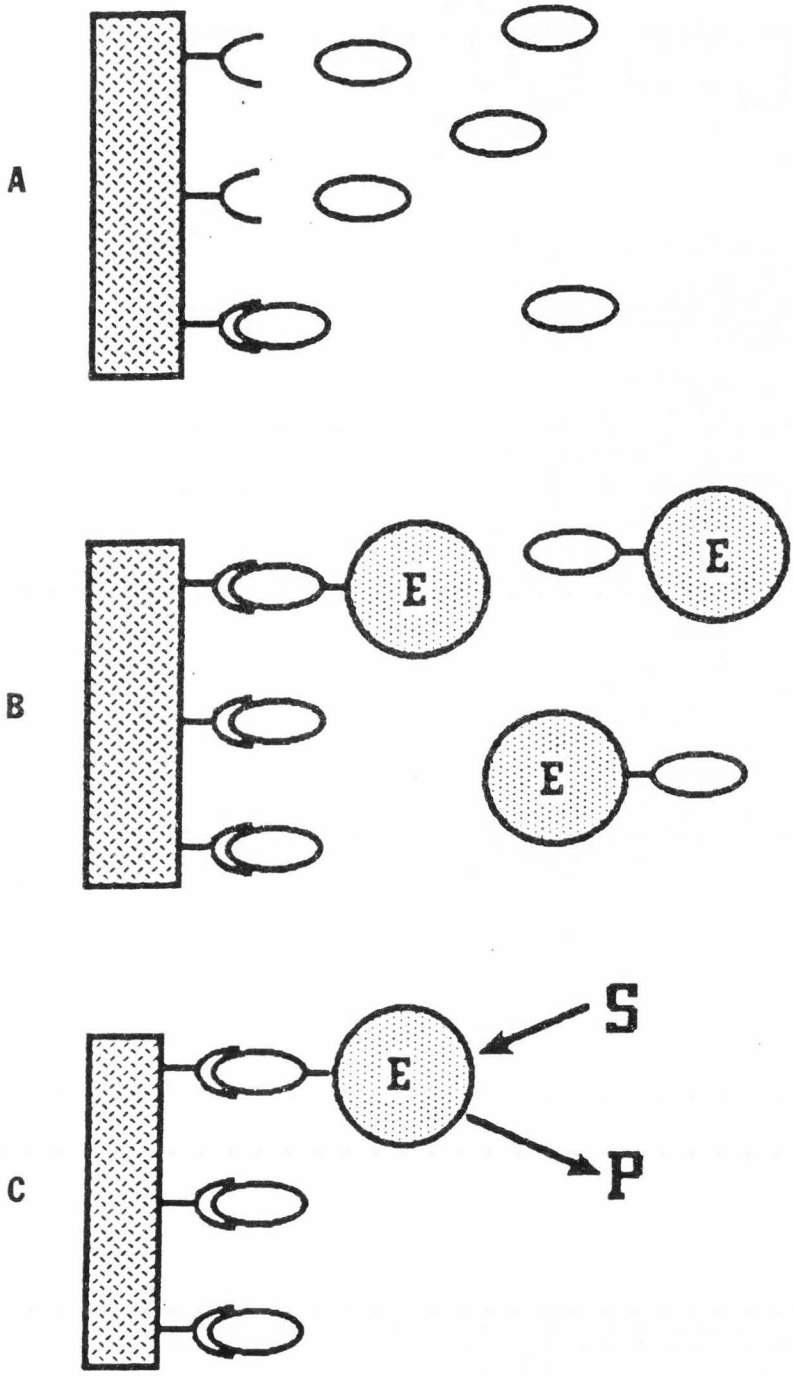


Figure 2. Schematic of competitive EIA. (A) Elipsoids represent haptens binding to antibodies attached to solid support. (B) Enzyme-hapten conjugates are bound by antibodies not bound to free hapten. (C) Substrate is added and enzymic activity is measured by production of product. Enzymatic activity is inversely proportional to free hapten concentration.

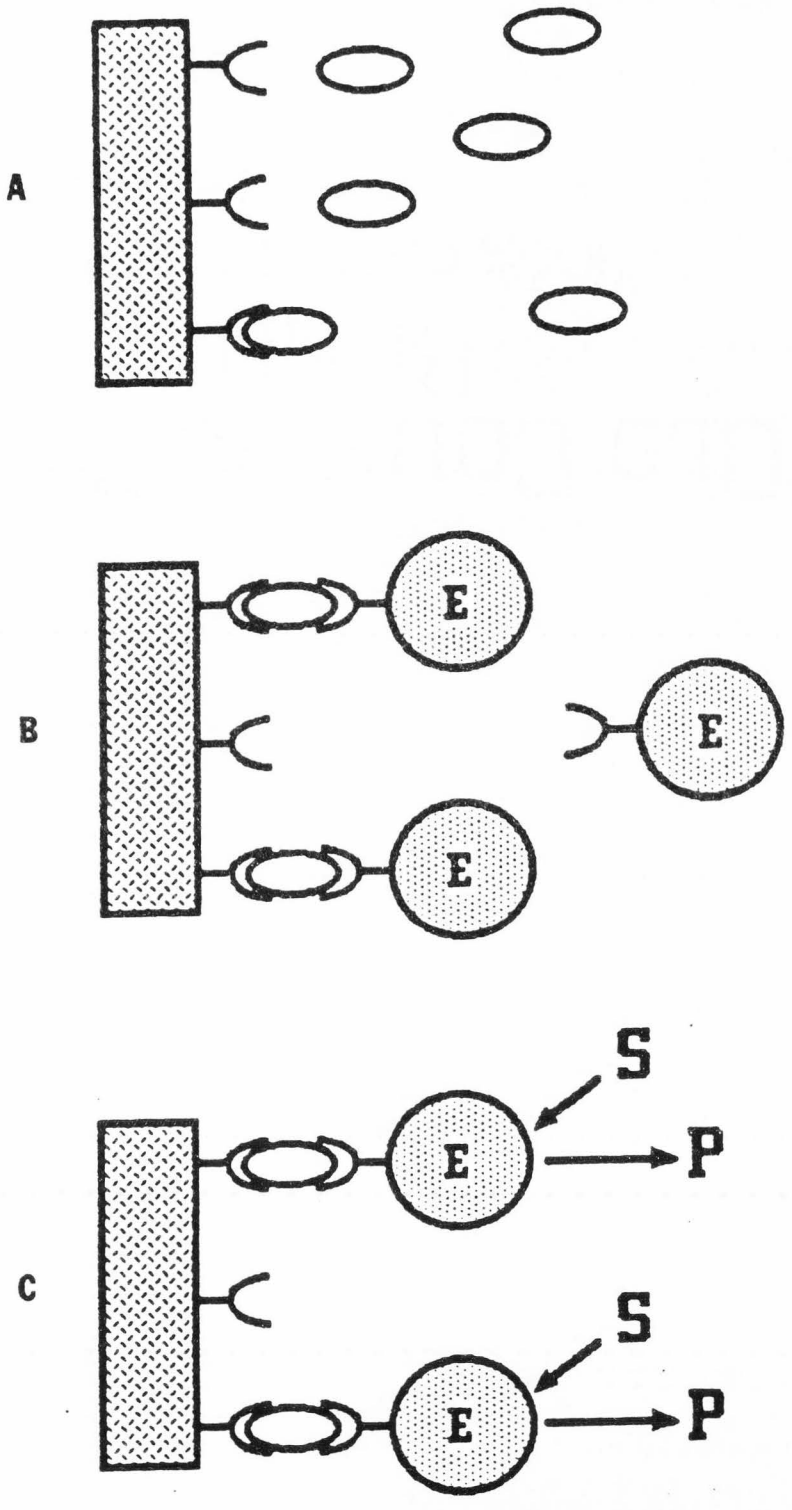


Figure 3. Schematic of noncompetitive EIA. (A) Ellipsoids represent haptens binding to antibodies attached to solid support; (B) Antibodies covalently linked to enzymes bind to haptens bound to solid support by antibodies; (C) Substrate is added and enzymic activity is determined. Hapten, concentration is proportional to enzyme activity.

that the antigen have two antigenic determinants. Antigen is exposed to immobilized antibodies. After rinsing, enzyme labeled antibodies are added. Enzyme activity associated with the solid phase is determined. The main advantage of sandwich assays is that satisfactory results can be obtained without precise adjustment of concentrations of reactants (Schall and Tenoso, 1981).

Homogeneous EIA

Homogeneous enzyme immunoassays do not require separation of bound and free label. In classic homogenous assays binding of antibody to enzyme-labeled hapten causes activation or inhibition of the enzyme (Figure 4). Lysozyme, malate dehydrogenase, and glucose-6-phosphate dehydrogenase are enzymes used for homogeneous EIA (Bastiani and Wilcox-Thole, 1982). Other designs such as substrate-labeled antigen, cofactor-labeled antigen or enzyme-labeled antibody can also alter catalytic rates of enzymes (Bastiani and Wilcox-Thole, 1982). The main advantage of homogeneous EIA is the plausibility of automation.

Methods of Protein Conjugation for EIA

Small molecular weight molecules (haptens) do not illicit antigenic response. Production of antibodies to haptens requires that they be covalently bound to macromolecules. Also, depending on the type of EIA to be used, preparation of enzyme-hapten conjugates of antibody-enzyme (protein-protein) conjugates are required. Many articles and books have been written on methods of chemical modification of proteins which can be employed to synthesize

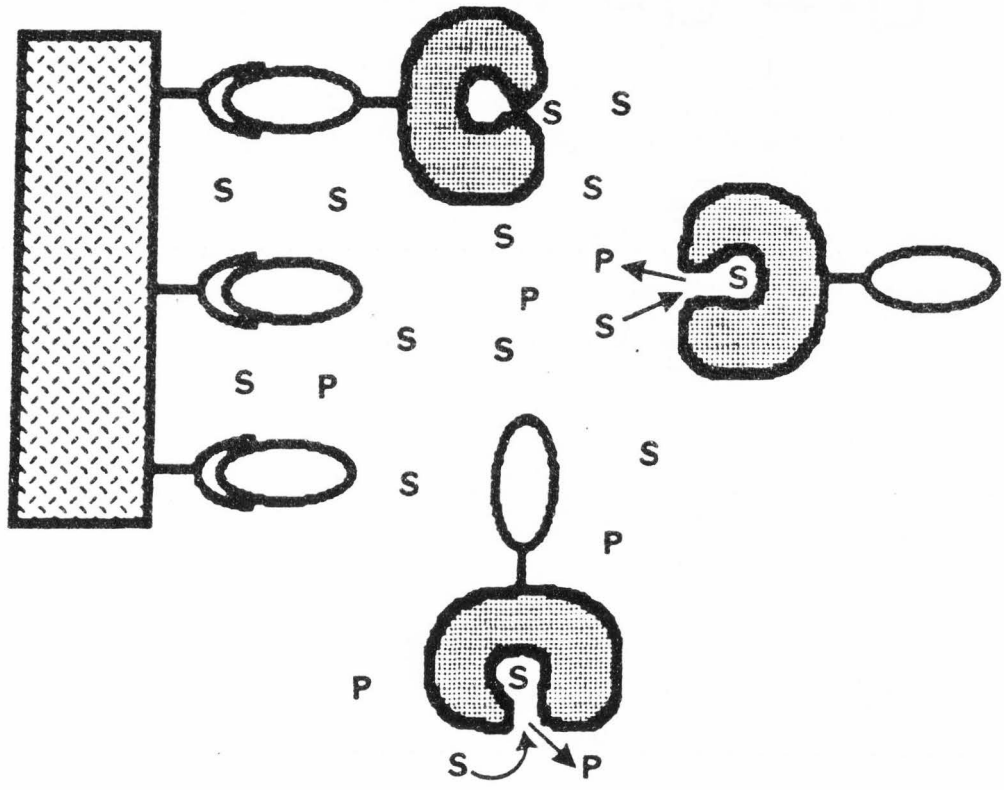


Figure 4. Schematic of homogeneous EIA. Antibodies are attached to a solid support. Free haptens (ellipsoids) and hapten-enzyme conjugates compete for antibody. Antibodies binding to hapten-enzyme conjugates alter catalytic activity of the enzyme.

protein-protein or protein-hapten conjugates (Kabakoff, 1980; Kennedy et al., 1976; Glazer et al., 1975; Means and Feeney, 1971).

Covalent attachment is made via nucleophilic functional groups, i.e., amino, imidizole, guanidino, carboxyl, hydroxyl, and phenolic side chains, or carbohydrate moieties on proteins and enzymes. A general classification of modifing reactions is acylating reagents, alkalating reagents, oxidation and reduction reactions and electrophilic reactions (Kabakoff, 1980). Acylating reagents are used to couple activated carboxyls with free amino groups and phenolic side chains. Deacylation of tyrosine can be achieved by subsequent treatment with hydroxylamine. Acetic anhydride, mixed anhydrides and N-hydroxysuccinimide esters are commonly used acylating reagents.

Alkylating reagents, haloacetate derivatives at alkaline pH and maleimides at slightly acidic pH are used to modify protein sulfhydryls. Maleimides can be used to introduce fluorogenic labels into proteins and bismaleimides to crosslink proteins.

Arylhalides are another group of protein alkylating compounds (Kabakoff, 1980). Fluorodinitrobenzene (FDNB) and trinitrofluorobenzene (TNFB) react with free amino groups at slightly alkaline pH. FDNB and TNBS were among the first haptens attached to proteins for immunological studies and have been used for model studies. Protein crosslinking can be achieved using difluorodinitrobenzene. Another frequently used reaction is reductive alkylation of amino groups with carbonyl compounds using sodium borohydride. This two-step reaction couples carbohydrates to proteins or haptens to glycoproteins.

Oxidative and reductive reactions are used to reduce disulfide bounds in proteins. Non-protein disulfide bonds can be reduced by protein sulfhydryls resulting in linkage via cysteine moieties.

Treatment of amines with sodium nitrite under acidic conditions forms diazonium ions which react by electrophilic substitution with phenolic and imidazol protein side chains, and to a lesser extent with lysine. Optimum pH for the reactions is 9 (Means and Feeney, 1971). Bis diazonium ions can be used to crosslink proteins.

Hapten-Protein Conjugates

Hapten-protein conjugates are used to produce antibodies in animals. Choice of protein is based on cost, availability, and ability to elicit antigenic response in the host animal. Hemocyanins, high molecular weight, copper-containing proteins, isolated from invertebrates are frequently used for this purpose. Haptens may have to be modified to introduce reactive groups or spacer arms prior to conjugation. Selection of methods for conjugation depends upon type of hapten and protein stability (Kabakoff, 1980). In preparation of hapten-enzyme conjugates mild procedures are used to minimize loss of enzymic activity. It is advantageous to use different procedures for synthesis of hapten-protein and hapten-enzyme conjugates. Heterology of hapten conjugates improves sensitivity of immunoassays by increasing competition for free antigen (VanWeeman and Schuurs, 1975). Antibodies specific for haptens are sensitive to hapten-protein linkage. Heterology can be achieved by using two different, but similar haptens; bridge heterology, using two methods for hapten

linkage to the same protein functional group; or site heterology, attaching haptens to different sites on the protein.

The type of reaction used for coupling depends on the chemical nature of the hapten. Methods used to link haptens containing acidic groups (natural or introduced chemically) include mixed anhydide reactions, carbodiimide and N-hydroxysuccinimide ester methods. Periodate cleavage and maleimide compounds are two-step linking procedures commonly employed for synthesis of hapten-protein complexes (Kabakoff, 1980).

Penicillin-Protein Conjugates

Reactions of penicillin with proteins were initiated in the early 1960 is for studying penicillin allergies (Williams and Chase, 1967). Primary methods for formation of penicillin-protein conjugates are acylation of protein amino groups and disulfide bonds with cysteine. Penicilloyl conjugates (Figure 5) are formed at alkaline pH by reaction with protein amines, primarily lysine and are believed to be primarily responsible for human allergic response to penicillin.

Benzylpenicillin is first converted to penicillanic acid which is used to prepare penicilloyl-protein conjugates at pH 11.0 in 1 M carbonate buffer (Williams and Chase, 1967). Penicilloyl conjugates can also be synthesized by a similar procedure reacting penicillin-G directly with protein (Brown and Swaisgood, 1982).

Penicillenate-protein conjugates are formed at neutral pH (Figure 5). Free amine groups are blocked to inhibit formation of penicilloyl

(Williams and Chase, 1967). Penicillamine can also be conjugated to proteins via a disulfide linkage (Figure 5).

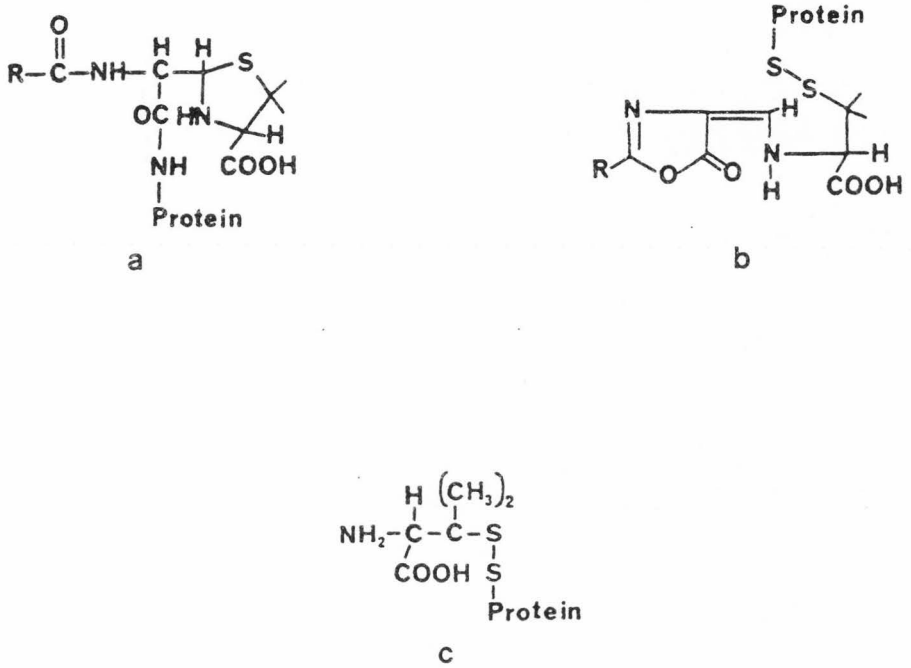


Figure 5. Chemical structures of penicillin bound to protein: (a) penicilloyl; (b) penicillenate and (c) penicillamine.

METHODS AND MATERIALS

Preparation of Antibiotic-Protein Conjugates

Penicillin and ampicillin were attached via the β-lactam ring to free amine groups on proteins (Brown and Swaisgood, 1982; Williams and Chase, 1967). It was necessary to use acid to adjust pH of the protein solution to pH 9.6 when using pH 10.4 sodium carbonate buffer (1.0 M). Addition of acid to highly concentrated protein solution caused precipitation of some protein which decreased yield; therefore, 1.0 M sodium carbonate buffer was adjusted to pH 9.6 prior to addition of protein. Use of sodium or potassium salts of antibiotic rather than penicillin-HCl probably necessitated the pH change. In the final step samples were dialyzed against deionized H_2O using either dialysis tubing or an ultrafiltration cell with 10,000 dalton exclusion membrane. This decreased laboratory time and dialysis using ultrafiltration membranes is as rapid as gel filtration. The protein-antibiotic conjugates prepared; penicillin-G-bovine gamma globulin (Pen-BGG), penicillin-G-rabbit serum albumin (Pen-RSA), and ampicillin-bovine gamma globulin (AMPC-BGG), were used in cross reactivity studies with anti-APA antibodies.

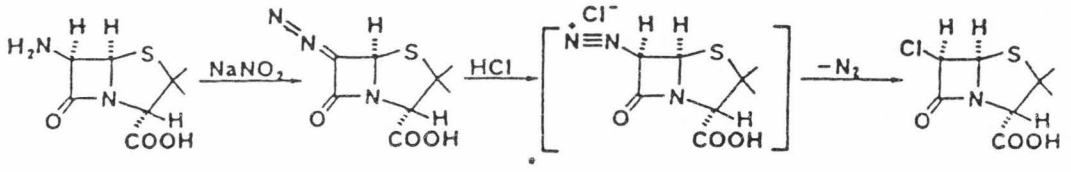
Preparation of APA-Protein Conjugates

Two procedures were used to prepare conjugates of 6-aminopenicillanic acid (APA) and protein used for antibody production. Diazonium derivatives of APA were used for attachment to bovine gamma globulin (BGG), and a sodium periodate procedure was used

to attach APA to egg white albumin (ovalbumin) (OVA) via its carbohydrate moiety.

Diazonium ions react with histidine, tyrosine, and lysine residues of proteins at alkaline pH (Means and Feeney, 1971; Williams and Chase, 1967). Procedures of Cignarella et al. (1962) and McMillan and Stoodley (1968) for synthesis of 6-chloropenicillanic acid and 6-bromopenicillanic acid were modified for synthesis of a diazonium intermediate to react with BGG.

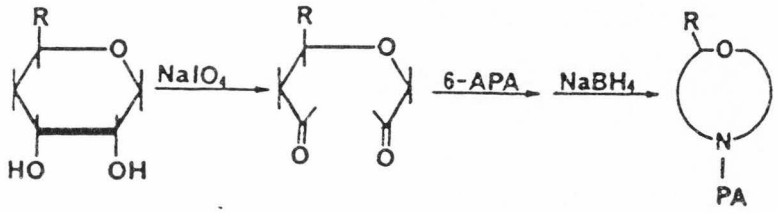
6-Chloropenicillanic acid is synthesized from APA with HCl and sodium nitrite at 0°C. In the proposed mechanism, 6-diazopenicillanic acid is converted to 6-chloropenicillanic acid with diazonium ions as an unstable intermediate (Sheehan et al., 1977; McMillan and Stoodley, 1968; Cignarella et al., 1962).



Equation 1. Synthesis of 6-chloropenicillanic acid.

In preparation of conjugates, 400 mg APA was suspended in 40 ml distilled water, 140 ml methanol, and 20 ml HCl at 0°C. While stirring 2.073 (0.03m) sodium nitrite was added and held for 30 min. The reaction mixture was added dropwise to 1.0 g BGG in 20 ml 0.13 M NaCl and 0.16 M boric acid buffer (pH 9.0). The pH was adjusted to 9.0 with 0.5 N NaOH and held for 1 h. Following dialysis the conjugate was lyophilized and stored at -20°C.

Sodium periodate ($NaIO_4$) cleaves vicinal glycols of carbohydrate moieties forming dialdehydes. Amines can then be coupled by reductive alkylation using sodium borohydride (Kabakoff, 1980).



Equation 2. Sodium periodate method of attaching APA to glycoproteins.

This procedure can be used to attach haptens with free amines to glycoproteins, crosslink glycoproteins to other proteins, or attach carbohydrates to proteins.

Modification of $NaIO_4$ procedures by Nakane and Kawoi (1974) for peroxidase-antibody conjugates and adenosine-protein conjugates (Lauer and Erlanger, 1974) were used to produce APA-OVA conjugates. This procedure was also used to attempt attachment of horsecrab hemocyanin to APA.

Ovalbumin (500 mg) was dissolved in 20 ml 0.1M $NaIO_4$ and stirred in the dark for 30 min at 4°C, after which 200 µl of ethylene glycol was added and stirred for 30 min. Two hundred fifty milligrams of APA dissolved in 0.15 M NaCl and 0.01 M MgCl buffer (adjusted to pH 9.0 with 3% K_2CO_2.) was added dropwise to protein solution. The pH was adjusted to 9.0 and held at 4°C for 1.5 h. Sodium borohydride (250 mg/2 ml H_2O) was added dropwise and the reaction mixture was

refrigerated overnight. Following dialysis the protein-hapten complex was dialyzed, lyophilized and stored at -20°C.

Determination of Bound Antibiotics

Bound haptens with ultraviolet (UV) absorbances can be determined by comparing spectra of conjugates to free protein and free hapten (Garvey et al., 1977). UV spectra of 1.0 or 2.0 mg per ml solutions of penicillin, ampicillin, BGG and antibiotic protein conjugates were obtained using a Beckman DU8-B spectrophotometer interfaced to a Textronics computer for data collection and storage. By comparison of non-overlapping maximum absorbances of haptens and protein solutions to conjugates, concentrations of each were calculated utilizing a computer program (Appendix I).

Preparation of Antibodies

Time for production of useful concentration of antibodies by rabbits is affected by the number of haptens attached to the protein carrier, protein used as the carrier, and also can vary greatly between rabbits (Odell and Franchimont, 1983). Several methods of injections and schedules for antibody production have been suggested, but due to antigen and animal variability no "best" procedure can be determined (Garvey et al., 1977; Odell and Franchimont, 1983).

APA-OVA and APA-BGG conjugates were used as antigens for the production of antibodies in rabbits. Injection schedules and dosages were varied throughout the project. The procedure which proved most successful follows. For initial injections, hapten-protein conjugates

were dissolved in PBS buffer, which was emulsified one to one with Freud's complete adjuvant with a final conjugate concentration of 25 mg/ml emulsion. One milliliter emulsion was injected intramuscularly at 5-6 sites. A 2 mg conjugate/ml emulsion of one part conjugate in buffer and one part Freud's incomplete adjuvant was used for boosting injections. One milliliter of emulsion was injected intramuscular and subcutaneous at several sites. The boosting injections were given 2 weeks after initial injection. Serum was collected and checked for titer 10 days after an injection.

Titer of the antibody against the antigen was determined using Ouchterlony gel diffusion plates. A 1% agarose gel was prepared with 3% polyethylene glycol incorporated into the gel to enhance bands of precipitation (Harrington et al., 1971; Wallenborg and Andersson, 1978). Doubling dilutions of a starting solution of 1 mg protein or conjugate per ml tris barbituate buffer (TBS) were used for antigens in outer wells. Ten microliters were added to each well.

Purification of Antibodies

Affinity chromatography has been used to isolate antibodies to specific antigens (Brown and Swaisgood, 1982; Heth and Brown, 1983). An affinity column for isolation of anti-APA immunoglobulins was prepared by attaching APA to thioester-derivatized glass beads (Brown et al., 1979). Thioester-derivatized glass beads attach to compounds via free amines. Penicillin-protein conjugates were used in preparing an affinity column by this method. APA was attached directly to thioester-derivatized glass beads (Figure 6).

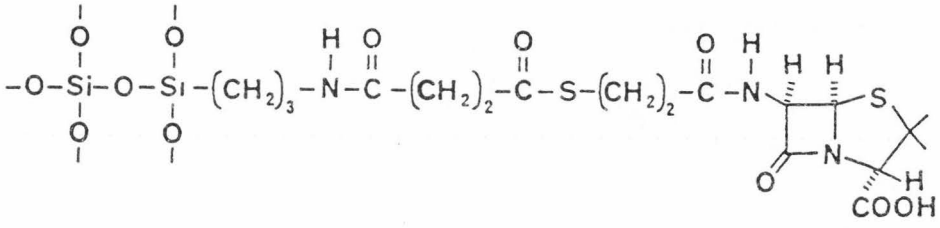


Figure 6. Chemical structure of APA covalently bound to thioester-derivatized glass beads used for affinity columns.

Two hundred seventy-five milligrams of APA were dissolved in 10 ml 0.01M phosphate buffered saline (0.15 M NaCl, pH 7.5) (PBS), to which 5.5 g of thioester-derivatized glass beads were added and degassed for 30 min. The beads in solution were then mixed using a shaker overnight at 4°C.

Ten centimeter columns with an interior diameter of 1 cm were filled with 6 ml of APA-thioester glass beads and equilibrated with PBS. Serum (1 or 0.5 ml) was allowed to filter into the beads, and then mixed through the column using a pasteur pipette. After addition of approximately 1 ml of degassed PBS to remove any air pockets, the column was held at room temperature for 1-2 h.

Affinity columns using glass beads have very rapid flow rates, therefore if the serum is not allowed to stand in the column the total exposure time of antibodies to antigen is only 5 to 10 min. Incorporating a holding step into the procedure increased the amount of specific antibody obtained. This would not be necessary if larger columns with slower flow rates were used.

A peristaltic pump adjusted to maintain a flow rate of about 1.5 ml/per min was used. After elution of the non-binding serum components from the column, antibodies were eluted using 0.1 M acetic acid. Decrease in pH interferes with antigen-antibody bonds. Peaks containing antibodies were collected and diluted with an equal volume of 0.1 M sodium carbonate to increase the pH. The solution of antibodies was dialyzed, lyophilized and stored at -20°C.

Affinity columns for isolation of anti-penicillin and anti-ampicillin antibodies were prepared by attaching Pen-RSA or

AMPC-BGG to thioester-derivatized glass beads by the above procedure except 25 ml of a 10 mg antibiotic-protein conjugates per 1 ml PBS were added to 5.5 g beads.

Affinity columns were used repeatedly without appreciable loss of binding capacity and rinsed with 25 ml of 4 M urea (filtered through 0.45 µm filters) after 3 or 4 uses to remove adhered protein. Elution profiles were monitored at 280 nm using a Beckman DU8-B spectrophotometer connected to a Textronic computer which plotted and stored the data (Appendix II).

Affinity Purification of Antibodies with DTT

Antibody avidity is a prime factor in the sensitivity of enzyme immunoassays. It has been reported that exposure of antibodies to dithiothreitol (DTT) increases avidity by cleaving disulfide bonds which give immunoglobulins their characteristic shape and allows for free rotation of both immunoglobin binding areas (Thompson and Jackson, 1984). DTT was added to 500 µl anti-APA sera to a final concentration of 2 mM and held at room temperature for 1.0 h (Reidler et al., 1982). The antibodies were isolated using APA attached to thioester-derivatized glass beads. Anti-APA antibodies purified from 500 µl untreated serum were used for comparison. Antibody peaks were collected and protein content was determined by Bio-Rad protein assays (Bio-Rad Lab., Richmond, CA).

APA-Enzyme Conjugates

Hapten-enzyme conjugates were prepared by three methods. Penicillin-enzyme conjugates was synthesized by a carbodiimide procedure (Brown and Swaisgood, 1982). APA was bound to HRP using modified periodate carbodiimide, and fluorodinitrobenzene (FDNB) periodate procedures (Brown and Swaisgood, 1982; Lauer and Erlanger, 1974; Nakane and Kawai, 1974).

HRP (200 mg) was dissolved 10 ml 0.05 M $NaIO_4$ in PBS and was stirred, protected from light, for 1 h at room temperature. Ethylene glycol (200 µl) was added and stirred for 1 h. The solution was then dialyzed at 4°C in 0.01 M sodium carbonate buffer (pH 9.5). Next 200 mg APA was added and stirred at 4°C until dissolved. Then 200 mg sodium borohydride in 1 ml H_2O was added dropwize. The reaction was held overnight prior to dialysis.

In preparation of FDNB-HRP conjugates, 200 mg HRP was dissolved in 10 ml 0.3 M sodium carbonate buffer to which 4 ml of a 1 percent solution of FDNB in absolute ethanol was added. After 1 h of mixing, 10.0 ml of 0.05 M $NaIO_4$ was added, held at room temperature and protected from light for 1 h. Ethylene glycol (100 µl) was added and mixed for 1 h prior to dialysis against 0.01 M sodium carbonate buffer (pH 9.5). Next 200 mg APA was added and mixed at room temperature for 2-3 h. The reaction was cooled to 4°C and 200 mg sodium borohydride in 1 ml sodium carbonate buffer was added. After 12-16 h the protein solution was dialyzed and lyophylized.

The carbodiimide used to attach HRP-APA was 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). HRP (100

mg) was dissolved in PBS buffer adjusted to pH 4.75. EDC (190 mg) and 250 mg APA were added in that order and stirred for 1.5 h at room temperature. The solution was then dialyzed to remove any unbound APA and lyophilized. HRP-PEN conjugates were prepared by the above procedure using 350 mg penicillin-G.

Enzymic activities were compared using 1.4 mM m-phenylenediamine in 2.8 mM hydrogen peroxide as substrate (Brown and Swaisgood, 1982). Absorbance maximum of 500 nm was determined by UV scan of substrate with enzyme after color had developed for 10 min. APA-enzyme solutions were prepared by adding 25 mg conjugate to 25 ml PBS. After stirring for several hours at 4°C the samples were filtered through glass fiber filters to remove insoluble particles.

Bio-Rad protein assays were used to determine protein content of enzyme solutions after filtration. Three milliliters of substrate and 75 µl enzyme solutions were used for the assay. Change in absorbance at 500 nm was recorded for 5 min at 5 s intervals using a Beckman DU8-B spectrophotometer interfaced with a Textronics computer.

RESULTS AND DISCUSSION

APA-Protein Conjugates

Both APA-conjugates resulted in production of anti-APA antibodies, but the APA-OVA conjugate was more desirable. APA-OVA conjugates dissolved readily in buffer leaving no particulate matter. Also, high titer antisera from rabbits injected with APA-OVA could be obtained 3-4 weeks after the initial injection, whereas it took several months to obtain a high titer from rabbit injected with APA-BGG. This could be due to several factors. APA-OVA could have more haptens attached per mole protein, and the number of haptens attached to protein influences antigenic response to haptens. Also BGG is a mammalian protein which is more closely related to rabbit serum proteins than OVA, an avian protein, and therefore less antigenic in rabbits than OVA. Hemocyanin, a copper protein isolated from invertebrates, is commonly used to bind haptens for antibody production because of its high antigenicity (Garvey et al., 1977). Attempts to utilize horsecrab hemocyanin for conjugation to APA were unsuccessful due to its insolubility during conjugation procedures.

Antibiotic-Protein Conjugates

The number of antibiotics, penicillin-G and ampicillin, attached to proteins were determined spectrophotometrically (Beech, 1975; Appendix I). This procedure cannot be applied to 6-APA since it doesn't have an absorbance band in the UV-region. Figures 7 and 8 are UV-scans of 1.0 mg penicillin per 1.0 ml methanol and 1.0 mg

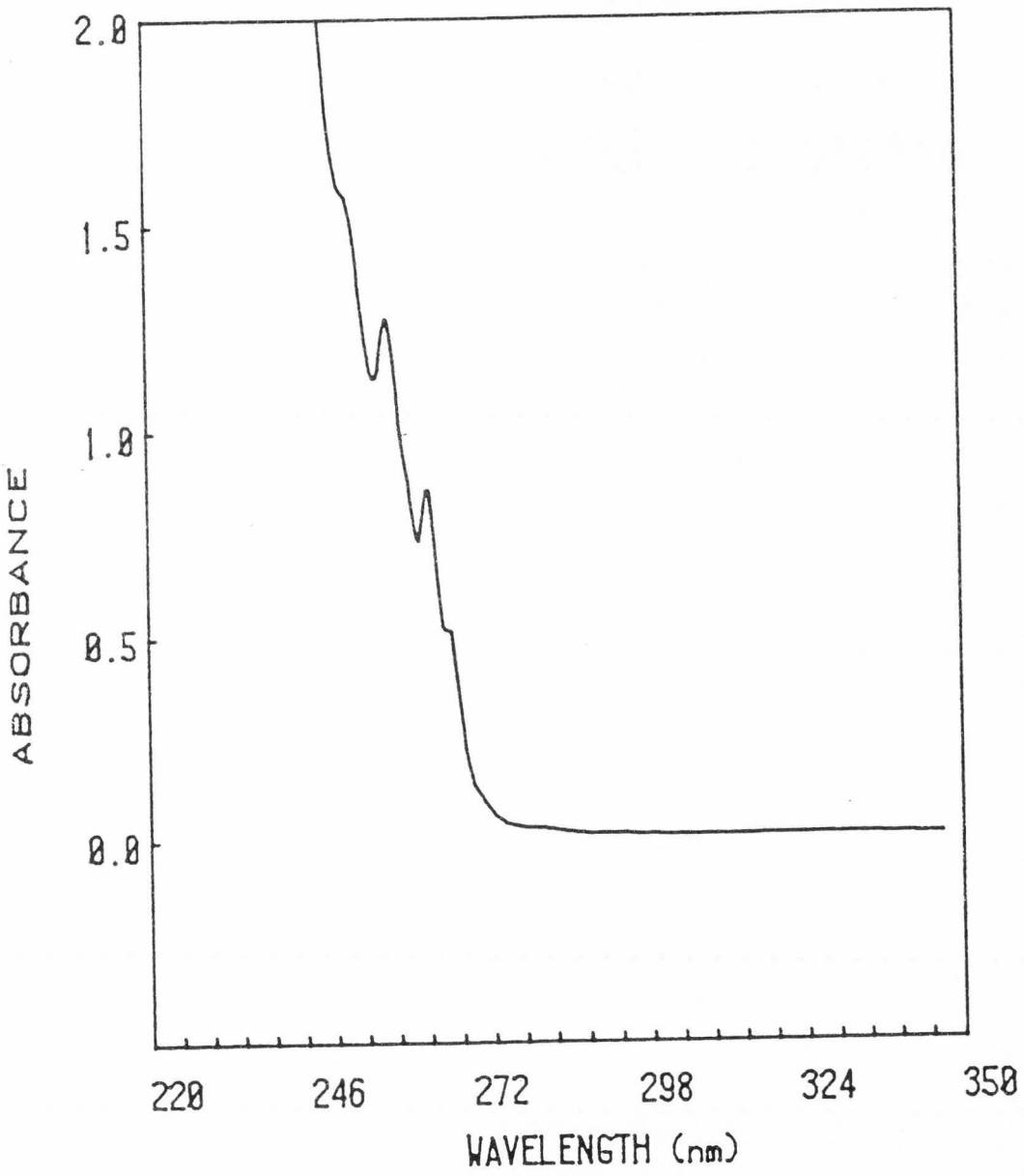


Figure 7. UV scan of penicillin (1.0 mg/ml MeOH). Absorbance peaks are at 259 and 265 nm, and inflection points are 254 and 268 nm.

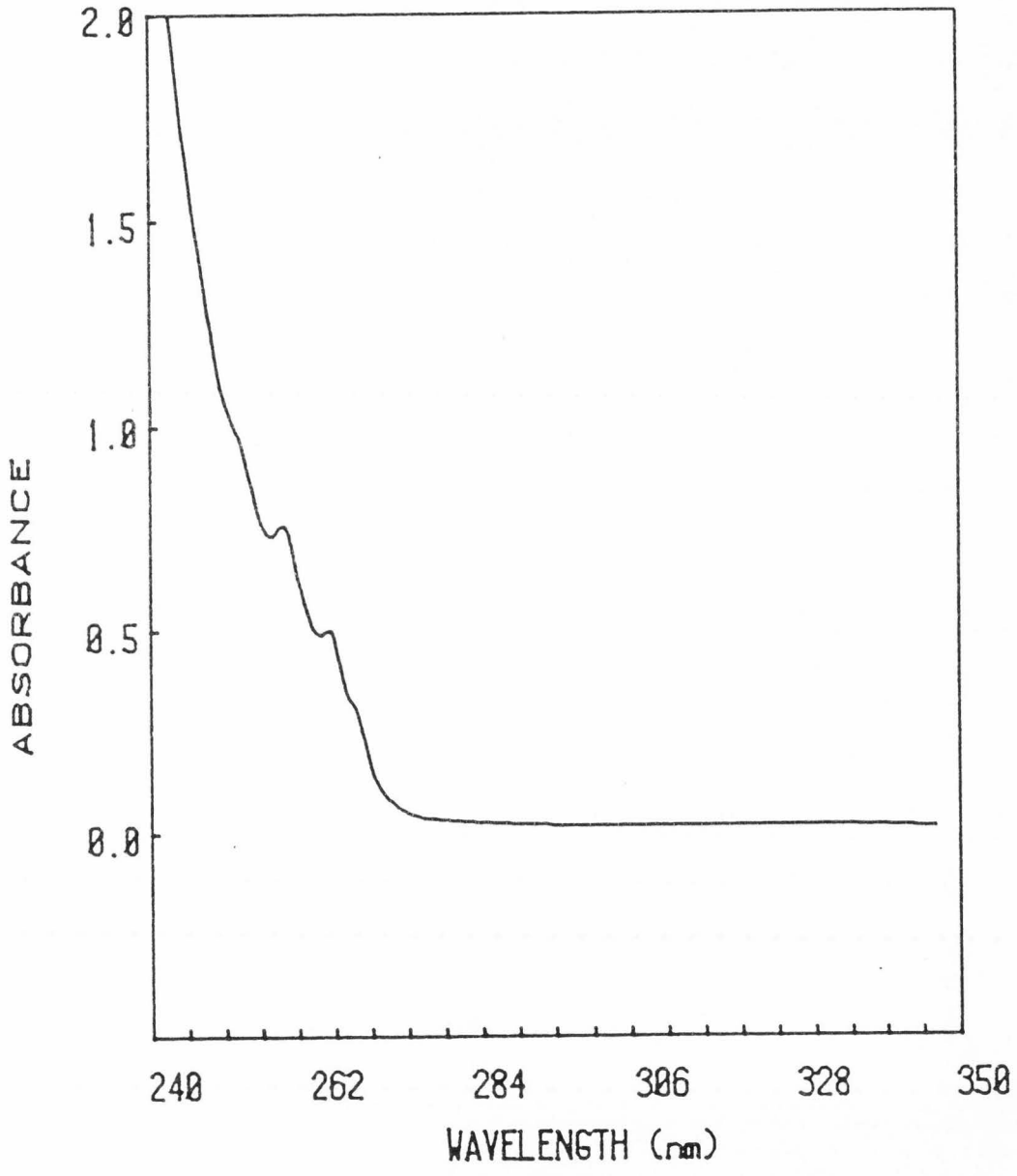


Figure 8. UV-scan of penicillin (1.0 mg/ml H_2O).

penicillin per 1.0 ml water respectively. The peaks are sharper in methanol, but still distinguishable in water. Presence of bound Pen can be determined quantitatively using absorbance at 259 nm for penicillin and 279 nm for BGG by comparison with absorbances of the conjugate at these wavelengths (Figures 8, 9 and 10). Pen-BGG had about 95 moles Pen per mole of BGG. Antibiotic covalently linked to protein can be qualitatively determined by visual inspection of UV-scans. Figures 11 and 12 are ultra violet (UV) scan profiles of AMPC and AMPC-BGG. Inflections of the absorbance profile between 250 nm and 270 nm are visual proof of bound AMPC.

Effect of DTT on Anti-APA Antibodies

Addition of DTT to serum did not improve binding of anti-APA antibodies and actually decreased quantity of antibody isolates. Protein analysis of antibody peaks showed a 25 percent decrease in protein (immunoglobulins) in serum exposed to DTT. Therefore addition of DTT does not improve avidity of anti-APA antibodies and would not be beneficial in EIA using these antibodies.

Immunodiffusion Analysis of Antisera

Ouchterlony (double diffusion-double dimension) technique was used to analyze anti-APA and anti-Pen antisera (Garvey et al.,, 1977). Reactivity of antisera to hapten-proteins conjugates and proteins used in this study were compared. Anti-APA-BGG serum reacted with BGG and APA-BGG but not with Pen-BGG, showing specificity had developed for APA-conjugate and specificity was diminishing for BGG (Figure 13).

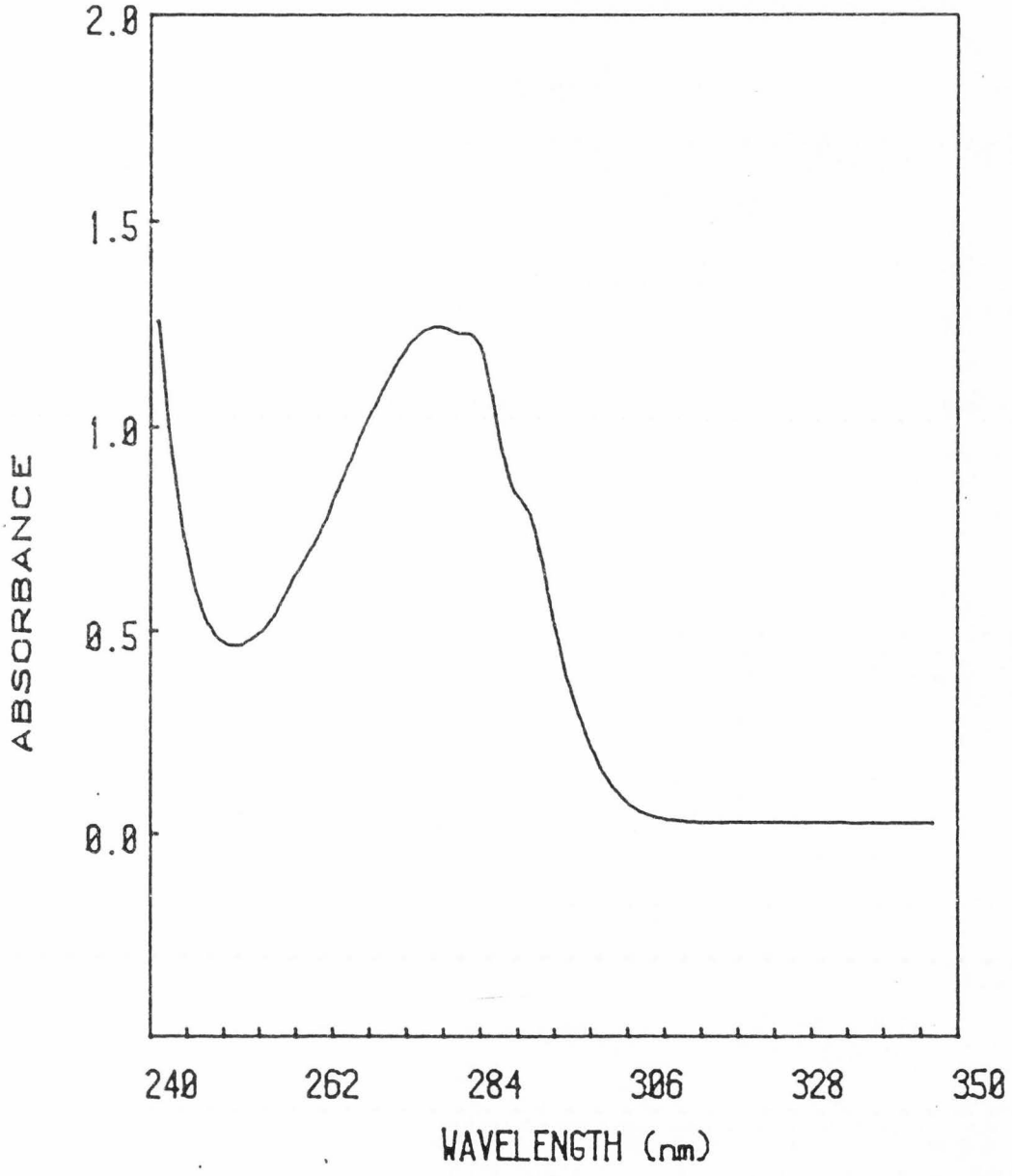


Figure 9. UV-scan profile of BGG (1 mg/ml H_2O).

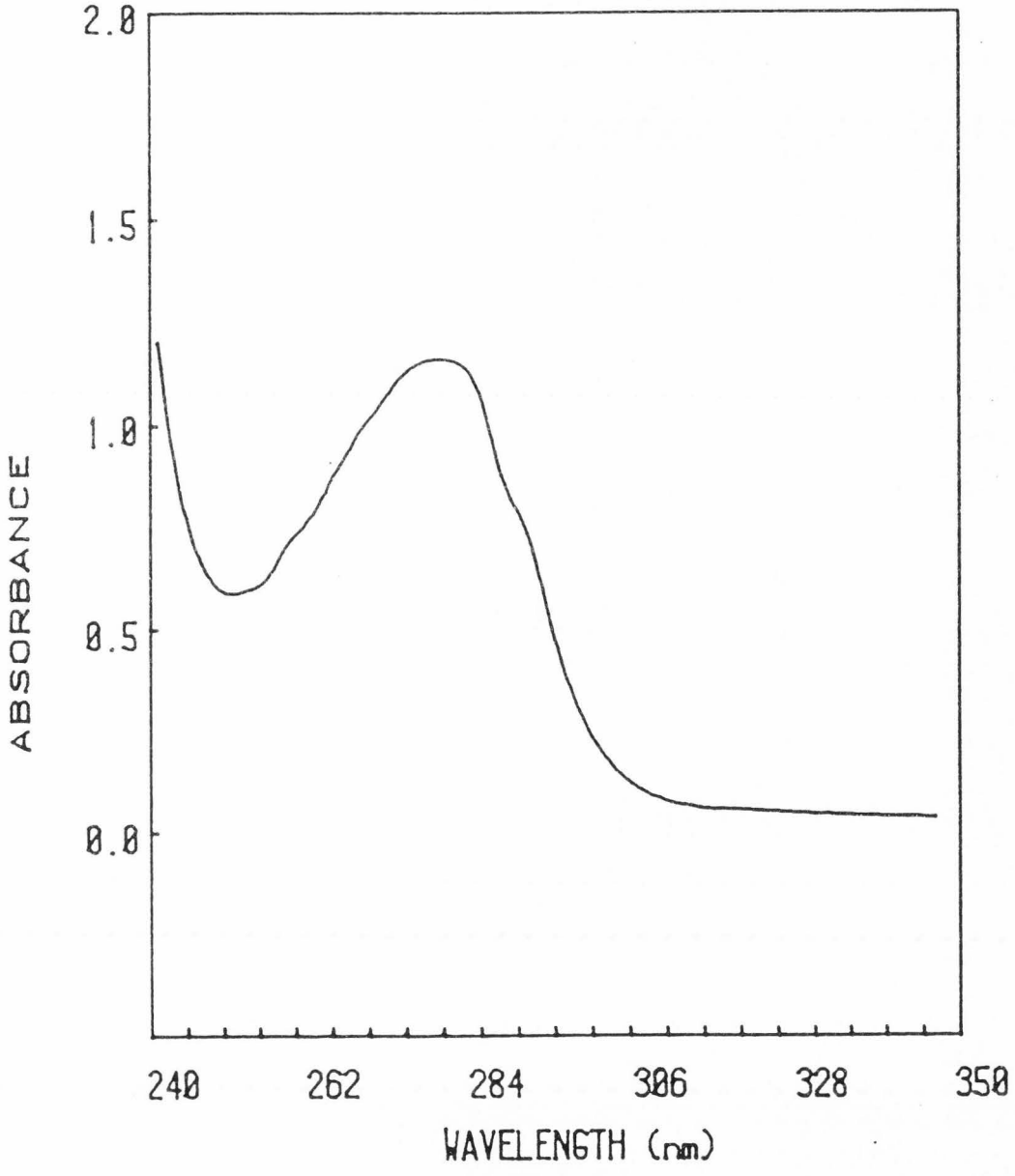


Figure 10. UV-scan of penicillin-BGG (1 mg/ml H_2O).

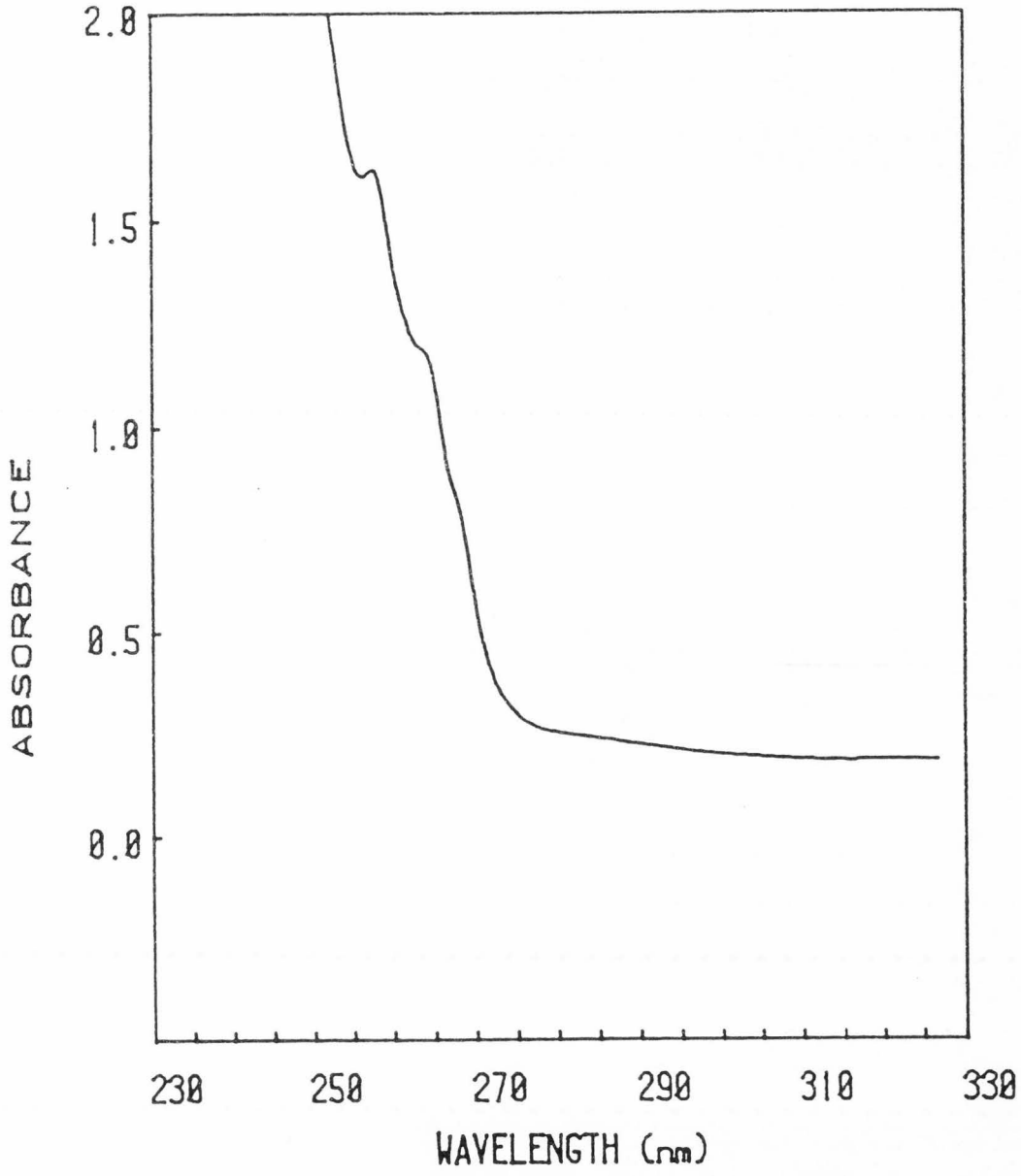


Figure 11. UV-scan of ampicillin (2 mg/ml PBS).

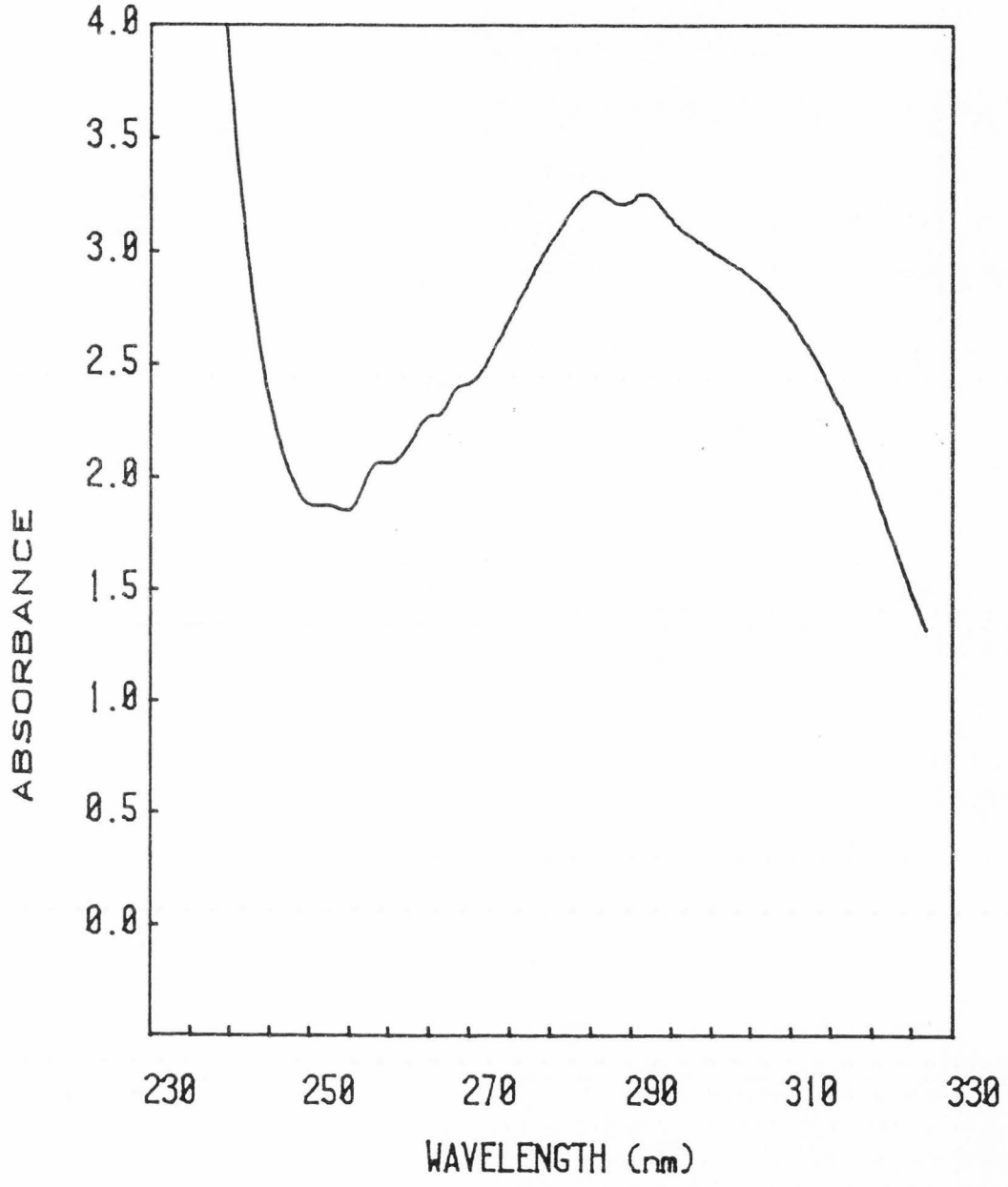


Figure 12. UV-scan of AMPC-BGG (2 mg/ml PBS). The absorbance inflections between 250 nm and 270 nm are visual proof of the presence of bound AMPC.

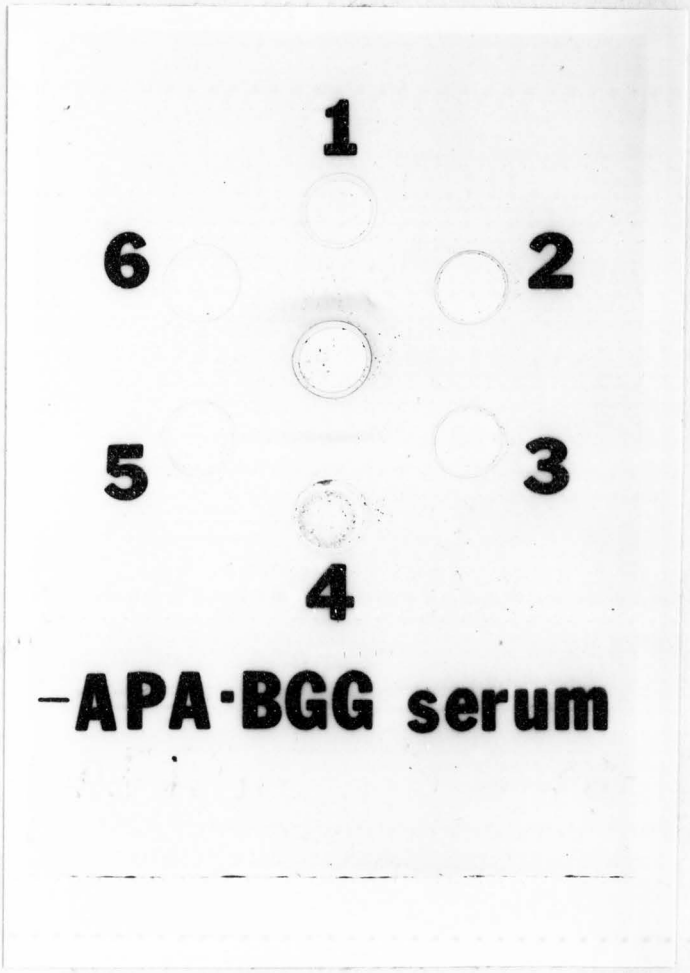


Figure 13. Ouchterlony immunodiffusion gel of anti-APA serum obtained from rabbits injected with APA-BGG. Antigens (1 mg/ml) in outer wells are 1) BGG, 2) Pen-BGG, 3) Pen-RSA, 4) APA-BGG, 5) APA-OVA, and 6) OVA.

APA-OVA antiserum only caused bands of precipitation to OVA and APA-OVA (Figure 14). Level of antigen (1 mg/1 ml TBS) was not appropriate to initiate precipitate to hapten only. Pen-BGG antiserum (Figure 15) precipitates with BGG, Pen-BGG and APA-BGG.

Ouchterlony immunodiffusion was also used to compare titers of hapten-proteins to proteins used for conjugates. Higher titer for hapten-protein than for uncoupled protein indicates development of antibody specificity for haptens. More than one band of precipitation or spurs indicate more than one antibody against the antigen. Narrow sharp precipitate bands indicate near equal concentrations of antigen and antibody and broader bands result from unequivalent concentrations. Figure 17 is the best example of this, as concentration of antibodies and APA-BGG become closer bands are more distinct (wells 3 and 4). This is also an indication of the molecular weight of antigens. In comparison of APA-BGG (Figure 17) and APA-OVA (Figure 18), bands of precipitation to APA-OVA were never as distinct as APA-BGG. The molecular weight of OVA is about 40,000 and BGG is 150,000 which is approximately the same as immunoglobulin-G. Both antisera have titers to the seventh doubling dilution, 0.015625 (not shown) to APA-protein. Antigenicity to hapten-protein was increased above antigenicity to proteins used for conjugation. Bands of precipitation of APA antiserum to BGG were distinct to fifth doubling dilution (Figure 16). APA antiserum produced from APA-OVA only had bands of precipitation against OVA to the third doubling dilution (Figure 19). The high titer of APA-OVA serum for APA-OVA and not OVA indicated its increased specificity for APA (Figures 18 and 19).

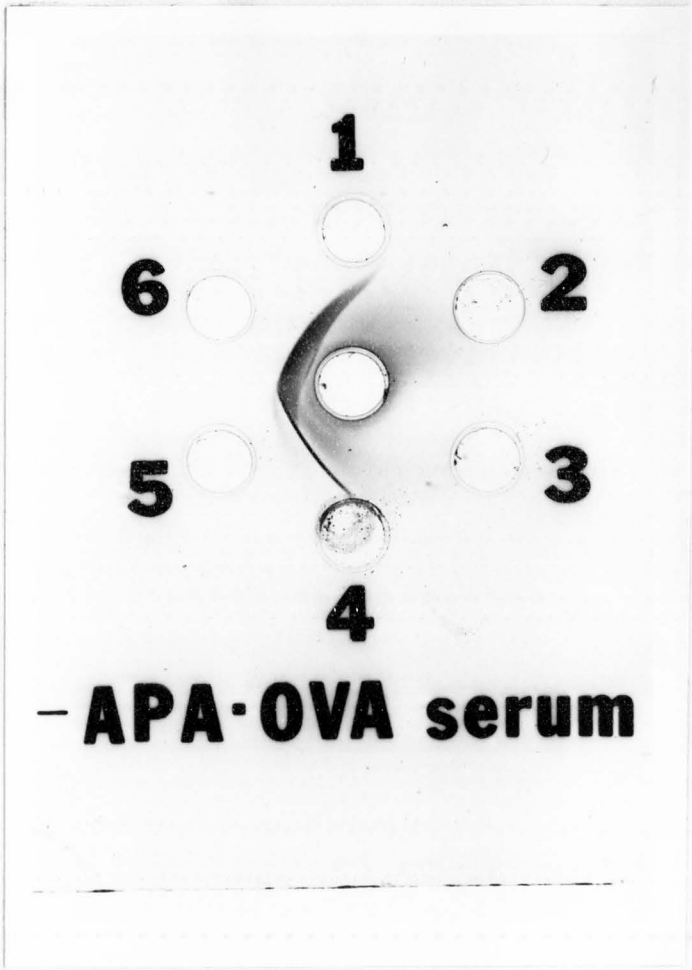


Figure 14. Ouchterlony immunodiffusion gel of anti-APA serum obtained using APA-OVA as antigen. Antigens (1 mg/ml) in outer wells are 1) BGG, 2) Pen-BGG, 3) Pen-RSA, 4) APA-BGG, 5) APA-OVA and 6) OVA.

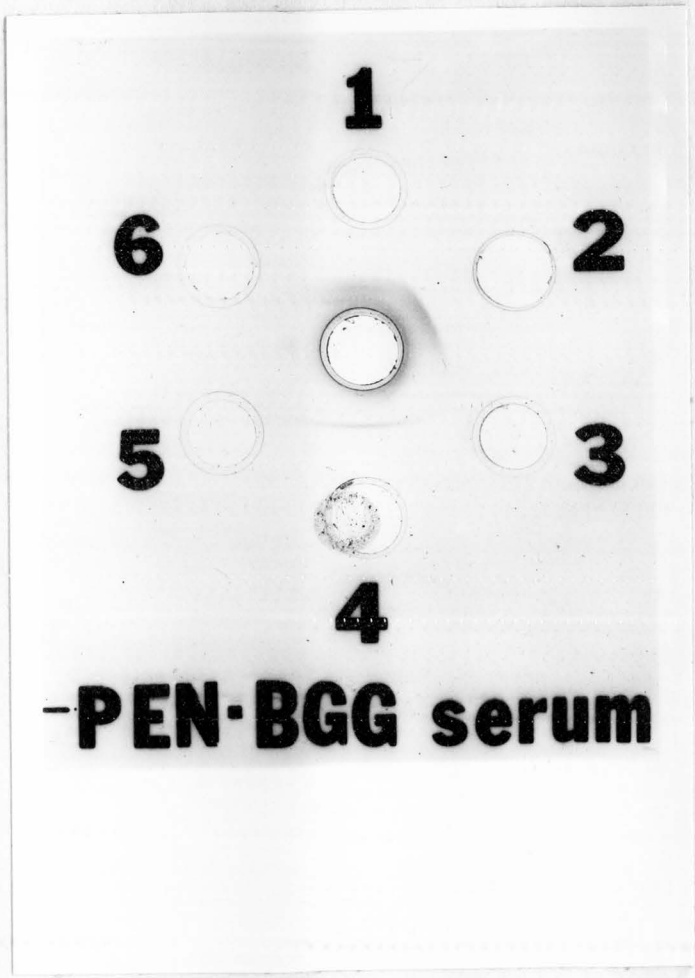


Figure 15. Ouchterlony immunodiffusion gel of anti-Pen serum obtained using Pen-BGG as antigen. Antigens (1 mg/ml) in outer wells are 1) BGG, 2) Pen-BGG, 3) Pen-RSA, 4) APA-BGG, 5) APA-OVA and 6) OVA.

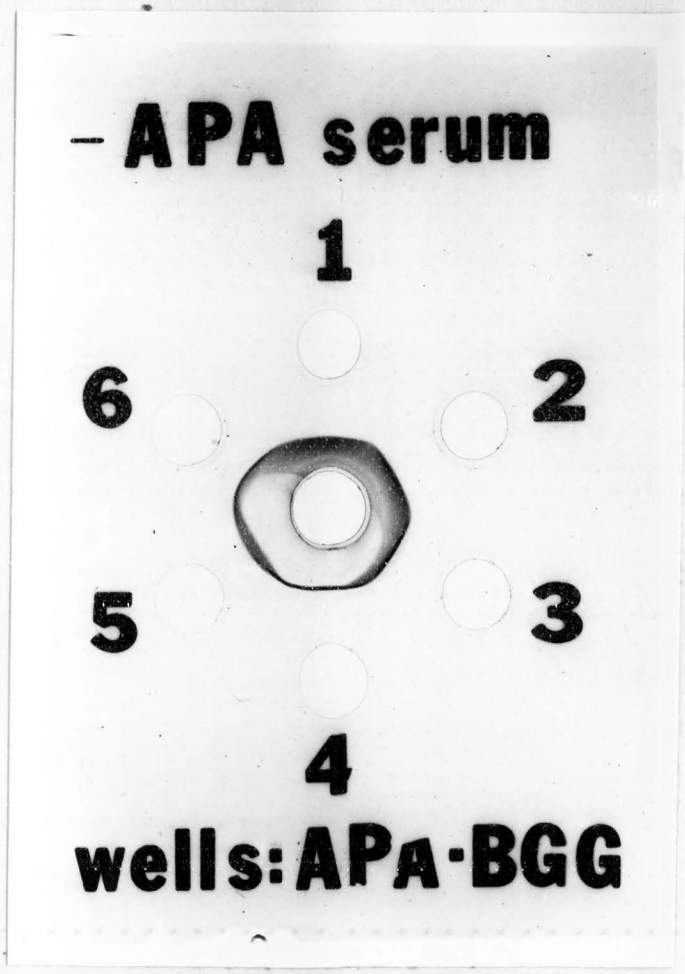


Figure 16. Titer of anti-APA serum (APA-BGG antigen) against doubling dilutions of BGG in TBS. BGG concentrations in outer wells are 1) 1.0 mg/ml, 2) 0.5 mg/ml, 3) 0.25 mg/ml, 4) 0.125 mg/ml, 5) 0.0625 mg/ml and 6) 0.03125 mg/ml.

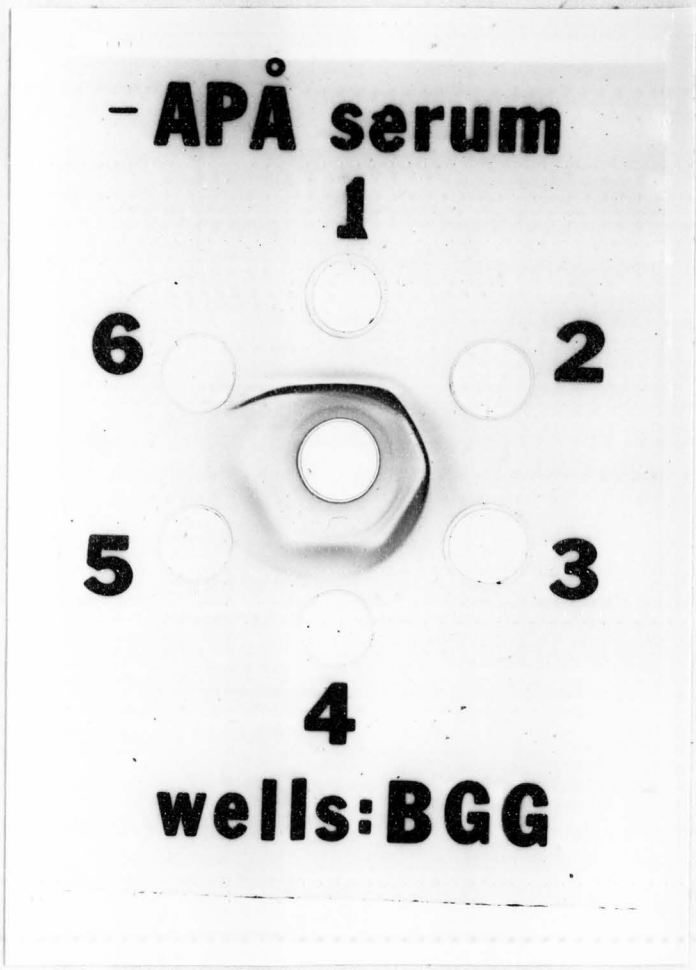


Figure 17. Titer of anti-APA serum (APA-BGG antigen) against doubling dilutions of APA-BGG in TBS. APA-BGG concentrations in outer wells are 1) 1.0 mg/ml, 2) 0.5 mg/ml, 3) 0.25 mg/ml, 4) 0.125 mg/ml, 5) 0.0625 mg/ml and 6) 0.03125 mg/ml.

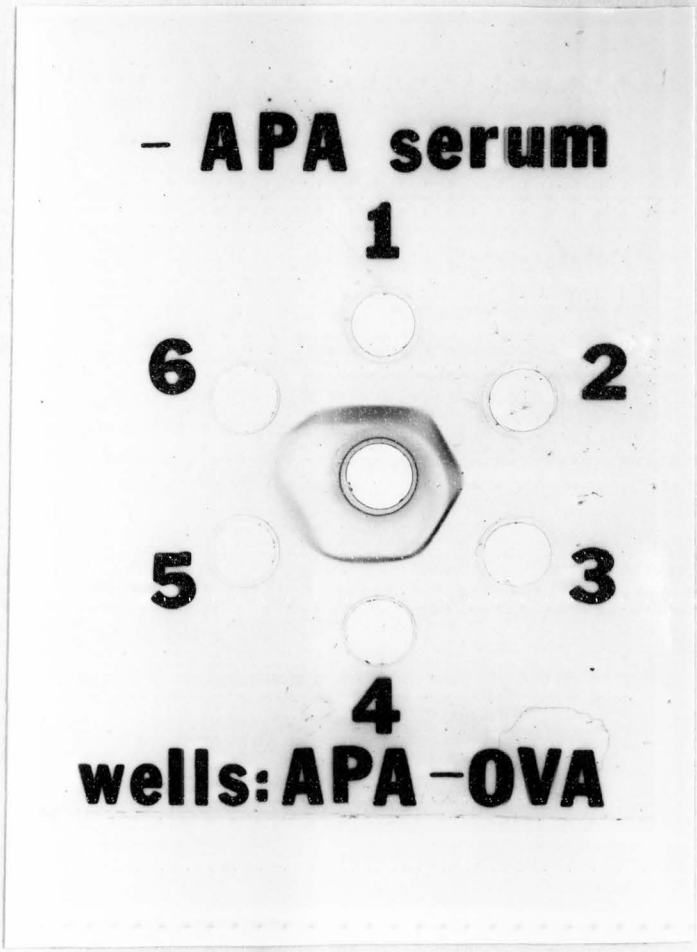


Figure 18. Titer of anti-APA serum (APA-OVA antigen) against doubling dilutions of APA-OVA in TBS. APA-OVA concentrations in outer wells are 1) 1.0 mg/ml, 2) 0.5 mg/ml, 3) 0.25 mg/ml, 4) 0.125 mg/ml, 5) 0.0625 mg/ml and 6) 0.03125 mg/ml.

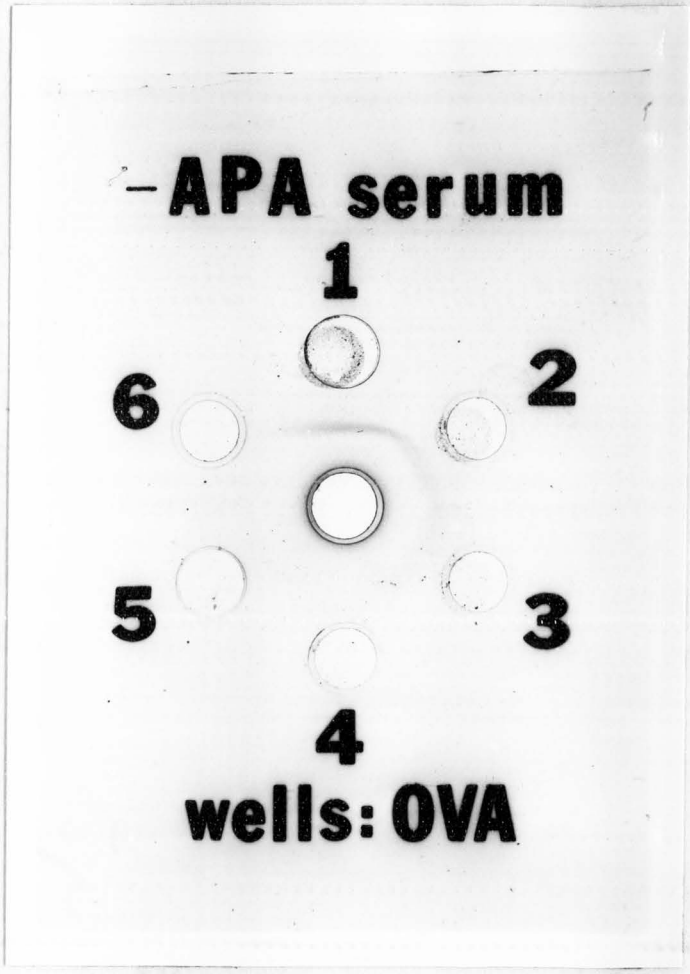


Figure 19. Titer of anti-APA serum (APA-OVA antigen) against doubling dilutions of OVA in TBS. OVA concentrations in outer wells are 1) 1.0 mg/ml, 2) 0.5 mg/ml, 3) 0.25 mg/ml, 4) 0.125 mg/ml, 5) 0.0625 mg/ml and 6) 0.03125 mg/ml.

Purification and Crossreactivity of Antibodies

Antigens covalently linked to glass beads were used to isolate antibodies and to show antigenicity towards antibiotics. Isolation of antibodies to bound antigen verifies existence of specific antibodies and provides proof that newly developed conjugation methods were successful.

APA affinity columns were used to verify presence of anti-APA antibodies (Figures 20 and 21). Presence of anti-penicillin antibodies from antiserum produced from Pen-BGG was verified using a Pen-RSA affinity column (Figure 22). Purified antibodies would aid in calibration of EIA because exact concentration of antibodies used could be determined and controlled.

Avidity of APA-OVA antiserum to Pen and AMPC was determined using a Pen-RSA affinity column and a AMPC-BGG affinity column (Figures 23 and 24). Ability to obtain purified antibodies on these columns from APA antiserum showed that antibodies were specific for the thiazolidine ring of penicillins.

Figure 25 is the elution profile of Pen-BGG antiserum over a AMPC-BGG affinity column. Purified antibodies were obtained, but since BGG was used in preparation of antiserum and affinity column the extent of cross-reactivity based on penicillin cannot be distinctly shown. Although it is surmised that Pen antiserum would cross-react to AMPC to some extent.

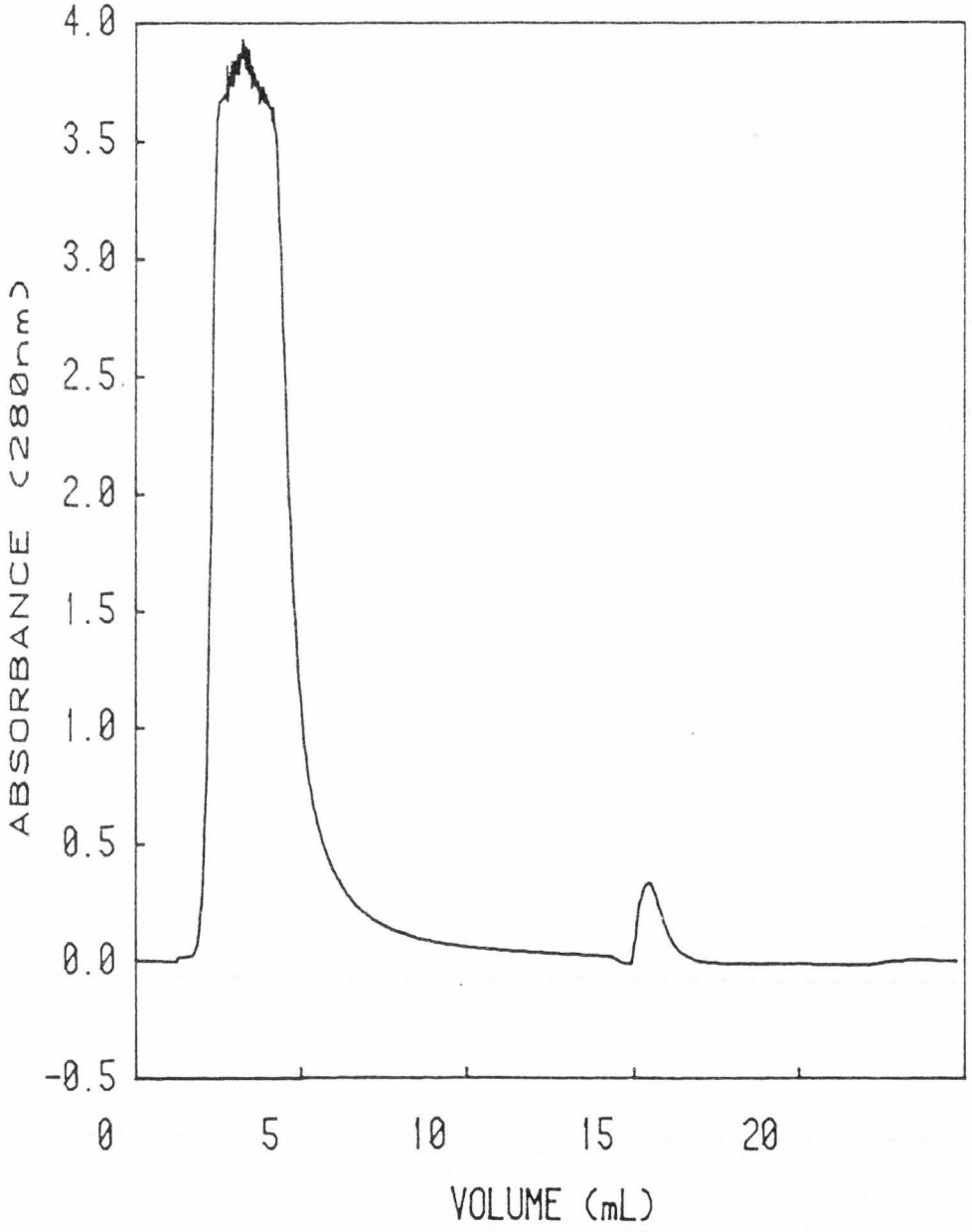


Figure 20. Affinity purification of anti-APA antibodies from APA-BGG antiserum using APA affinity column.

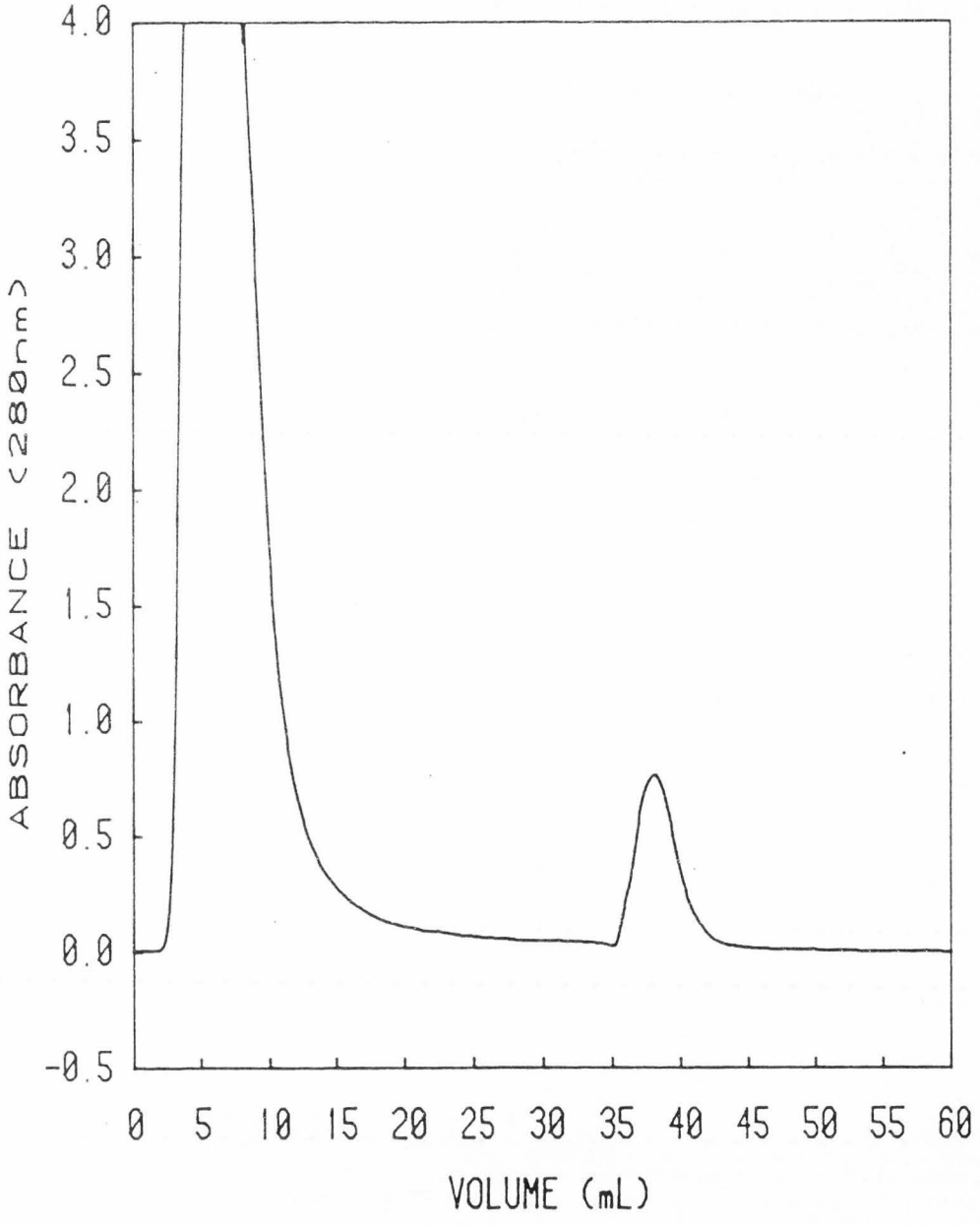


Figure 21. Affinity purification of anti-APA antibodies from APA-OVA antiserum using APA affinity column.

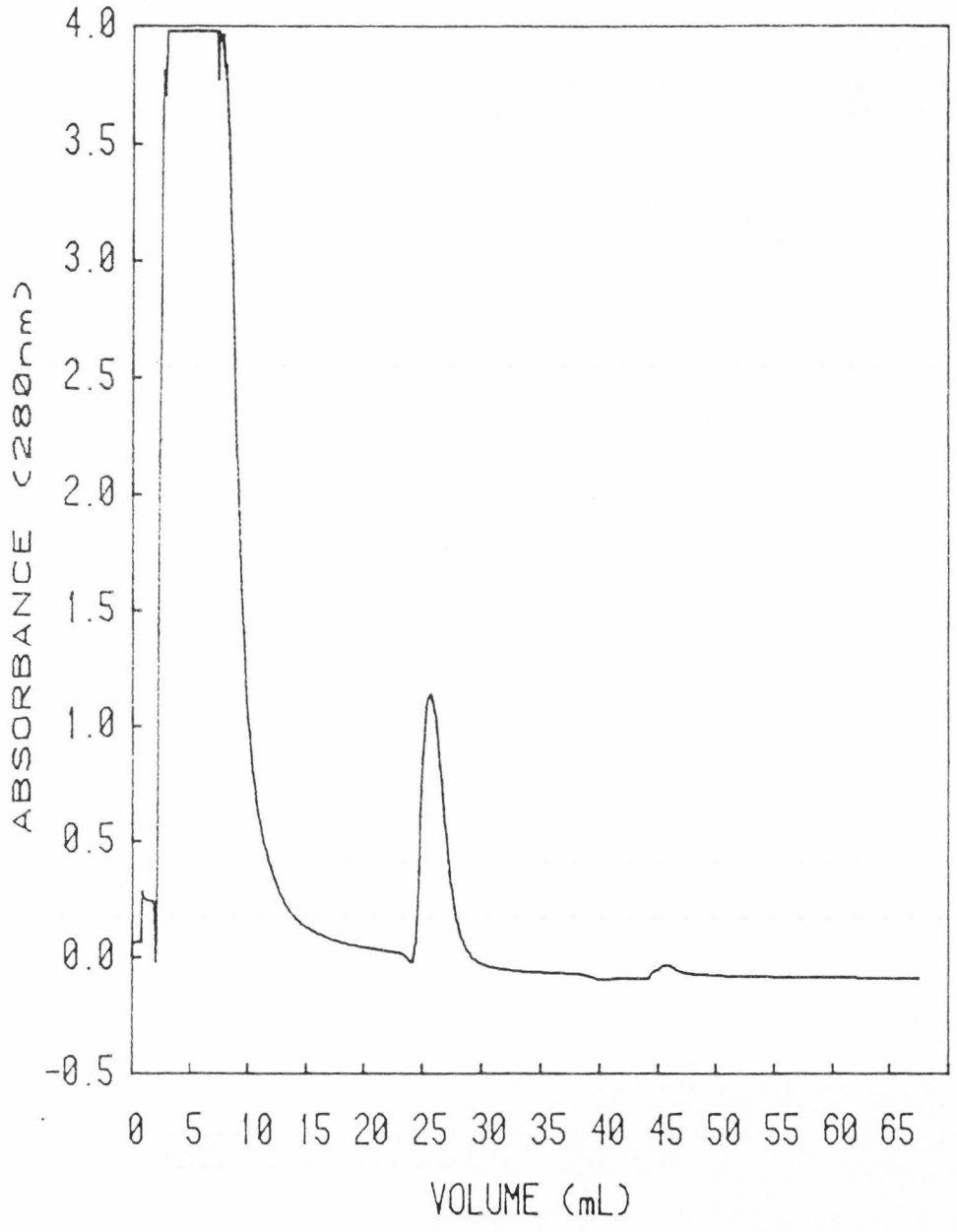


Figure 22. Affinity purification of anti-Pen antibodies from Pen-BGG antiserum using Pen-RSA affinity column.

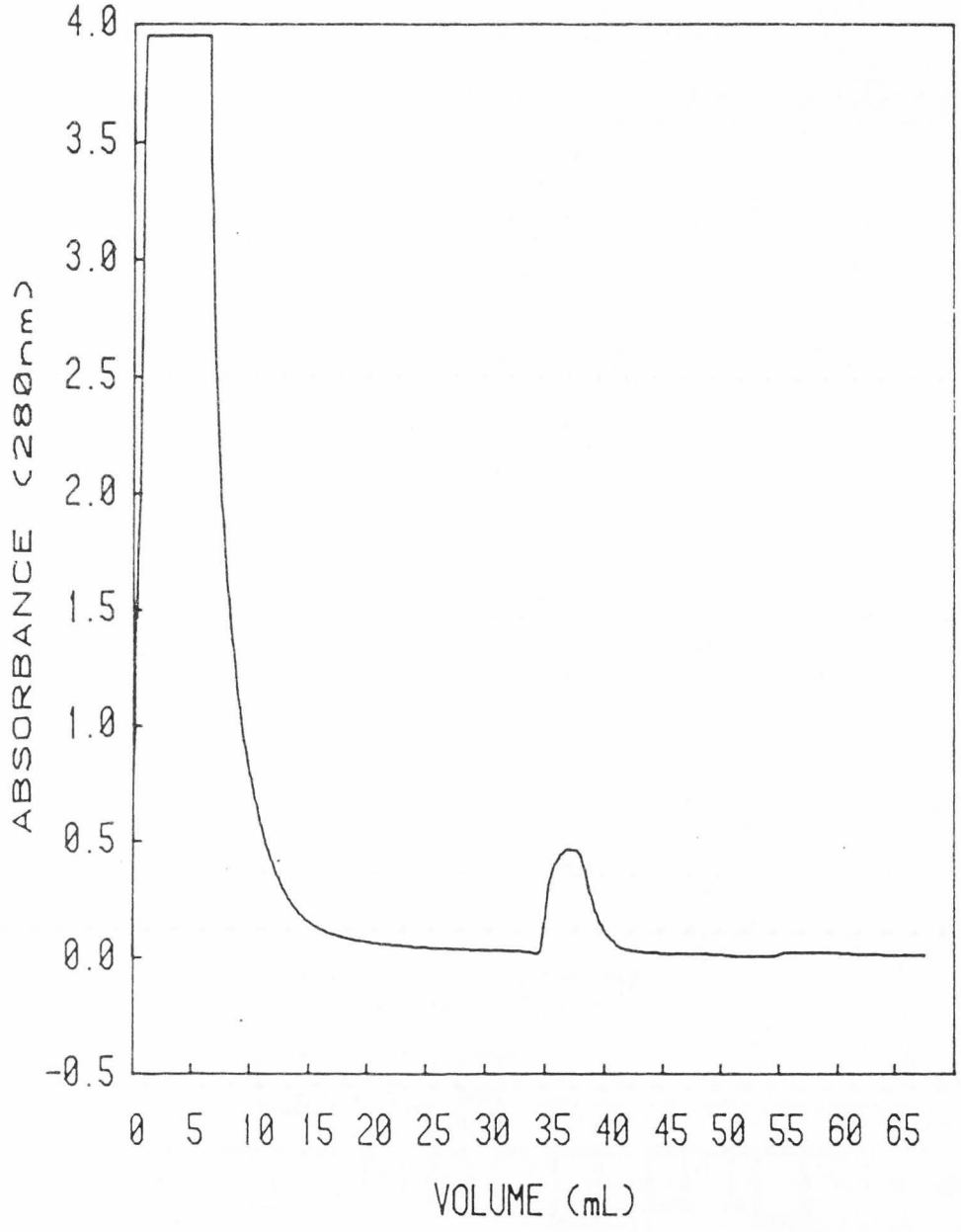


Figure 23. Affinity purification of anti-Pen antibodies from APA-OVA antiserum using Pen-RSA affinity column.

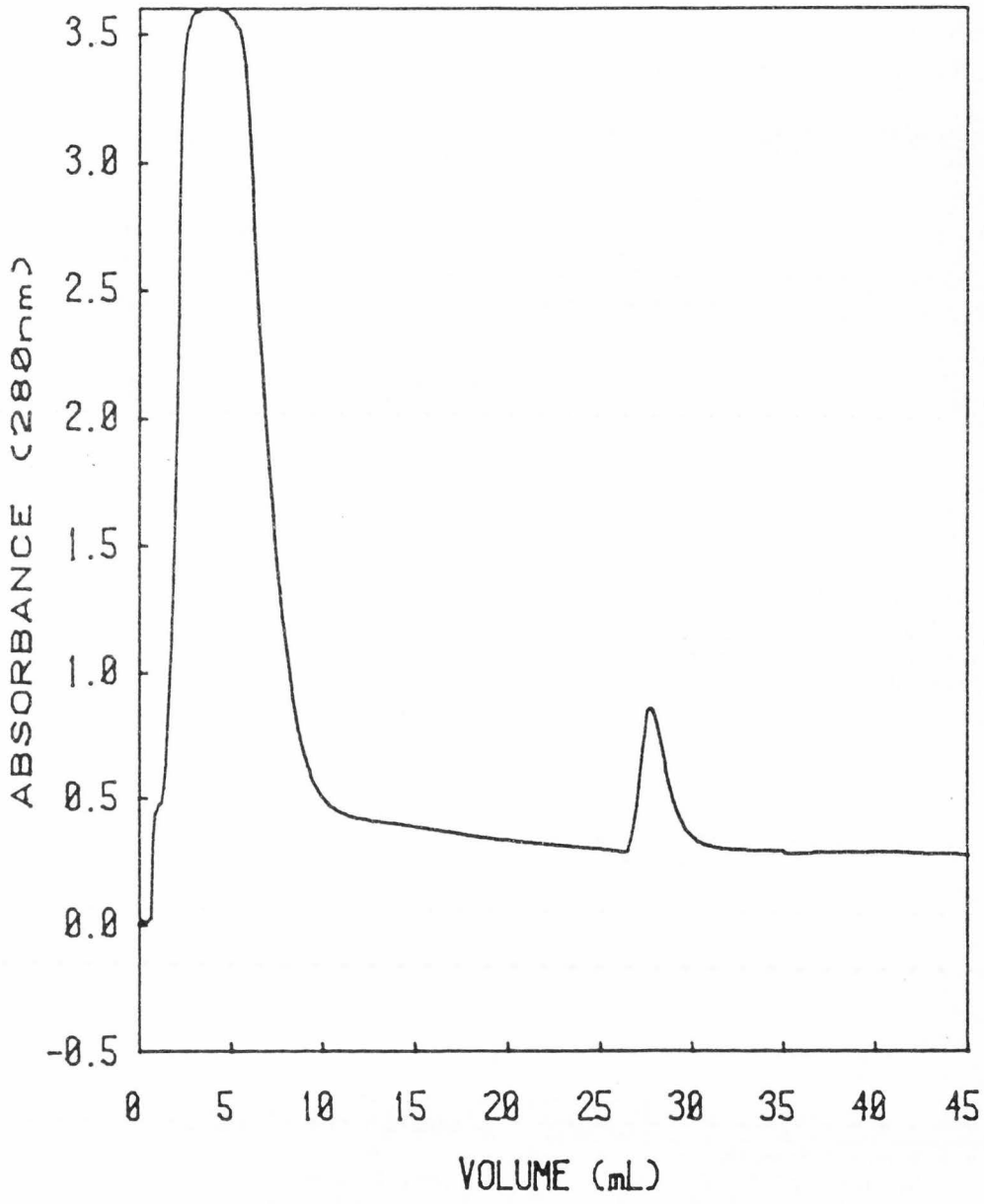


Figure 24. Affinity purification of anti-AMPC antibodies from APA-OVA antiserum using AMPC-BGG affinity column.

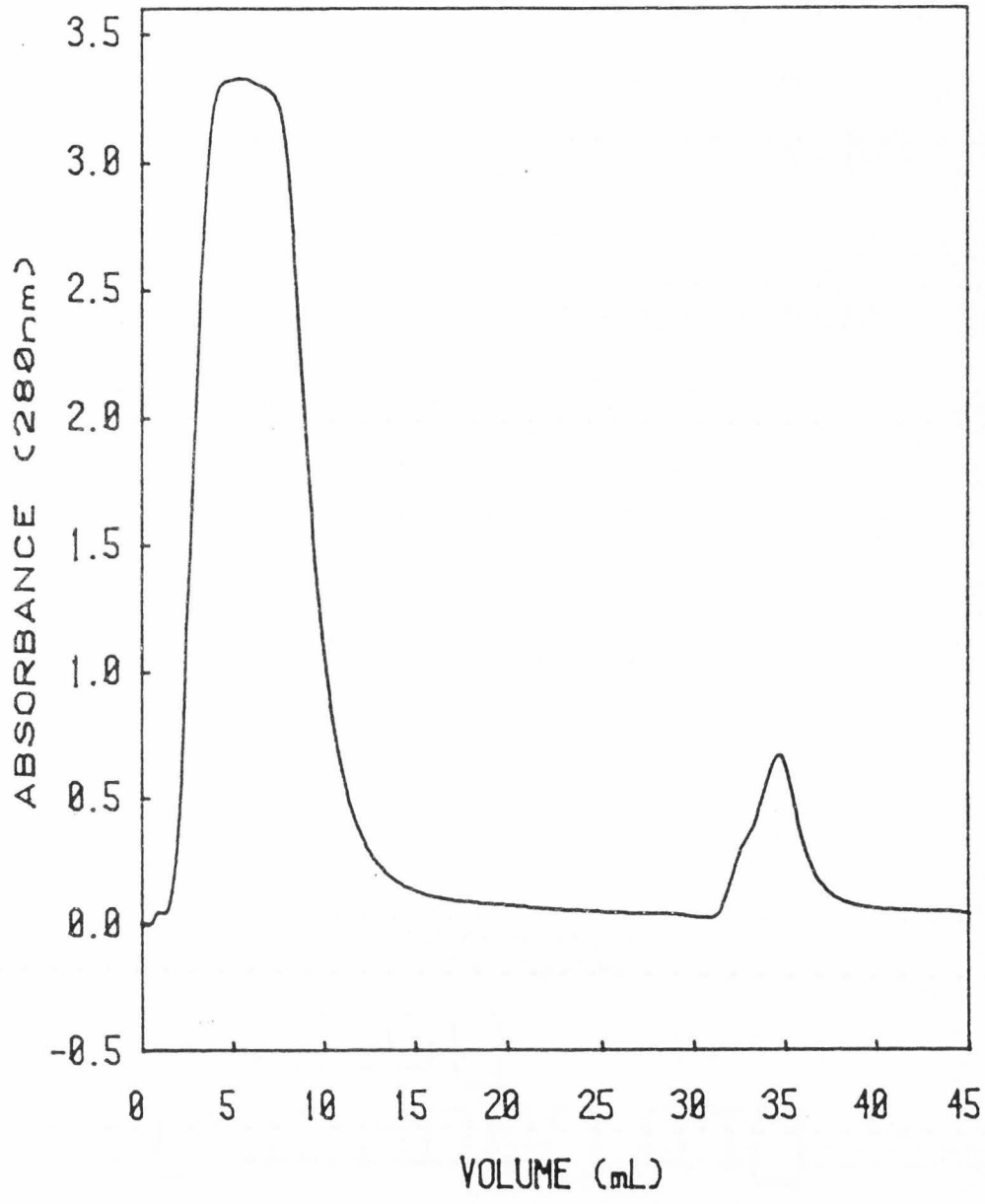


Figure 25. Affinity purification of anti AMPC-BGG antibodies from Pen-BGG antiserum using AMPC-BGG affinity column.

Enzyme-Hapen Conjugates

The relative activities of APA-HRP conjugates are shown in Table 2. Combination of $NaIO_4$ conjugation and the FDNB amine-blocking procedures resulted in total inactivation of HRP and also greatly decreased HRP solubility. Although similar procedures have previously been used successfully, modification of the procedure would be needed for use with APA conjugation. $NaIO_4$ procedure also caused a decrease in enzymic activity, which is expected, but 41% loss of activity is larger than desired. Further modification of this enzyme-hapten conjugation procedure to decrease activity loss would be needed to make the process economically feasible for EIA. EDC coupling procedure showed increase in enzymic activity. The mild procedure obviously did not inhibit enzymic activity and dialysis during the procedure may have removed any inhibiting substances that may have been present in the grade of HRP used. Crosslinking of HRP to HRP may have also affected activity of the enzyme.

Table 2. Relative activities of enzyme conjugates.

Enzyme	Concentration µg/75 µl	ΔAbsorbance[a]	% of Uncoupled HRP Activity
HRP	20.55	14.5×10^{-5}	100
HRP-APA (EDC-Procedure)	20.625	17.9×10^{-5}	123
HRP-APA ($NaIO_4$ Procedure)	30.3	8.6×10^{-5}	59
DNB-HRP-APA ($NaIO_4$ Procedure)	2.25	0	0

a) Δ Absorbance is change in absorbance per s at 500 nm.

CONCLUSIONS

Methods for preparation of APA-conjugates and antibodies needed for EIA have been developed. APA-OVA synthesized by periodate procedure is the preferred conjugate for production of APA-specific antibodies in rabbits, but APA-BGG should be used for antibody production if a periodate procedure is used in the assay for conjugation of APA to enzymes. Using a different protein (OVA) in conjugating APA by the diazonium procedure could increase antigenicity. Different methods of conjugation for APA-protein and APA-enzymes are desired because antibodies are antigenic toward protein-hapten linkage which decreases sensitivity in EIA.

APA-HRP prepared by EDC procedure is the preferred enzyme for EIA because enzymic activity is retained. It would be advantageous to refine the method by using an enzyme with protected amines or carboxyl groups to prevent protein-protein crosslinking during the procedure. FDNB for blocking amines affects enzymatic activity and (t-butyloxycarbonyloxyimino)2-phenylacetonitrile (BOC) used to protect amine groups on proteins decreased protein solubility too greatly to be used. It is suggested that the EDC procedure for hapten-enzyme be modified by first attaching protein to thioester-derivatized glass which would bind free amines and prevent enzyme-enzyme linkage after addition of EDC and hapten. Unbound hapten and crosslinked haptens could easily be removed by washing the beads. Hapten-HRP could then be released by hydroxylamine cleavage of the thioester bound on glass beads. Cross-linked enzymes utilized for EIA would decrease sensitivity of the assay and make it difficult to calibrate.

Antibodies produced against APA are antigenic toward the thiazolidine ring of penicillins as shown by their affinity towards ampicillin and penicillin. Anti-APA antibodies should therefore be antigenic towards other semisynthetic penicillins because APA is usually used in their synthesis.

The goal to develop an EIA for analysis of a class of compounds is different than the goal of most EIA which desire specificity for particular drugs or hormones. Determination of the presence of β-lactam antibiotics is wanted and identification of the antibiotic is not needed.

Development of an antibiotic EIA for use in milk is complicated by the complexity of milk. Methods of conjugation of penicillin and APA to protein in this laboratory have concentrated on attachment via the β-lactam ring. APA was attached to proteins via its free amine and penicillin conjugates are penicilloyl linkages, i.e. attachment to protein lysine by cleavage of the lactam ring at alkaline pH. Penicilloyl (Figure 5) is considered the predominant protein attachment which causes allergic reaction, but penicillenate (Figure 5) a disulfide linkage to protein by cleavage of the thiazole ring also occurs spontaneously at neutral pH. The pH of fresh milk is 6.65. Sulfhydryl proteins in milk (κ-casein and β-lactoglobulin) may bind some penicillin. Antibodies produced against penicilloyl linkage may not be antigenic towards penicillinate due to structural change. All penicillin in milk would not be bound in this form, but some would still cause interference in EIA. This probably would not be evident in initial laboratory studies, because penicillin spiked milk would

typically not be held for several days prior to assaying for penicillin.

The potential for anti-APA antibodies to be used to detect β-lactam antibiotics was shown. The methods for antibody production and conjugates synthesized can now be used to develop EIA procedure for APA and β-lactam antibiotics.

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APPENDICES

Appendix I

Computer Program: UV-Scans

```
 4 PAGE
 5 GOSUB 110
 6 END
 8 PAGE
 9 GOSUB 210
 10 END
 12 PAGE
 13 GOSUB 1190
 14 END
 16 PAGE
 17 W=32
 18 GOSUB 1070
 19 END
 20 PAGE
 21 W=1
 22 GOSUB 1070
 23 END
 24 PAGE
 25 GOSUB 1360
 26 END
 28 PAGE
 29 Q9=32
 30 GOSUB 1360
 31 END
 32 PAGE
 33 Q9=41
 34 GOSUB 1360
 35 END
 36 PAGE
 37 GOSUB 2020
 38 END
 100 INPUT H
 110 REM ** INITIALIZE **
 120 INIT
 130 W=32
 140 CALL "RATE",9600,1,2
 150 G$=""
 160 G=0
 170 DIM Y(2000),X(2000)
 180 PAGE
 190 R1=120
 200 RETURN
 210 REM ** ENTER PARAMETERS AND RUN **
 220 GOSUB 110
 230 PRINT "JJJJJDATE..............  ";
 240 INPUT D$
 250 PRINT
 260 PRINT "Enter 'H' (1=scale 0 to 4)";
 270 INPUT H
 280 PRINT
 290 PRINT "Now push STOP twice, then RUN, then the file"
 300 PRINT "number where the data should be saved followed"
```

```
310 PRINT "by ENTER."
320 I=1
330 INPUT @40:X$
340 PRINT @40:"F"
350 INPUT @40:Z$
360 T$=SEG(Z$,9,6)
370 T=VAL(T$)
380 R$=SEG(Z$,16,6)
390 R=VAL(R$)
400 P$=SEG(Z$,23,4)
410 R2=VAL(P$)
420 GOSUB 660
430 FOR I=1 TO 4000
440 INPUT @40:Y$
450 Y(I)=VAL(Y$)
460 IF I<>1 THEN 480
470 MOVE @32:T-R2*I/R1,Y(I)
480 DRAW @32:T-R2*I/R1,Y(I)
490 IF T-R2*I/R1<R+1 THEN 510
500 NEXT I
510 F$=SEG(Z$,7,2)
520 F=VAL(F$)
530 IF F=1 THEN 550
540 IF F<>2 THEN 580
550 PRINT "GGGGGGGGThis file contains a program!"
560 PRINT "Enter a new file number. ";
570 INPUT F
580 FIND F
590 WRITE @33:X$
600 WRITE @33:Z$
610 FOR J=I-1 TO 1 STEP -1
620 WRITE @33:T-R2*J/R1,Y(J)
630 NEXT J
640 PRINT "GGGGGGGGGGGGGGGGGGGGGGG"
650 RETURN
660 REM ** PLOT **
670 PAGE
680 VIEWPORT 40,120,20,80
690 WINDOW R,T,-0.5,4*H
700 AXIS @W:5,0.5,R,-0.5
710 MOVE @W:R,4*H
720 Z=0
730 DRAW @W:T,4*H
740 DRAW @W:T,-0.5
750 MOVE @W:0,R
760 GOSUB 980
770 A1=0.6
780 FOR J=R TO T STEP (T-R)/5
790 MOVE @W:J-1+A1,-0.5-0.3*H
800 PRINT "H";
810 PRINT @W: USING "3D":J
820 NEXT J
830 FOR J=0 TO 4*H STEP 0.5
```

```
840 MOVE @W:R-0.1*(T-R),J-0.05
850 IF W<>32 THEN 870
860 PRINT @32:"HH";
870 PRINT @W: USING "2D.1D":J
880 NEXT J
890 MOVE @W:(T-R)/2+R-15,-0.5-0.6*H
900 PRINT @W:"WAVELENGTH (nm)"
910 IF W=32 THEN 970
920 MOVE @W:R-0.15*(T-R),1.75*H
930 PRINT @W,25:90
940 PRINT @W:"HHHHHHHHHABSORBANCE";
950 MOVE @W:-5.5,-0.11
960 PRINT @W,25:0
970 RETURN
980 MOVE @W:5,-0.5
990 RDRAW @W:0,0.15*H
1000 MOVE @W:10,-0.5
1010 RDRAW @W:0,0.15*H
1020 FOR I=0.5 TO 4*H STEP 0.5
1030 MOVE @W:0,I
1040 RDRAW @W:0.4,0
1050 NEXT I
1060 RETURN
1070 REM ** PLOT DATA **
1080 PAGE
1090 PRINT "JJJJJJEnter 'H' (1 = 0 to 4 scale). ";
1100 INPUT H
1110 PAGE
1120 GOSUB 660
1130 FOR I=1 TO (T-R)*(R1/R2)-10
1140 IF I<>1 THEN 1160
1150 MOVE @W:X(1),Y(1)
1160 DRAW @W:X(I),Y(I)
1170 NEXT I
1180 RETURN
1190 REM ** LOAD DATA FROM TAPE **
1200 GOSUB 110
1210 PRINT "JJWHICH FILE ON TAPE? ";
1220 INPUT F1
1230 FIND F1
1240 READ @33:X$
1250 READ @33:Z$
1260 I$=SEG(Z$,9,6)
1270 I=VAL(T$)
1280 R$=SEG(Z$,16,6)
1290 R=VAL(R$)
1300 P$=SEG(Z$,23,4)
1310 R2=VAL(P$)
1320 FOR I=1 TO (T-R)*(R1/R2)-5
1330 READ @33:X(I),Y(I)
1340 NEXT I
1350 RETURN
1360 REM ** CALCULATE AND PRINT PARAMETERS **
```

```
1370 PRINT "JJJSTARTING FILE NUMBER... ";
1380 INPUT B9
1390 PRINT "JENDING FILE NUMBER..... ";
1400 INPUT E9
1410 FOR L=B9 TO E9
1420 F1=L
1430 GOSUB 1230
1440 A=INT(4*R2/R1)
1450 E=INT(13*R2/R1)
1460 IF (E-A)/2<>INT((E-A)/2) THEN 1480
1470 E=E+1
1480 M=0
1490 FOR I=1 TO INT(7.5*R2/R1)
1500 IF Y(I)<M THEN 1530
1510 M=Y(I)
1520 B=I
1530 NEXT I
1540 M=0
1550 FOR I=INT(7.5*R2/R1) TO R2*T
1560 IF Y(I)<M THEN 1590
1570 M=Y(I)
1580 D=I
1590 NEXT I
1600 C=B+0.5*(D-B)
1610 IF (C-A)/2<>INT((C-A)/2) THEN 1630
1620 C=C+1
1630 A1=0
1640 FOR I=A TO C
1650 A1=A1+R1/R2*(Y(I+1)+Y(I))
1660 NEXT I
1670 A1=A1*0.5
1680 A2=0
1690 FOR I=C TO E
1700 A2=A2+R1/R2*(Y(I+1)+Y(I))
1710 NEXT I
1720 A2=A2*0.5
1730 S=0
1740 S=Y(A)+Y(C)
1750 FOR I=A+1 TO C-1
1760 S=S+2*Y(I)
1770 NEXT I
1780 FOR I=A+2 TO C-2 STEP 2
1790 S=S+2*Y(I)
1800 NEXT I
1810 A3=S/(R2/R1*3)
1820 S=0
1830 S=Y(C)+Y(E)
1840 FOR I=C+1 TO E-1
1850 S=S+2*Y(I)
1860 NEXT I
1870 FOR I=C+2 TO E-2 STEP 2
1880 S=S+2*Y(I)
1890 NEXT I
```

```
1900 A4=S/(R2/R1*3)
1910 PRINT @Q9:"JJSAMPLE NO. ";S$;"    FILE NO. ";F
1920 PRINT @Q9:"J     VOLUME  ABSORBANCE   AREA"
1930 PRINT @Q9: USING 1980:A/R2*R1,Y(A)
1940 PRINT @Q9: USING 1990:B/R2*R1,Y(B),A1,A3
1950 PRINT @Q9: USING 1980:C/R2*R1,Y(C)
1960 PRINT @Q9: USING 1990:D/R2*R1,Y(D),A2,A4
1970 PRINT @Q9: USING 1980:E/R2*R1,Y(E)
1980 IMAGE 3X,2D.2D,5X,2D.2D
1990 IMAGE 3X,2D.2D,5X,2D.2D,5X,2D.3D,5X,2D.3D
2000 NEXT L
2010 RETURN
2020 REM ** PRINT DATA FROM TAPE **
2030 PAGE
2040 PRINT "TYPE '32' FOR SCREEN OR '41' FOR PRINTER."
2050 INPUT Q9
2060 PAGE
2070 S$=SEG(Z$,28,2)
2075 PRINT @Q9:"L"
2080 PRINT @Q9:"File #..... ";F1
2090 PRINT @Q9:"Slit #..... ";S$
2100 PRINT @Q9:"Points/min..... ";R1
2110 PRINT @Q9:"nm/min..... ";R2
2120 PRINT @Q9:"Low Wavelength. ";R
2130 PRINT @Q9:"High Wavelength ";T
2140 PRINT @Q9:
2150 PRINT @Q9:" nm   Absorbance"
2160 PRINT @Q9:
2170 FOR I=1 TO (T-R)*(R1/R2)-10
2180 PRINT @Q9: USING "3d.1d,2x,2d.4d":X(I),Y(I)
2190 NEXT I
2200 RETURN
```

Appendix II

Computer Program: Elution Profiles of Chromatography Columns

```
 4 PAGE
 5 GOSUB 1000
 6 END
 8 PAGE
 9 GOSUB 2000
 10 END
 12 PAGE
 13 GOSUB 5000
 14 END
 16 PAGE
 17 W=32
 18 GOSUB 4000
 19 END
 20 PAGE
 21 W=1
 22 GOSUB 4000
 23 END
 24 PAGE
 25 GOSUB 6000
 26 END
 28 PAGE
 29 Q9=32
 30 GOSUB 6000
 31 END
 32 PAGE
 33 Q9=41
 34 GOSUB 6000
 35 END
 36 PAGE
 37 GOSUB 7000
 38 END
 180 INPUT H
 1000 REM ** INITIALIZE **
 1010 INIT
 1020 W=32
 1025 CALL "RATE",1200,0,2
 1030 G$=""
 1040 G=0
 1050 DIM Y(1000)
 1060 PAGE
 1070 PRINT "WHAT IS H (try 1)? ";
 1080 INPUT H
 1499 RETURN
 2000 REM ** ENTER PARAMETERS AND RUN COLUMN **
 2005 GOSUB 1000
 2010 PRINT "JJJJJDATE..... ";
 2020 INPUT D$
 2030 PRINT "JFILE NUMBER..... ";
 2040 INPUT F
 2045 IF F=1 THEN 8
 2050 PRINT "JSAMPLE NUMBER..... ";
 2060 INPUT S$
 2070 PRINT "JSAMPLE SIZE..... ";
```

```
2080 INPUT S
2090 PRINT "JFLOW RATE..... ";
2100 INPUT R1
2110 PRINT "JREADINGS PER MIN... ";
2120 INPUT R2
2122 PRINT "JRUN TIME..... ";
2125 INPUT T
2130 FIND F
2132 GOSUB 3000
2135 MOVE @32:0,0
2140 FOR I=1 TO T*R2
2150 INPUT @40:X1,X2,Y(I)
2155 DRAW @32:I/R2*R1,Y(I)
2160 NEXT I
2170 WRITE @33:D$
2180 WRITE @33:F
2190 WRITE @33:S$
2200 WRITE @33:S
2210 WRITE @33:R1
2220 WRITE @33:R2
2230 WRITE @33:T
2240 FOR I=1 TO T*R2
2250 WRITE @33:Y(I)
2260 NEXT I
2270 PRINT "GGGGGGGGGGGGGGGGGGGGG"
2499 RETURN
3000 REM ** PLOT **
3010 IF W=1 THEN 3040
3020 VIEWPORT 70,128,15,95
3030 GO TO 3050
3040 VIEWPORT 40,120,20,80
3050 WINDOW 0,5*INT((T*R1+4.95)/5),-0.5,4*H
3060 AXIS @W:5,0.5,0,-0.5
3070 MOVE @W:0,-0.5
3075 Z=0
3080 FOR I=1 TO 1
3090 DRAW @W:0+Z*4.35,-0.5+Z*H
3100 DRAW @W:5*INT((T*R1+4.95)/5)-Z*4.35,-0.5+Z*H
3110 DRAW @W:5*INT((T*R1+4.95)/5)-Z*4.35,(4-Z)*H
3120 DRAW @W:0+Z*4.35,(4-Z)*H
3125 Z=Z+0.007
3130 NEXT I
3140 A1=-0.7
3150 FOR J=0 TO INT(T*R1) STEP 5
3160 IF J<10 THEN 3180
3170 A1=-1.2
3180 MOVE @W:J-1+A1,-0.5-0.3*H
3200 PRINT "H";
3210 PRINT @W:J;
3220 NEXT J
3230 FOR J=-0.5 TO 4*H STEP 0.5
3240 MOVE @W:-T*(R1/2)/4.5,J-0.05
3250 IF W<>32 THEN 3270
```

```
3260 PRINT @32:"HH";
3270 PRINT @W: USING "2D.1D":J
3280 NEXT J
3420 MOVE @W:T*(R1/2),-0.5-0.6*H
3430 PRINT @W:"HHHHHVOLUME (mL)"
3440 IF W=32 THEN 3530
3450 MOVE @W:-T*(R1/2)/3.75,1.75*H
3460 PRINT @W,25:90
3470 PRINT @W:"HHHHHHHHHABSORBANCE (280nm)";
3480 MOVE @W:-5.5,-0.11
3500 PRINT @W,25:0
3530 RETURN
3600 MOVE @W:5,-0.5
3610 RDRAW @W:0,0.15*H
3620 MOVE @W:10,-0.5
3630 RDRAW @W:0,0.15*H
3640 FOR I=0.5 TO 4*H STEP 0.5
3650 MOVE @W:0,I
3660 RDRAW @W:0.4,0
3670 NEXT I
3680 RETURN
4000 REM ** PLOT DATA **
4010 GOSUB 3000
4015 Z=0
4020 MOVE @W:0,0+Z
4030 FOR I=1 TO T*R2
4040 DRAW @W:I/R2*R1,Y(I)+Z
4050 NEXT I
4055 Z=Z+0.01*H
4060 RETURN
5000 REM ** LOAD DATA FROM TAPE **
5005 GOSUB 1000
5010 PRINT "JJWHICH FILE ON TAPE? ";
5020 INPUT F1
5021 PRINT
5030 FIND F1
5040 READ @33:D$
5050 READ @33:F
5060 READ @33:S$
5070 READ @33:S
5080 READ @33:R1
5090 READ @33:R2
5100 READ @33:T
5110 FOR I=1 TO T*R2
5120 READ @33:Y(I)
5130 NEXT I
5140 RETURN
6000 REM ** CALCULATE AND PRINT PARAMETERS **
6010 PRINT "JJJSTARTING FILE NUMBER... ";
6020 INPUT B9
6030 PRINT "JENDING FILE NUMBER..... ";
6040 INPUT E9
6050 FOR L=B9 TO E9
```

```
6060 F1=L
6070 GOSUB 5030
6080 A=INT(4*R2/R1)
6090 E=INT(13*R2/R1)
6095 IF (E-A)/2<>INT((E-A)/2) THEN 6100
6097 E=E+1
6100 M=0
6110 FOR I=1 TO INT(7.5*R2/R1)
6120 IF Y(I)<M THEN 6150
6130 M=Y(I)
6140 B=I
6150 NEXT I
6160 M=0
6170 FOR I=INT(7.5*R2/R1) TO R2*T
6180 IF Y(I)<M THEN 6210
6190 M=Y(I)
6200 D=I
6210 NEXT I
6220 C=B+0.5*(D-B)
6230 IF (C-A)/2<>INT((C-A)/2) THEN 6250
6240 C=C+1
6250 A1=0
6260 FOR I=A TO C
6270 A1=A1+R1/R2*(Y(I+1)+Y(I))
6280 NEXT I
6290 A1=A1*0.5
6300 A2=0
6310 FOR I=C TO E
6320 A2=A2+R1/R2*(Y(I+1)+Y(I))
6330 NEXT I
6340 A2=A2*0.5
6350 S=0
6360 S=Y(A)+Y(C)
6370 FOR I=A+1 TO C-1
6380 S=S+2*Y(I)
6390 NEXT I
6400 FOR I=A+2 TO C-2 STEP 2
6410 S=S+2*Y(I)
6420 NEXT I
6430 A3=S/(R2/R1*3)
6440 S=0
6450 S=Y(C)+Y(E)
6460 FOR I=C+1 TO E-1
6470 S=S+2*Y(I)
6480 NEXT I
6490 FOR I=C+2 TO E-2 STEP 2
6500 S=S+2*Y(I)
6510 NEXT I
6520 A4=S/(R2/R1*3)
6530 PRINT @Q9:"JJSAMPLE NO. ";S$;"    FILE NO. ";F
6550 PRINT @Q9:"J     VOLUME  ABSORBANCE   AREA"
6560 PRINT @Q9: USING 6610:A/R2*R1,Y(A)
6570 PRINT @Q9: USING 6620:B/R2*R1,Y(B),A1,A3
```

```
6580 PRINT @Q9: USING 6610:C/R2*R1,Y(C)
6590 PRINT @Q9: USING 6620:D/R2*R1,Y(D),A2,A4
6600 PRINT @Q9: USING 6610:E/R2*R1,Y(E)
6610 IMAGE 3X,2D.2D,5X,2D.2D
6620 IMAGE 3X,2D.2D,5X,2D.2D,5X,2D.3D,5X,2D.3D
6630 NEXT L
6640 RETURN
7000 REM ** PRINT DATA FROM TAPE **
7005 PAGE
7010 PRINT "JJWHICH FILE ON TAPE? ";
7020 INPUT F1
7021 PRINT
7030 FIND F1
7035 PAGE
7036 PRINT "TYPE '32' FOR SCREEN OR '41' FOR PRINTER."
7037 INPUT Q9
7040 READ @33:D$
7050 READ @33:F
7060 READ @33:S$
7070 READ @33:S
7080 READ @33:R1
7090 READ @33:R2
7100 READ @33:T
7101 PAGE
7102 PRINT @Q9:"DATE ..........";D$
7103 PRINT @Q9:"FILE # .........";F
7104 PRINT @Q9:"SAMPLE # .......";S$
7105 PRINT @Q9:"SAMPLE SIZE ....";S
7106 PRINT @Q9:"FLOW RATE ......";R1
7107 PRINT @Q9:"READINGS/MIN ...";R2
7108 PRINT @Q9:"RUN TIME .......";T
7109 PRINT
7110 FOR I=1 TO T*R2
7120 READ @33:Y(I)
7125 PRINT @Q9:60/R2*I/60*R1,Y(I)
7130 NEXT I
7140 RETURN
```

VITA

Alice Ann Heth

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Study of Enzyme Immunoassay for β-Lactam Antibiotics

Major Field: Nutrition and Food Sciences

Biographical Information:

Personal Data: Born at Streator, Illinois, January 27, 1955, daughter of Virgil and Pearl Heth and raised in Grand Ridge, Illinois.

Education: Attended Ottawa Township High School, Ottawa, Illinois, graduating in May, 1973. Received the Bachelor of Science degree in Food Science from University of Illinois-Urbana-Champaign in May 1977; 1981 received Master of Science degree from North Carolina State University; 1984; completed requirements of the Doctor of Philosophy degree at Utah State University, with a major in Nutrition and Food Sciences.