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INDUCTION OF HUMORAL IMMUNITY TO SOLUBLE PROTEIN TOXINS IN THE AMERICAN COCKROACH: THE INTERACTION OF COCKROACH HEMOLYMPH WITH BEE VENOM AND WITH BEE VENOM PHOSPHOLIPASE A_2

By

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B.S., University of Montana, 1976

Presented in partial fulfillment of
the requirements for the degree of
Master of Science

UNIVERSITY OF MONTANA

1982

Approved by:

Jon A. Rudbach
Chair, Board of Examiners

Dean, Graduate School

12-13-82
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Finn, Donald James, M.S., November, 1982 Microbiology

Induction of Humoral Immunity to Soluble Protein Toxins in the American Cockroach: the Interaction of Cockroach Hemolymph with Bee Venom and with Bee Venom Phospholipase A_2 (123 pp.)

Director: Jon A. Rudbach

Previous investigations (Karp, R.D., and L.A. Rheins, 1980, Developmental and Comparative Immunology, 4:447-458, 629-639) demonstrated that American cockroaches immunized with a toxoid derived from honeybee venom were protected on challenge with a dose of bee venom that constituted an LD_{100} for nonimmunized insects. The primary protective response peaked within two weeks of immunization, and gradually subsided by the fifth week. A second, subimmunizing dose of toxoid was shown to elicit a secondary protective response. Both responses appeared to be specific. The present study employed agarose and polyacrylamide gel electrophoresis to establish that the hemolymph of both normal cockroaches and of insects immunized with bee venom toxoid contained components capable of binding to two distinct fractions of whole bee venom: melittin and phospholipase A_2. The nature of the binding interaction was investigated in a series of immunodiffusion gels run at various levels of pH and ionic strength. The formation of the melittin-hemolymph complex appeared to represent a form of hydrophobic bonding; the mechanism of hemolymph-phospholipase A_2 binding was not resolved. The binding of hemolymph to venom components was not inhibited by a variety of simple sugars; therefore the binding was not due to the action of an insect lectin specific for any of those carbohydrates. A radial diffusion assay was devised to demonstrate that the hemolytic activity of melittin could be inhibited by both immune and normal hemolymph and by polyanions, but not by polycations. It was postulated that cationic melittin might bind to the anionic surfaces of cells via electrostatic attraction, and that hemolymph and polyanions might inhibit that activity by providing alternate ionic binding sites. A separate assay demonstrated that the enzymatic activity of phospholipase A_2 was not inhibited by polyanions, polycations, or hemolymph; hemolymph exhibited some capacity to serve as a substrate for the enzyme. The results of a radioimmunoprecipitation procedure suggested that both immune and normal hemolymph contained a protein of approximately 66,000 daltons that exhibited a binding affinity for phospholipase A_2.

ACKNOWLEDGEMENT

I would like to thank my thesis director, Dr. Jon Rudbach, for his advice and for his generous support of this project. I thank my collaborators, Dr. Richard Karp, Larry Rheins, and their associates at the University of Cincinnati, for providing me with the immunized animals and many helpful suggestions that made this project possible. Thanks are also due to my committee members, Dr. George Card, Dr. Richard Fevold, Dr. Gary Gustafson, and Dr. Walter Hill, for their encouragement and advice; and to Drs. Card and Gustafson for sharing their equipment and reagents. Val Kuntz performed the agarose electrophoresis experiments that laid the groundwork for subsequent investigations of bee venom-hemolymph binding; I am grateful for her contribution. I appreciate the suggestions and the friendship of my fellow students and co-workers, from whom I have learned much.

TABLE OF CONTENTS

Page

ABSTRACT....................................ii

ACKNOWLEDGEMENT.............................iii

LIST OF TABLES..............................vi

LIST OF FIGURES.............................vii

ABBREVIATIONS...............................ix

I. INTRODUCTION.............................1

Historical..................................1

Statement of thesis 5

II. MATERIALS AND METHODS...................8

Animals.....................................8

Hemolymph...................................8

Antigens and antisera.......................9

Preparation of ^{125}I-labeled PLA................10

Protein assays..............................11

Agarose gel electrophoresis.................11

Polyacrylamide gel electrophoresis..........12

Autoradiography.............................13

Electroblotting.............................14

Immunodiffusion.............................15

Carbohydrate inhibition assays..............16

Immunoelectrophoresis.......................17

Immunoprecipitation assay...................17

Radial diffusion assay......................18

Phospholipase A_2 inhibition assays..........20
  First method..............................20
  Second method.............................22
Radioimmunoprecipitation procedure..........23
III. RESULTS....................................25
Agarose electrophoresis.....................25
Polyacrylamide gel electrophoresis..........26
Electroblotting.............................32
Immunodiffusion.............................33
Carbohydrate inhibition assays..............48
Immunoelectrophoresis.......................60
Immunoprecipitation assay...................66
Radial diffusion assay......................68
Phospholipase A_2 inhibition assays..........88
  First method..............................88
  Second method.............................95
Radioimmunoprecipitation procedure..........97
IV. DISCUSSION..................................103
Interaction of hemolymph with melittin.....105
Interaction of melittin with polyanions....107
Interaction of hemolymph and polyions with phospholipase A_2..110
Significance of radioimmunoprecipitation...112
Conclusion..................................112
LITERATURE CITED................................119

LIST OF TABLES

Table Page

1. Sample preparation protocol for phospholipase A_2 hemolysis-inhibition assays..................90

2. Effect of polyionic compounds on phospholipase A_2-mediated sheep erythrocyte lysis.....91

3. Effect of hemolymph and normal rabbit serum on phospholipase A_2-mediated sheep erythrocyte lysis.....................................93

4. Effect of utilizing hemolymph or normal rabbit serum as sole substrate on phospholipase A_2-mediated sheep erythrocyte lysis.....94

5. Colorimetric assay for inhibition of phospholipase A_2 activity by hemolymph, normal rabbit serum, and rabbit anti-phospholipase A_2........96

LIST OF FIGURES

Figure Page

1. Agarose electrophoresis of hemolymph, bee venom, and ^{125}I-phospholipase A_2....28

2. Acrylamide gel electrophoresis of hemolymph and ^{125}I-phospholipase A_2....31

3. Immunodiffusion of hemolymph vs. bee venom and phospholipase A_2 at pH 9.0....37

4. Immunodiffusion of hemolymph vs. bee venom and phospholipase A_2 at pH 7.5....40

5. Immunodiffusion of hemolymph vs. bee venom and phospholipase A_2 at pH 5.0....43

6. Immunodiffusion of hemolymph vs. bee venom and phospholipase A_2 at pH 4.0....46

7. Effect of carbohydrates on bee venom-hemolymph precipitate formation....51

8. Comparison of agar vs. agarose as a gel support.55

8b. Composite diagram of precipitate patterns seen in figure 8....57

9. Immunoelectrophoresis of normal and immune hemolymph at pH 8.6....63

10. Immunoelectrophoresis of bee venom and phospholipase A_2 at pH 8.6....65

11. Radial diffusion assay for bee venom-mediated hemolysis of sheep erythrocytes....73

12. Radial diffusion assay for inhibition of bee venom-mediated sheep erythrocyte lysis by serum and serum components....76

13. Radial diffusion assay for inhibition of bee venom-mediated sheep erythrocyte lysis by polycations....80

14. Radial diffusion assay for inhibition of bee venom-mediated sheep erythrocyte lysis by polyanions....83

LIST OF FIGURES (CONTINUED)

Figure Page

15. Radial diffusion assay for inhibition of bee venom-mediated sheep erythrocyte lysis by hemolymph..86

16. Results of radioimmunoprecipitation procedure..100

Abbreviations

BV	bee venom
CFA	complete Freund adjuvant
HL	hemolymph
IHL	immune hemolymph
NHL	normal hemolymph
NRS	normal rabbit serum
PBS	phosphate buffered saline
PBS-Ca	phosphate buffered saline with calcium
PLA	phospholipase A_2 from bee venom
TGB	Tris-gelatin buffer

Introduction

The science of comparative immunology has its origins in the work of Metchnikoff who, during the 1880's, reported his observations on the phagocytosis of yeast cells by hemocytes of the water flea, *Daphnia* (15). Early papers by Noguchi (1903) and Cantacuzene (1923) described the presence of inducible agglutinins, lysins, and precipitins in a variety of invertebrate phyla (30). Insect immunity seemed to have been extensively investigated first by Metalnikov during the 1920's (15).

It is well established that insects possess phagocytic cells (12, 17); cellular responses such as phagocytosis, encapsulation, and cytotoxicity have been observed frequently (25). For example, Schmit and Ratcliffe (37) described the encapsulation of implanted nerve cord fragments by hemocytes of the wax moth *Galleria mellonella*. Other workers demonstrated the encapsulation of reciprocal xenografts in the American cockroach and the desert locust; allografts were not recognized as foreign (24). Vinson (52) postulated that the ionic nature of the surface of an implant may be important in eliciting a phagocytic response. He found that particles of a weakly basic anion exchanger injected into the hemocele of *Heliothis* species were readily encapsulated, whereas weakly acidic cation exchangers were not enveloped.

Nonspecific humoral components likely to serve as defense against bacterial pathogens are found in many insects; the presence of lysozyme in insect hemolymph has been noted repeatedly (25). Hemagglutinins, lectin-like proteins with specificity for certain carbohydrate determinants, have been observed in insects and in many other invertebrates (25, 29).

Attempts to demonstrate the typical humoral immune response of vertebrates in members of the invertebrate phyla have met with little success (17, 34). Attempts were made to correlate early observations of insect serological activity to mammalian-type antibody responses, despite a lack of knowledge of the capabilities of insects to form antibodies (7). Recent searches for insect immunoglobulins have proved abortive (3, 7, 25). Good and Papermaster pointed out that one of the most important distinctions between immunity in vertebrates and immunity in invertebrates rests on the presence and absence, respectively, of definitive lymphoid tissues (15). It is now generally believed that the humoral responses of insects differ from those of mammals, and that the mechanisms of those responses may vary greatly even among the Insecta.

Many investigators have attempted to use a variety of particulate antigens to induce specific, acquired immunity in insects (15, 17). The results have been varied, as the following examples demonstrate.

Stephens (44) observed that the hemolymph of wax moth larvae became moderately bactericidal when the larvae were actively immunized against *Pseudomonas aeruginosa*. She found the protective effect to be short-lived, diminishing by the third day after vaccination. Subsequent investigation suggested that the "-cidal" factor was an acidic substance of relatively low molecular weight, nonprotein, and heat stable (45). Vaccinated animals did not exhibit cross protection against *Shigella dysenteriae*. A number of nonspecific agents, both protein and nonprotein, did not stimulate bactericidal activity in hemolymph after their injection into normal larvae.

Gingrich (12) described the appearance of a lytic substance in the hemolymph of the large milkweed bug *Oncopeltus fasciatus* in response to an injection of *Pseudomonas aeruginosa*. Both the lytic substance and the accompanying acquired resistance were detected 4 h after vaccination, reached a maximum level after 24 h, and disappeared within 5 days. He concluded that the substance was not a protein. Cross protection was observed against *Escherichia coli*, but not against *Proteus vulgaris*, *Bacillus subtilis*, or *Micrococcus* species. A weak, transitory resistance to *Pseudomonas* infection and a low titer of the lysin could be evoked by the injection of heterologous substances (bovine serum albumin, human erythrocytes, and saline).

Seaman and Roberts (38) observed that American cockroaches immunized with the ciliated protozoan Tetrahymena produced a hemolymph factor that immobilized the parasite. The factor was sensitive to heat, precipitated by ammonium sulfate, and seemed to represent the active form of a nonreactive normal protein component present in the hemolymph of nonimmunized insects.

Boman and Steiner (3) induced the production of antibacterial proteins in Cecropia (giant silk moth) pupae by injecting live Enterobacter cloacae or several different "nonpathogenic" bacteria. Ultraviolet-killed preparations of the same organisms elicited a response no greater than the injury reaction caused by the injection of sterile saline. The antibacterial proteins were active against a variety of both Gram positive and Gram negative organisms. The response was first evident 8 to 10 h after vaccination, peaked in 8 days, then gradually declined. Two active proteins, termed cecropins, were subsequently isolated from immune hemolymph and sequenced. They proved to be small, basic peptides which share some architectural and functional features with the bee venom lysin, melittin. The cecropins, however, appear to be specific for bacteria, whereas melittin lyses both bacteria and eukaryotic cells (42).

In general, invertebrates appeared unable to acquire a specific immune response (7, 15, 25). They tended to respond either at once or not at all; repeated exposure to a specific antigen would not eventually stimulate immunity if none existed initially (25). The relatively nonspecific responses usually observed tended to peak rapidly and were of short duration. No anamnestic response was observed upon repeated injection of antigen (30, 35).

In contrast to the findings discussed previously are the results obtained by Dr. Richard Karp and Lawrence Rheins of the University of Cincinnati in their investigation of the immune response of the American cockroach (23, 35, 36). These researchers employed honeybee toxin and Western Cottonmouth Moccasin venom as antigens. These soluble protein complexes were chosen because first, they would not be cleared rapidly from the hemolymph by the insects' phagocytic system; second, since they were both lethal for cockroaches, they provided a natural biologic assay. Insects were vaccinated with 5 µl of 1% honeybee toxoid prepared from heat- and formalin-treated toxin, then rested for various periods of time before being challenged with 7 µl of 1% honeybee toxin (the LD_{100} for normal cockroaches). The protection generated in response to the vaccine was apparent at 3 days post injection, peaked after 2 weeks, and declined between the third and fourth weeks. Animals which were vaccinated with honeybee toxoid and challenged after 2 weeks with cottonmouth venom were not protected. Thus, the response of cockroaches to the bee toxin vaccine differed from the results reported by most other investigators of insect immunity, both in terms of kinetics and of specificity. In subsequent experiments, Rheins and Karp (36) demonstrated that protection could be transferred passively from immunized to normal animals via cell-free hemolymph. In addition, they found that immunized cockroaches produced a secondary response after injection with an additional dose of homologous toxoid. The secondary response reached higher levels than the primary response, had a shorter lag time, and could be triggered with subimmunizing doses of antigen. It was also specific, and could not be induced in bee toxin-immunized cockroaches by a secondary injection of cottonmouth venom. Rheins (36) obtained a strong precipitin line when he combined immune hemolymph with homologous toxin in gel diffusion tests. He also noted that proteolytic enzymes destroyed both the *in vivo* and *in vitro* activity of the inducible substance. Additional tests conducted by these researchers (unpublished data) suggested that phospholipase A_2 was the bee venom component responsible for inducing the protective response, and that its mode of action might be related to its highly cationic nature.

The purpose of the present study was to investigate further the interaction of bee venom with the protective component induced in the hemolymph of cockroaches vaccinated with bee venom toxoid. One tentative goal was to isolate and to purify a quantity of the immune factor so that its structure and amino acid sequence could be determined; another was to devise an *in vitro* assay to quantitate the difference in immune activity between normal and immunized cockroaches. As the project evolved, it became apparent that the former objective was hindered by a paucity of working material (the combined yield of hemolymph from eight to twelve adult cockroaches generally ranged from 200 to 800 µl), whereas the latter was complicated by the observation that there are components in both immune and normal hemolymph that are capable of binding to phospholipase A_2 as well as to another bee venom peptide, melittin. Therefore, the plan to isolate and to characterize a specific immune substance was set aside, and effort was concentrated on elucidating the nature of the interaction of both immune and normal hemolymph with melittin and with phospholipase A_2.

This project was undertaken in collaboration with Dr. Rich Karp and Larry Rheins of the University of Cincinnati, Cincinnati, Ohio. All insects used in the study were immunized at the U. of C.: 5 µl of 1% honeybee toxoid was injected between the fourth and fifth abdominal sternites of each animal. The immunized insects were rested for several days, then shipped to the University of Montana where they were bled on the day of projected peak titer, as described under Materials and Methods.

MATERIALS AND METHODS

Maintenance of animals

All insects used in this study were adult *Periplaneta americana* obtained from Carolina Biological Supply Co., Burlington, NC. The animals were housed in plastic wastebaskets containing a layer of clean wood shavings, and provided with water and Purina Lab Chow. The top 6 to 8 cm of the interior of each container were coated with vaseline to prevent escape. Ambient temperature was approximately 25°C. Cockroaches were immunized with bee venom toxoid at the University of Cincinnati as described previously and shipped to the University of Montana for subsequent processing.

Hemolymph

Bleeding was accomplished via a centrifugation procedure adapted from a method described by Sternburg and Corrigan (46). Lids (2.5 cm in diameter) from plastic vials were perforated with a # 11 cork borer (approximately 1.7 cm in diameter) and inserted top down into 50 ml plastic centrifuge tubes to serve as support for a 2 cm disc of fine wire screen. Cockroaches were anaesthetized with carbon dioxide and placed on ice to prevent coagulation of their hemolymph (HL). The head and the abdominal tip of each insect were dipped in melted paraffin to seal the oral and anal openings. The antennae and legs were clipped off close to the body and the animals were placed head down on screens in the modified tubes, two to four per container, and centrifuged at 500 X *g* for 10 min at 4°C. The expressed hemolymph was transferred to 1.5 ml disposable centrifuge tubes (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY.) and centrifuged for 5 min in an Eppendorf microcentrifuge to remove cellular material. Clarified samples were stored at 4°C and used within 48 h, or combined with an equal volume of glycerol and held at -20°C until needed.

Antigens

Phospholipase A_2 (PLA) from bee venom was purchased from Sigma Chemical Co., St. Louis, MO. Honeybee venom (BV) was obtained from Sigma and from United States Biochemical Corp., Cleveland, OH.

Antisera

Antibodies against HL and PLA were produced in New Zealand White rabbits. Two animals were injected intramuscularly with approximately 0.8 ml of vaccine prepared by emulsifying 0.1 ml HL, 0.880 ml phosphate buffered saline (PBS; 0.15 M NaCl, 0.0033 M PO_4; pH 7.2), and 0.020 ml of 1% merthiolate (aqueous) in 1 ml Complete Freund Adjuvant (CFA; Difco Laboratories, Detroit, MI). Another pair of rabbits was vaccinated with approximately

1 ml of a mixture containing 0.2 mg PLA in 1.38 ml PBS plus 0.020 ml of 1% merthiolate emulsified in 1.75 ml CFA.

The animals were bled from the marginal ear vein and were re-vaccinated at intervals throughout the course of the study. Booster doses of vaccine contained Incomplete Freund Adjuvant in place of CFA to minimize the danger of adverse tissue reactions at the sites of injection.

Preparation of ^{125}I-labeled phospholipase A_2

Radiolabeling of PLA was performed with a Chloramine-T procedure (appendix, p. 116). Labeled protein was separated from residual radioactivity on a column of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). The elution buffer was 10 mM tricine (Sigma), pH 8.0. Approximately 0.8 ml fractions were collected, and their radioactivity was determined in a crystal scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, IL) set at optimal voltage. The protein fractions that demonstrated the highest activity (cpm) were pooled.

Specific activity of the pool was assessed by combining approximately 1 X 10^4 cpm of pool with 0.5 ml of bovine serum diluted 1:200 in PBS and 0.5 ml of 10% trichloracetic acid (aqueous) at 4°C. After determining total cpm, the mixture was centrifuged for 20 min (1000 X g) at 4°C. The precipitate was washed once with 1 ml of cold 5% trichloracetic acid and counted, and the percent counts precipitated was determined. The average total activity of

10 µl portions from lots of PLA labeled at three different times during this study was approximately 1.4 X 10^4 cpm. Specific activity as determined by trichloracetic acid precipitation averaged 64% of the total.

Protein assays

Protein content of HL was etimated either by the differential absorbance technique of Warburg and Christian (5) or by a dye-binding method using bovine serum albumin as a standard (4). The quantity and concentration of HL that can be obtained from a cockroach varies greatly from one animal to the next. Consequently, it was not always possible to run a protein assay on each batch of NHL and IHL extracted. When HL was plentiful; protein was determined. When the yield was meager, protein assessment was forfeited and the HL used for other assays. Three different lots of NHL had protein concentrations of approximately 8.2, 10.4, and 25.3 mg/ml (average: 14.6). Four lots of IHL contained 5.4, 10.6, 12.2, and 13.5 mg/ml protein respectively (average: 10.4).

Agarose gel electrophoresis

Agarose electrophoresis of HL, BV, and PLA was conducted in barbital buffer, pH 8.6 (21) in a Boehringer Mannheim Model HS Horizontal Electrophoresis Tank (Boehringer Mannheim Biochemicals, Indianapolis, IN). Gels (3 mm thick) of 0.8% Seakem agarose (Marine Colloids, Inc. Rockland, ME) in barbital buffer were cast on GelBond film (Marine Colloids). The general protocol for the preparation of samples was as follows:

1. HL alone: one part HL plus three parts glycerol-PBS (50% glycerol in PBS).
2. BV or PLA alone: one part BV (10 mg/ml, aqueous) or PLA (2 mg/ml, aqueous) plus three parts glycerol-PBS.
3. ^{125}I-PLA alone: one part label (in tricine elution buffer) plus three parts glycerol-PBS.
4. HL combined with BV or label: one part HL plus one part BV , PLA, or ^{125}I-PLA plus two parts glycerol-PBS.

All samples were mixed and incubated at approximately 25°C for 30 min. Electrophoresis of 10 to 20 µl portions was carried out at 100 volts (constant voltage) for 3 to 5 h at 25°C.

Gels were simultaneously fixed and stained overnight in a solution of Coomassie Brilliant Blue G250 containing 12% trichloracetic acid (2), destained for approximately 24 h in several changes of distilled water, and dried according to directions supplied with the GelBond film.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis and SDS-PAGE were performed using the Tris-glycine buffer system of Laemmli (26) in a standard vertical electrophoresis chamber (Model 100, Aquebogue Machine Shop, Aquebogue, NY).

Samples for non-SDS runs were prepared as described previously under Agarose gel electrophoresis. SDS-solublized samples were processed as directed in the Laemmli procedure. Briefly, equal volumes of samples and SDS solublizing solution were combined in 1.5 ml plastic centrifuge tubes (Eppendorf), heated in a boiling water bath for 5 min, cooled in ice, then centrifuged for 5 min in an Eppendorf microcentrifuge. Portions of 20 to 40 µl were applied to the gel and electrophoresed at 30 milliamps (constant current). Bromphenol blue (0.005%, aqueous) was used as a tracking dye. Gels were stained for protein with the Coomassie procedure described previously.

Glycoproteins were stained by a modified periodic acid-Schiff method (55).

Polyacrylamide gels were dried onto 0.33 mm thick chromatography paper (Whatman 33M; W. & R. Balston, London, England) in a home-made vacuum drying apparatus.

Both agarose and polyacrylamide gels were photographed on Kodak Tri X film; films were developed with Kodak chemicals according to standard procedures.

Autoradiography

Dried agarose and polyacrylamide gels containing radiolabeled components were laid on Kodak XAR-5 X-ray film in a Kodak X-Omatic Regular cassette and held at -70°C for 24 to 48 h. Films were developed in Kodak Liquid X-ray Developer.

Electroblotting

Electrophoretic transfer of PAGE-separated HL proteins to diazobenzyloxymethyl paper was done in a Biorad Trans-Blot chamber (Biorad Laboratories, Richmond, CA). Activated paper was prepared as directed in the Appendix, p. 117. Transfer was carried out in 25 mM sodium phosphate buffer, pH 6.6, at approximately 7°C. Complete transfer required 10 to 15 h at 30 volts, 0.6 amps. Unreacted diazonium groups on the blotting paper were inactivated by incubating the blots in 1% ethanolamine (aqueous) with 0.25% gelatin, pH 9.0, for 2 h at room temperature. Blots were then washed five times in distilled water, dried at 37°C, and stored at 4°C until needed.

Direct probing of blots with ^{125}I-labeled PLA was performed in Tris-gelatin buffer (TGB), pH 7.5 (Appendix, p. 118). Tracks containing electrophoresed HL proteins were cut from the blots and placed in 11 X 100 mm test tubes containing 2 ml of TGB and from 15 X 10^3 to 150 X 10^3 cpm of ^{125}I-PLA. The tubes were stoppered and incubated on a rocker at room temperature for 4 to 8 h. The label was decanted, and each blot was washed repeatedly in 2 ml of TGB until the wash fluid showed no reactivity above background level. The strips were dried at 37°C and autoradiographed as described previously.

Electroblots of HL electropherograms were also probed with 40 µg of unlabeled PLA in 2 ml of TGB in a fashion analogous to that described above. The strips were washed five times in TGB, then incubated overnight at 4°C with 2 ml of rabbit anti-PLA serum diluted in TGB at concentrations ranging from 1:10 to 1:500. Strips were washed further seven times with 2 ml portions of TGB, then probed with 2 X 10^6 cpm of ^{125}I-labeled staphylococcal protein A (New England Nuclear, Boston, MA) in 3 ml of TGB overnight at 4°C. The strips were washed again in multiple changes of TGB until the wash fluid was free of detectable radioactivity, dried at 37°C, and autoradiographed.

Immunodiffusion

Ouchterlony gel diffusion tests of IHL, NHL, normal rabbit serum (NRS), and rabbit anti-PLA versus BV and PLA were performed at pH 9.0 and pH 7.5 in 0.05 M Tris-HCl, and at pH 5.0 and pH 4.0 in citrate-phosphate buffer (14). All tests were carried out both with and without the inclusion of 0.6 M KCl. All gels contained 0.01% merthiolate as a preservative. Portions (3 ml) of 1% Noble agar in various buffers were dispensed onto glass microscope slides which had been coated previously with 0.5% Noble agar (aqueous) and air-dried. Subsequently, 3 mm wells were punched in the gels and they were filled with various dilutions of HL, BV, PLA, or rabbit sera. Components were allowed to diffuse overnight at room temperature (20 - 22°C). Precipitin lines were photographed in gels illuminated against a black background with oblique lighting. Gels were washed for 48 h in several changes of buffer composed of 0.85% NaCl and 0.05 M Tris, pH 8.0, to remove unprecipitated proteins. They were then air dried and stained with oil red O (18) followed by amido black (5).

Carbohydrate inhibition

Tests for the inhibition of HL-BV and HL-PLA interactions by carbohydrates were run in gels containing 1% Noble agar in 0.05 M Tris, both with and without 0.6 M KCl at pH 9.0 and in 0.05 M Tris with 0.6 M KCl at pH 7.5. Gels were poured and punched as described previously. HL was diffused against BV (5 mg/ml final concentration) and PLA (1 mg/ml final concentration) in combination with each of the following carbohydrates: D-galactose, D-glucose, D-mannose, alpha-L-fucose, alpha-L-rhamnose, and N-acetyl-D-glucosamine. Carbohydrate stock solutions were made in distilled water. Final carbohydrate concentrations of 0.25 M were employed.

HL was also diffused against BV (5 mg/ml final concentration) combined with concanavalin A at final concentrations of 1, 10, and 100 µg/ml. Precipitate lines were photographed after overnight incubation at room temperature and stained as described previously.

The effect of heparin on HL-BV and HL-PLA interactions was assayed in 1% Noble agar gels with 0.05 M Tris at pH 9.0 both with and without 0.6 M KCl, in 1% Noble agar in PBS, in 1% agarose in PBS, and in 1% agarose in veronal buffer, pH 8.6 (5). Dilutions of HL in distilled water and in 10 mg/ml aqueous sodium heparin (Sigma) were diffused overnight at room temperature against 5 mg/ml BV and 2 mg/ml PLA. Patterns were photographed and stained as described above.

Immunoelectrophoresis

Immunoelectrophoretic investigations were carried out in 1% agarose in veronal buffer, pH 8.6 (5). Merthiolate (0.01%) was included as a preservative. Slides were prepared as described under Immunodiffusion. Wells and troughs were cut with a custom-made template. Electrophoresis of 10 µl samples of BV, PLA, and HL was performed in an Egatonig type AlW chamber (Agafor-Gerät, Berne, Switzerland) at 2.5 milliamps per slide (constant current), 4°C, for 90 to 120 min. Troughs were filled with NHL, IHL, NRS, or rabbit anti-PLA and the components were allowed to diffuse overnight at room temperature. Gels were photographed, washed, dried, and stained for lipid and protein as described earlier.

Staphylococcal protein A immunoprecipitation assay

In order to check for specific binding of PLA by HL, the following assay was devised. Formalinized *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) were coated with rabbit anti-PLA or anti-HL as described in published procedures (10). Approximately 5×10^4 cpm of ^{125}I-PLA were incubated with 10 or 20 µl of HL in 50 µl of buffer (0.05 M Tris, 0.85% NaCl, pH 8.0) for 1 h at room temperature. Various amounts of anti-HL coated Pansorbin were added to precipitate any HL-^{125}I-PLA complexes. As a positive control, anti-PLA Pansorbin was substituted for the anti-HL form. Pansorbin alone (uncoated) and Pansorbin coated with NRS were employed to check for nonspecific binding. The mixtures were incubated at room temperature for an additional 20 min, then centrifuged for 5 min in an Eppendorf microcentrifuge at 4°C. The precipitates were washed three times in 1 ml portions of cold buffer, and their radioactivity was assessed in a gamma counter. Percent activity bound was calculated by comparing the cpm precipitated to the total cpm.

Radial diffusion assay

A hemolysis-inhibition radial diffusion assay was devised to investigate the inhibitory effect of HL and other substances on the hemolytic activity of whole BV. Sheep blood in modified Alsevers solution (Colorado Serum Co. Labs, Denver, CO) was washed twice in PBS, and the cells derived from 1 ml of preserved whole blood were suspended in 15 ml PBS to give an erythrocyte concentration of approximately 1.5%. Seakem agarose (2% in PBS) was melted in a steam cabinet. The melted agarose and the cell suspension were held in a water bath at 50°C until their temperatures had equilibrated. Equal volumes of each were combined and gently swirled. Then 13 ml portions of the final mixture were applied to 3¼ X 4 inch (8.3 X 10.2 cm) glass slides which had been coated previously with 0.5% agarose (aqueous), dried, and prewarmed to 50°C on a slide warmer. When the gels had hardened, four rows of five wells each (20 wells per plate) were made in each plate with a 3 mm punch.

Serial two-fold dilutions of BV in PBS were prepared using Microtiter plates and transfer pipets (Cooke Engineering Co., Alexandria, VA) to yield a venom concentration range from approximately 5000 µg/ml to 9.8 µg/ml. Subsequently, 10 µl portions of each dilution were added to the first ten wells of one assay plate; the venom in these wells served as uninhibited BV standards. Similar dilutions of PLA were prepared to give a concentration range from 2000 µg/ml to 3.9 µg/ml, and 10 µl volumes of each dilution were transferred to the appropriate wells.

Stock solutions of five polyanionic substances (sodium heparin, phosvitin, Gram-negative endotoxin, dextran sulfate, and sodium polyanethol sulfonate) and two polycations (lysozyme, protamine sulfate) were made in PBS at concentrations of 5 mg/ml. Stock solutions of bovine serum albumin and bovine gamma globulin in PBS were made at approximately physiological concentrations (40 mg/ml and 25 mg/ml respectively).

In order to evaluate the effect of polyionic and other substances on the hemolytic activity of BV, ten serial two-fold dilutions of each stock solution and of NRS, NHL, and IHL were made in PBS using Microtiter equipment. Then 10 µl of each dilution was mixed with 10 µl of 2500 µg/ml BV in separate microtiter wells, and 10 µl of that final mixture was transferred to appropriate wells in the diffusion plates. All plates were placed in a humid chamber at room temperature and allowed to incubate for 18 to 24 h. The diameters of the hemolytic zones were read in two dimensions with a calibrating viewer (Transidyne General Corp., Ann Arbor, MI) and recorded to the nearest 0.1 mm.

Assay for inhibition of PLA activity

Since purified PLA proved to be nonreactive in the radial diffusion assay described above, two standard procedures for the evaluation of PLA activity (40) were modified to examine the effect of HL, polyanions, polycations, and other compounds on PLA function. Both methods employed Asolectin, a mixture of 95% purified soy phosphatides (Associated Concentrates, Woodside L.I., NY) as substrate. A stable stock suspension of Asolectin (25 mg/ml in distilled water) was prepared by emulsifying the lipid with the aid of a Branson sonifier (Model W-350, Heat Systems-Ultrasonics, Inc., Plainview L.I., NY). The mixture was sonicated for approximately 10 min using a half-inch probe, with the power output control set at three (100 watts), then centrifuged at 10,000 X *g*, 4°C, for 30 min to remove any undispersed lipid.

For the first assay, sheep erythrocytes in Alsever's solution were washed twice in PBS. Working strength substrate was prepared by diluting stock Asolectin to a concentration of 2 mg/ml in PBS containing 36 µg/ml $CaCl_2 \cdot 2H_2O$ (PBS-Ca). One volume of dilute substrate was mixed with one volume of washed, packed sheep erythrocytes and ten volumes of PBS-Ca. Then 200 µl of this suspension was distributed into a series of 11 X 100 mm test tubes in an ice bath. Additional components were added according to the scheme that is presented in Results . The addition of glycerol to tubes that contained no HL proved necessary because the HL samples were preserved in 50% glycerol, which affects the osmotic fragility of the sheep cells.

When the addition of all components was complete, the tubes were removed from the ice bath, mixed, and placed in a 30°C water bath for 30 min. After the incubation, the reaction was stopped by returning the tubes to the ice bath and adding 2 ml of an ice-cold solution of 2.2% glycerol in PBS or, in the case of the 100% hemolysis control, 2 ml of distilled water. All tubes were centrifuged for 10 min at 4°C at 1000 X g. The optical density of the supernatant hemoglobin solution was read at 540 nm in a Coleman Junior spectrophotometer. Percent hemolysis was calculated by comparing the optical density of the sample hemolysates to that of the 100% hemolysis control.

In order to determine if HL or NRS could serve as a substrate for PLA in the absence of Asolectin, the assay was repeated on those samples with a mixture of one volume of packed sheep cells plus 11 volumes of PBS-Ca replacing the sheep cell-Asolectin suspension employed previously.

Alternate assay for PLA activity

This assay replaced the indicator function of sheep cells with a colorimetric reaction. It was run in 11 X 100 mm test tubes. Blanks contained 1.030 ml of PBS-Ca and 0.2 ml of diethyl ether. Standards consisted of 1 ml of 2 mg/ml Asolectin in PBS-Ca plus 30 µl of PBS-Ca and 0.2 ml ether. Uninhibited enzyme controls contained the same volumes of substrate and ether as the standards, plus 10 µl of 125 µg/ml PLA and 20 µl of PBS-Ca. To assess the effect of HL and other substances on PLA, 20 µl of the various compounds was mixed with 10 µl of 125 µg/ml PLA, then combined with 1 ml of substrate and 0.2 ml of ether. The addition of all of the components was carried out in an ice bath. The tubes were then mixed and transferred to a 30°C water bath for 20 min. The reaction was stopped by the addition of 3 ml of a mixture of 95% ethanol and hydroxylamine reagents to each tube (2 ml 95% ethanol, 0.5 ml hydroxylamine HCl solution (2.09 g in 15 ml distilled water) and 0.5 ml 3.5 N NaOH). The tubes were replaced in the 30°C water bath for 5 min. Color development was carried out by addition of 0.5 ml of ferrichloride reagent (10 g $FeCl_3 \cdot 6H_2O$ in 100 ml of 0.1 N HCl). After 5 min, the samples were read at 525 nm, and the percent hydrolysis of substrate was calculated from the optical density ratios of samples and standards.

Radioimmunoprecipitation

Radioimmunoprecipitation of ^{125}I-labeled HL was performed by a method adapted from that described by Swanson (48).

Separate lots of Pansorbin coated with anti-PLA or anti-HL were prepared by combining one volume of appropriate antiserum with two volumes of staphylococcal suspension. The mixtures were incubated 20 min at room temperature followed by 10 min on ice, then diluted with approximately ten volumes of cold PBS and centrifuged for 3 min in an Eppendorf microcentrifuge. The supernatant fluids were discarded; the pellets were washed twice more in cold PBS and finally restored with PBS to a volume equal to that of the initial amount of Pansorbin employed.

Iodination reaction vessels were prepared by coating the interior of 4 ml glass vials with 20 µl of a 1 mg/ml solution of Iodo-Gen (Pierce Chemical Co., Rockford, IL) in chloroform and allowing them to dry at room temperature overnight. Fresh IHL, IHL and NHL preserved in 50% glycerol, and NRS were labeled by placing 50 µl of each in separate reaction vessels along with 5 µl of 10^{-3} M KI in PBS. Approximately 100 µCi of ^{125}I in about 10 µl of PBS was added to each vial. The contents were held at room temperature for 6 min with occasional mixing. The reactions were terminated by transferring the contents of each vial to 1.5 ml microfuge tubes (Eppendorf). Subsequently, 20 µl of PLA (2 mg/ml in PBS) were added to the tubes, which were mixed, incubated at room temperature for 5 min, then placed on ice for 20 min; 50 µl of antibody-coated Pansorbin (either anti-HL or anti-PLA) were combined with each labeled sample. The mixtures were kept on ice for an additional 30 min with occasional agitation, diluted to approximately 1 ml with cold PBS, centrifuged, washed in three more changes of cold PBS, and resuspended to a final volume of 50 µl. Each sample was mixed with 50 µl of SDS sample solublizing solution (26), placed in a boiling water bath for 10 min, cooled on ice, then re-centrifuged for 5 min. Finally, 20 µl portions were applied to a polyacrylamide gradient gel that ranged in approximate acrylamide concentration from four to 18%. SDS-PAGE, staining, and autoradiography were carried out as described under Polyacrylamide Gel Electrophoresis.

RESULTS

Agarose electrophoresis

In order to resolve HL into its constituent proteins and to assess the effect of BV and PLA on the electrophoretic migration of NHL and IHL, agarose electrophoresis was performed. Figure 1 demonstrates the typical agarose electrophoresis patterns of NHL, BV, and IHL alone (A1, A3, and A5) and of NHL and IHL in combination with BV (A2 and A4). BV by itself was cationic at pH 8.6, whereas HL was anionic. Note that when BV and either NHL or IHL were run in combination, the cathodal migration of BV appeared to be markedly reduced, and the cathodal portion of both the NHL and IHL bands appeared to shift toward the origin. This suggested that some factor or factors in BV could combine with HL in a manner that altered the electrophoretic mobility of both components.

Bee venom is known to contain a variety of enzymes and biologically active peptides (33). Previous work by Karp and Rheins suggested that phospholipase A_2 was the BV component against which BV-immunized cockroaches mounted their protective response. In order to determine if PLA was capable of binding to HL, samples of NHL and IHL were mixed with ^{125}I-labeled PLA and subjected to agarose electrophoresis. Autoradiography of the electropherograms was performed to assess the migration of the label. Typical electrophoretic and autoradiograph patterns are shown in Figure 1 (B and C). No discernible displacement of the HL patterns was evident when NHL and IHL alone (B2 and B3) were compared with NHL and IHL run in combination with ^{125}I-PLA (B4 and B5). Perhaps the small amount of PLA incorporated into the HL samples in this experiment was insufficient to cause a noticeable shift in HL migration. Comparison of the autoradiogram of labeled PLA alone (C1) with the patterns produced by labeled PLA combined with NHL and IHL (C4 and C5) revealed that the label was concentrated in an area corresponding to the location of the HL bands. Therefore, PLA appeared capable of binding to both NHL and IHL, and the choice of PLA as a BV component implicated in the cockroach immune response was substantiated.

Polyacrylamide electrophoresis

Due to the large pore size of agarose gels, electrophoresis in such gels tends to separate components on the basis of their charge rather than according to their molecular size. When polyacrylamide is employed as an electrophoretic medium, pore size can be controlled by varying the concentration of acrylamide, thereby superimposing a molecular sieving effect on separation by charge. Incorporation of sodium dodecyl sulfate into an acrylamide gel system negates any intrinsic charge on the sample molecules and

Fig. 1: Agarose electrophoresis of HL, BV, and ^{125}I-PLA.

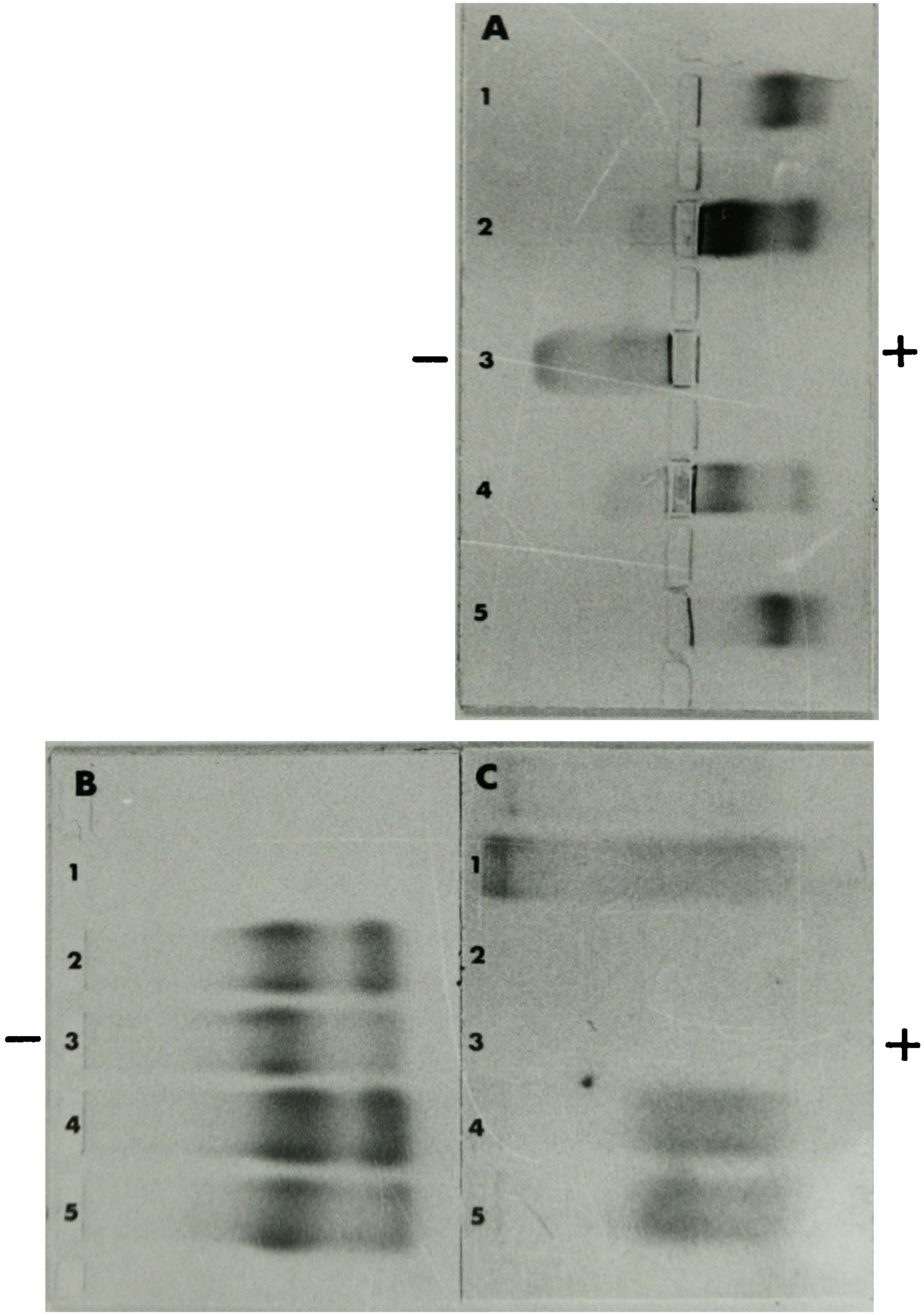
A: electrophoresis of HL and BV in 0.8% agarose, pH 8.6.

1. NHL
2. NHL + BV
3. BV
4. IHL + BV
5. IHL

B: electrophoresis of HL and ^{125}I-PLA in 0.8% agarose, pH 8.6.

1. ^{125}I PLA
2. NHL 9/7/81
3. IHL 9/7/81
4. NHL 9/7/81 + ^{125}I-PLA
5. IHL 9/7/81 + ^{125}I-PLA

C: autoradiogram of gel described in B.


A
1
2
−
3
+
4
5
B
1
2
−
3
4
5
C
1
2
3
+
4
5

permits their separation based on molecular size alone (5).

In order to take advantage of the greater resolving power of polyacrylamide electrophoresis, various combinations of HL and ^{125}I-PLA were subjected to PAGE or SDS-PAGE procedures. The use of a non-polysaccharide gel matrix also allowed the gels to be stained for glycoproteins with a periodic acid-Schiff technique. All of the bands that are pictured in this and subsequent sections were stained for protein with Coomassie Blue. Staining with the periodic acid-Schiff procedure yielded patterns which were indistinguishable from those produced by Coomassie Blue, thus suggesting that most HL proteins were glycoprotein in nature.

Figure 2A shows polyacrylamide electropherograms (7.5% acrylamide, pH 8.3) of single lots of IHL (A1 through A4) and NHL (A7 through A10) pre-incubated with various amounts of ^{125}I-PLA, and the pattern produced by ^{125}I-PLA alone (A5). The patterns displayed by IHL were representative of those usually seen in both immune and normal HL. The compression of the cathodal bands in the NHL patterns was uncharacteristic. Various atypical banding patterns were seen in occasional lots of both IHL and NHL throughout the course of this project. Previous work has shown that the electrophoretic patterns of cockroach hemolymph may vary with the sex of the insect and with its stage of development (39). Therefore, caution must be exercised in interpreting any differences in banding patterns between

Fig. 2: Acrylamide gel electrophoresis of HL and ^{125}I-PLA.

A: electrophoresis of HL and ^{125}I-PLA in 7.5% polyacrylamide gel with Tris-glycine buffer, pH 8.3.

1, 2, 3: IHL 10/6/81 with various dilutions of ^{125}I-PLA.

4: IHL 10/6/81

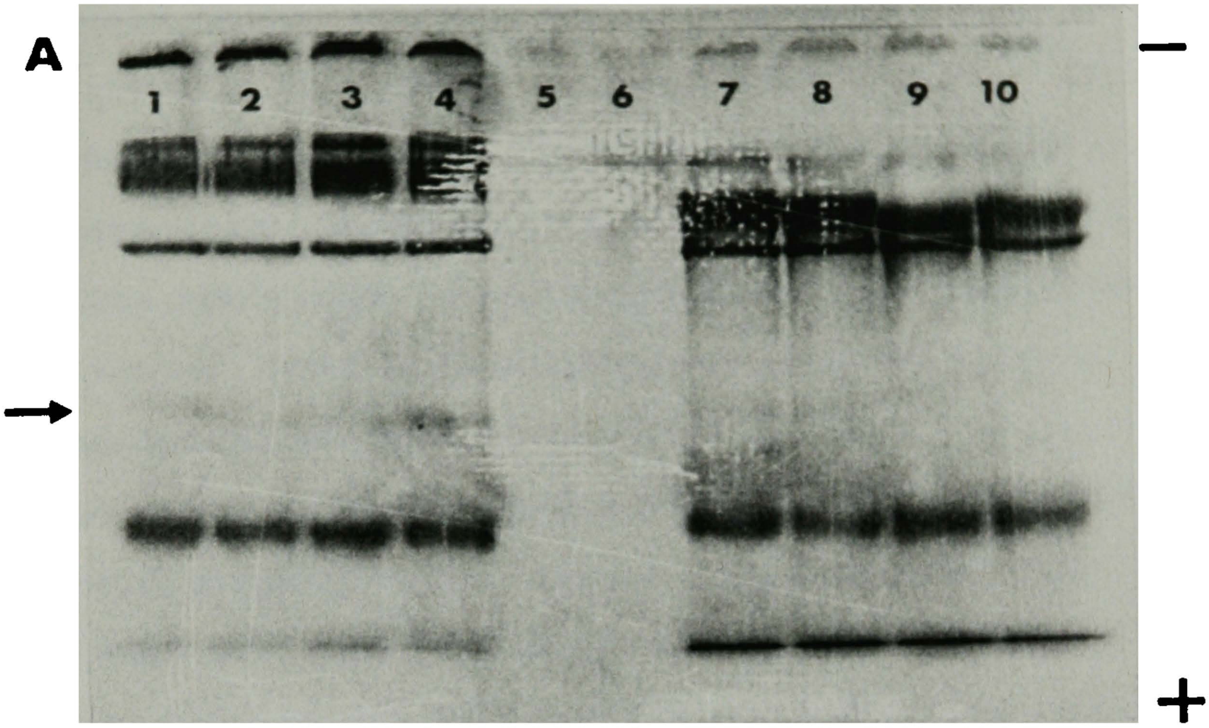
5: ^{125}I-PLA

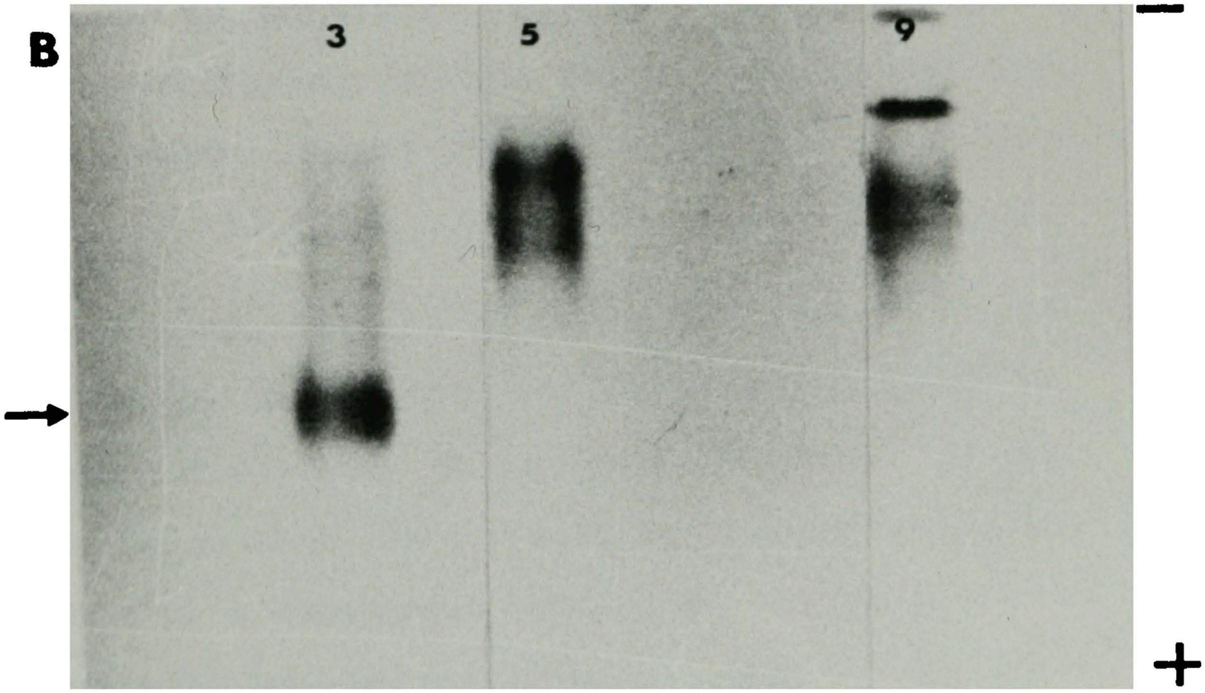
6: Unlabeled PLA

7, 8, 9: NHL 10/6/81 with various dilutions of ^{125}I-PLA

10: NHL 10/6/81

B: autoradiogram of gel described in A.


A
1
2
3
4
5
6
7
8
9
10
−
+


B
3
5
9
−
+

immune and normal HL.

Figure 2B is the autoradiogram taken from the gel in Figure 2A. Only tracks B3, B5, and B9 contained sufficient label to register on the film. Track B5 represented ^{125}I-PLA electrophoresed alone. In comparison, the label incorporated into track B3 along with IHL migrated closer to the anode. The positions indicated by the arrows suggested the association of label (B3) with a protein band faintly visible in tracks A1 through A4.

^{125}I-PLA mixed with NHL (track B9) was not displaced anodally; the distribution of label seen in track B9 suggested that much of the ^{125}I-PLA may have precipitated in the sample well and failed to enter the gel.

The results presented in Figure 2 illustrated one instance where a difference in the PLA-binding properties of immune vs. normal HL was clearly observed.

Electroblotting

As noted above, PAGE of HL pre-mixed with ^{125}I-PLA suggested that the PLA-binding activity of IHL might be associated with a specific HL protein fraction. It was desired to confirm that observation by demonstrating the binding of PLA to a hemolymph band after the HL proteins had been separated by electrophoresis. Due to the small pore size of acrylamide gels as compared to agarose gels, PAGE-separated components may be inaccessible to a probe molecule applied to the gel exterior. The electroblotting technique provides a convenient means of transferring the components of interest to a surface where they are covalently bound and may be freely exposed to the desired probe (9, 49).

Electroblots of NHL and IHL, both native and SDS-solublized, were taken from 10% and 15% acrylamide gels. Neither direct probing of the blots with ^{125}I-PLA nor indirect probing with cold PLA followed by rabbit anti-PLA and ^{125}I-protein A revealed any specific binding of label. As a control on the method, one track on each gel included either ^{125}I-PLA preincubated with IHL before electrophoresis or ^{125}I-PLA solublized in SDS. This radioactivity was successfully transferred to the blotting paper and produced the only clearly visible bands on the final autoradiograms. Various manipulations of blotting buffer pH, Tris-gelatin buffer components, washing protocol, and duration of electrophoretic transfer did not enhance the binding of label. There are at least two plausible reasons for this failure. First, the PLA-binding fraction of HL may be so labile that it failed to survive the two sessions of electrophoresis or the numerous washings. Second, some component involved in the blotting procedure (e.g. gelatin, ethanolamine, or detergents) may have blocked the reaction of HL with label.

Immunodiffusion

Previous work by Rheins and Karp (36) demonstrated that IHL, diffused against BV in Ouchterlony immunodiffusion gels at pH 7.5, produced a strong precipitin band. This suggested that the protective factor in IHL behaved like a precipitating antibody. NHL occasionally produced precipitin lines when diffused against BV; these were attributed to the reaction of some BV component with an unidentified substance innate to HL.

The electrophoretic evidence presented earlier indicated that BV and HL are oppositely charged at pH 8.6. The published isoelectric point of PLA from BV is 10.5 (50); the pI of most HL proteins has been estimated to range from pH 5.0 to pH 5.5 (39). In order to determine if the binding of BV and PLA to HL might be due to an electrostatic interaction dependent on the environmental pH, BV and PLA were diffused against IHL and NHL in a series of immunodiffusion gels of differing pH's that covered a range from 4.0 to 9.0. The immunodiffusion system employed by Rheins and Karp contained 0.6 M KCl. In order to assess the effect of a high ionic environment on HL-BV interactions, each gel in the present experiment was run at two levels of ionic strength. One gel at each pH level included 0.6 M KCl, and one did not.

Results of the immunodiffusion assays are presented in Figures 3 through 6. As a preface to the slide-by-slide description, the following observations may be useful. 1) The slides are arranged in order of decreasing pH, one pH level per page. 2) The top two slides on each page included 0.6 M KCl; the bottom two did not. 3) All wells in the top row (1) of the body of each slide contained BV 5 mg/ml (aqueous). Wells in the bottom rows (2) contained PLA 2 mg/ml (aqueous). 4) Wells in the center of each slide contained serial two-fold dilutions of NHL or IHL in distilled water. 5) Control wells in the right-hand portion of each slide contained BV, PLA, rabbit anti-HL, or NHL as indicated. The central control well always contained NHL. Unlabeled wells were empty.

In Figure 3, slide A (pH 9.0; 0.6 M KCl), a dense precipitin line was formed by the reaction of NHL with BV. As the dilution of NHL increased, the line moved closer to the HL wells. This behavior was typical of a true antigen-antibody reaction. A faint precipitin line formed between NHL in the first two wells and PLA in row two. Inspection of the first NHL well suggested that an analogous band was present between NHL and the PLA fraction of whole BV.

In Figure 3, slide B (pH 9.0, 0.6 M KCl), a faint band developed between BV in row one and the lowest dilution of IHL. The low reactivity of IHL may have been due to a low total protein content; unfortunately, no protein determinations were run on the samples used in this experiment.

Figure 3 (C and D) demonstrated the interaction of HL with BV and PLA at pH 9.0 in the absence of KCl. In comparison with Figure 3A, the BV-NHL line shown in Figure 3C was markedly reduced in intensity and appeared to be displaced toward the BV wells. The precipitates surrounding the NHL wells were more difficult to interpret. They may have

Fig. 3: Immunodiffusion of HL vs. BV and PLA.

1% Noble agar, 0.05 M Tris; pH 9.0 Slides 3A to 3D

With

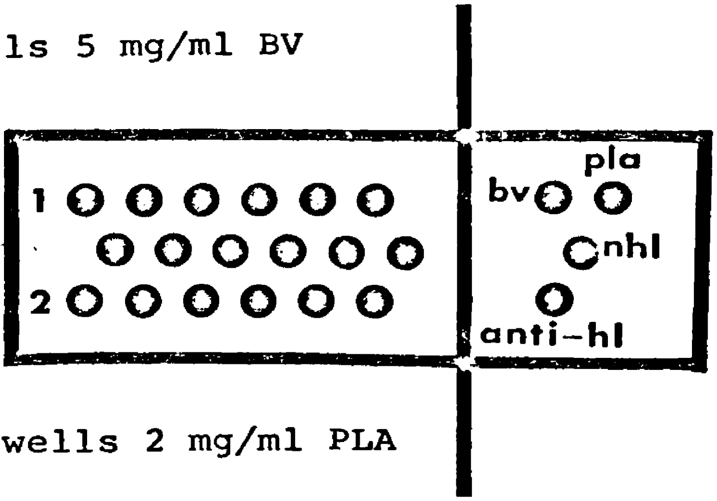
0.6 M KCl

With

0.6 M KCl

Top row: all wells 5 mg/ml BV

Center row:
twofold serial
dilutions HL in
water; first
well is HL 1:2
in glycerol



Controls

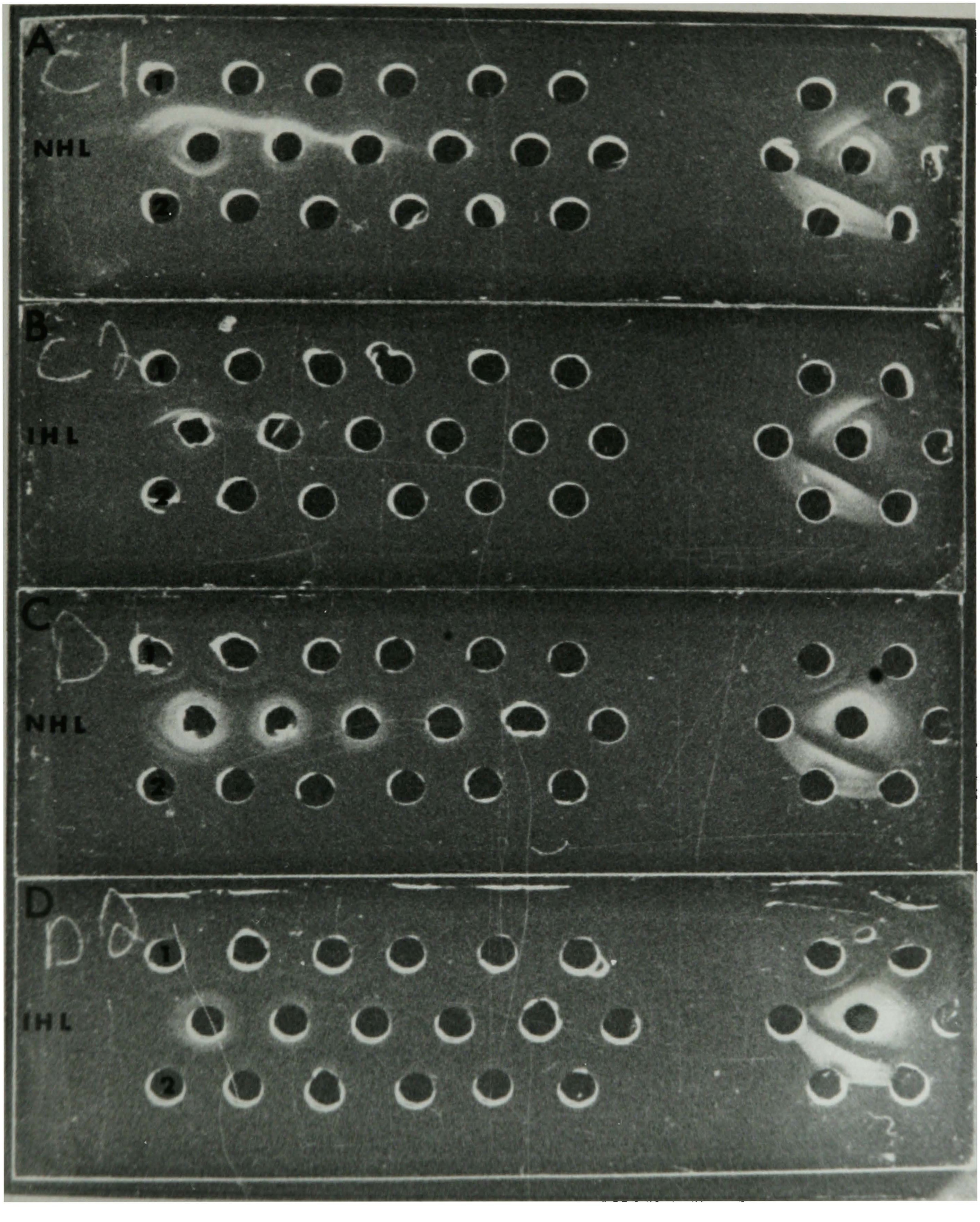
Bottom row: all wells 2 mg/ml PLA

Without

0.6 M KCl

Without

0.6 M KCl


A
Cl
1
NHL
2
B
1
IHL
2
C
D
1
NHL
2
D
1
IHL
2

been due to the reaction of NHL with PLA, or they may have represented a nonspecific reaction of NHL with some component of the supporting medium; the shape of the precipitin rings tended to suggest the former. Figure 3D indicated that IHL was also affected by a decrease in ionic strength; the changes in precipitin pattern were analogous to those described for NHL.

Overall, the results presented in Figure 3 indicated that a) both normal and immune HL were capable of reacting with BV and PLA to form antigen-antibody-like precipitin lines, b) that the reaction of both NHL and IHL with an as yet unidentified component of BV appeared to be enhanced in a high ionic strength environment, and c) that the reaction of IHL and NHL with PLA and the PLA component of BV may be more evident at lower ion concentrations.

The immunodiffusion reactions shown in Figure 4 were run at pH 7.5. Slides 4A and 4B included 0.6M KCl. Slide 4A displayed separate precipitin lines formed by the reaction of NHL with BV and with PLA. The patterns were very similar to those seen at pH 9.0, but were somewhat less intense, particularly in the case of the BV-HL band. In Figure 4B, the only discernible reaction was a very faint halo around the well containing the lowest dilution of IHL. Slides C and D contained no KCl. As was noted in the gels run at pH 9.0, the most striking effect of the reduction in ionic strength was the enhancement of the HL-PLA precipitate zones surrounding the wells containing NHL (C) and IHL (D).

Fig. 4: Immunodiffusion of HL vs. BV and PLA.

1% Noble agar, 0.05 M Tris; pH 7.5 Slides 4A to 4D

With

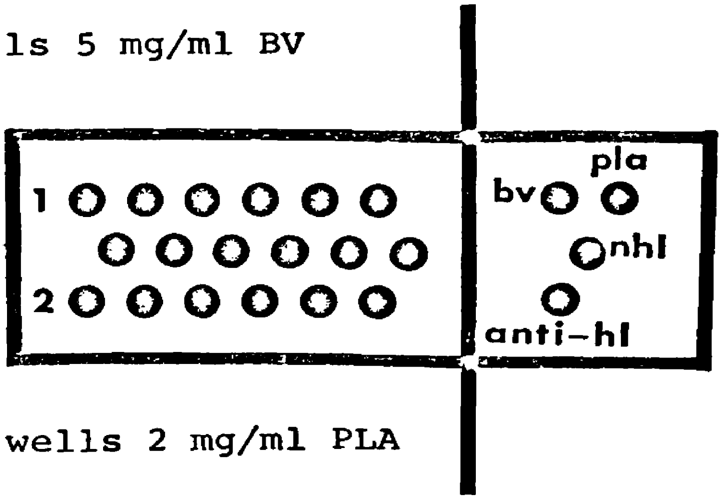
0.6 M KCl

With

0.6 M KCl

Top row: all wells 5 mg/ml BV

Center row:
twofold serial
dilutions HL in
water; first
well is HL 1:2
in glycerol



Controls

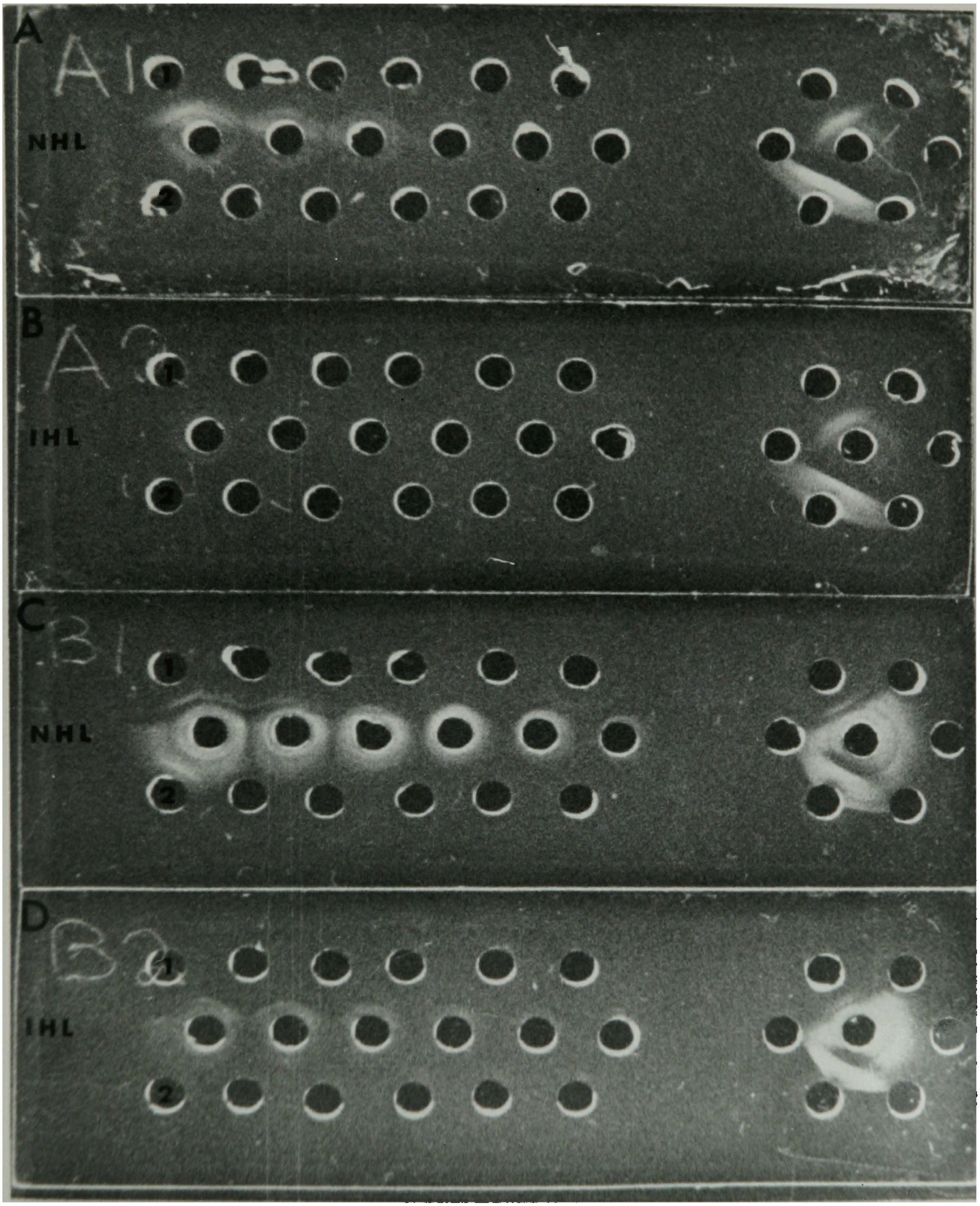
Bottom row: all wells 2 mg/ml PLA

Without

0.6 M KCl

Without

0.6 M KCl


A
A1
1
NHL
2
B
A2
1
IHL
2
C
B1
1
NHL
2
D
B2
1
IHL
2

Overall, the reduction in pH seemed to have decreased the intensity of the HL-BV reaction in the presence of KCl (Figures 3A and 4A) and to have increased the reactivity of HL with PLA in the absence of KCl (Figures 3C and 4C).

In contrast are the results presented in Figure 5. These gels were run at pH 5.0. The gels in slides A and B contained 0.6 M KCl. Neither NHL (A) nor IHL (B) exhibited any precipitin reaction with BV or PLA. However, the precipitin band formed between NHL and rabbit anti-HL in the control wells appeared much as it did in previous gels. Therefore, in the presence of a high salt concentration, a pH of 5.0 is not inimical to a true antigen-antibody precipitin reaction, but it does appear to interfere with the HL-BV system. Since the pI of the majority of HL proteins is estimated to be about 5, the HL molecules incorporated into the gels in Figure 5 should have carried little or no net charge. Therefore, it is unlikely that they would have bound by electrostatic interaction to the positively charged BV components, which were demonstrated earlier (under Agarose Electrophoresis) to behave as cations at pH 8.6.

Figures 5C and 5D show the results obtained when NHL (C) and IHL (D) were diffused against BV and PLA at pH 5.0 without KCl. As before, the primary effect of reducing the salt content was the enhancement of the HL-PLA precipitin zones surrounding the HL wells. The effect on the antigen-antibody control reaction was interesting: whereas this

Fig. 5: Immunodiffusion of HL vs. BV and PLA.

1% Noble agar, 0.1 M citrate-phosphate; pH 5.0; Slides 5A to 5D

With

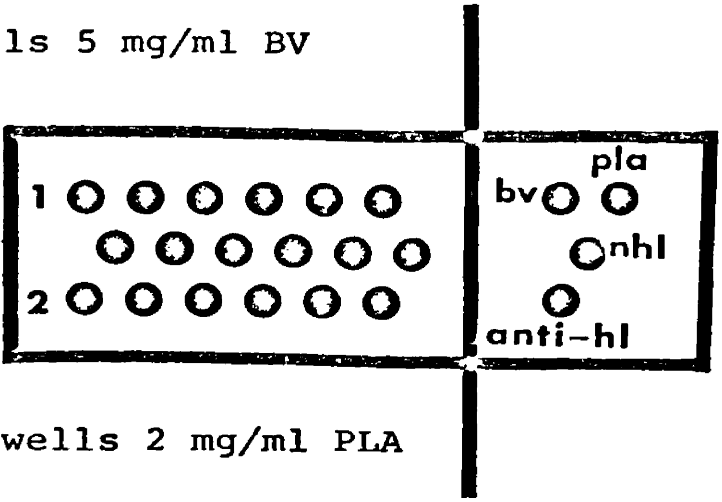
0.6 M KCl

With

0.6 M KCl

Top row: all wells 5 mg/ml BV

Center row: twofold serial dilutions HL in water; first well is HL 1:2 in glycerol



Controls

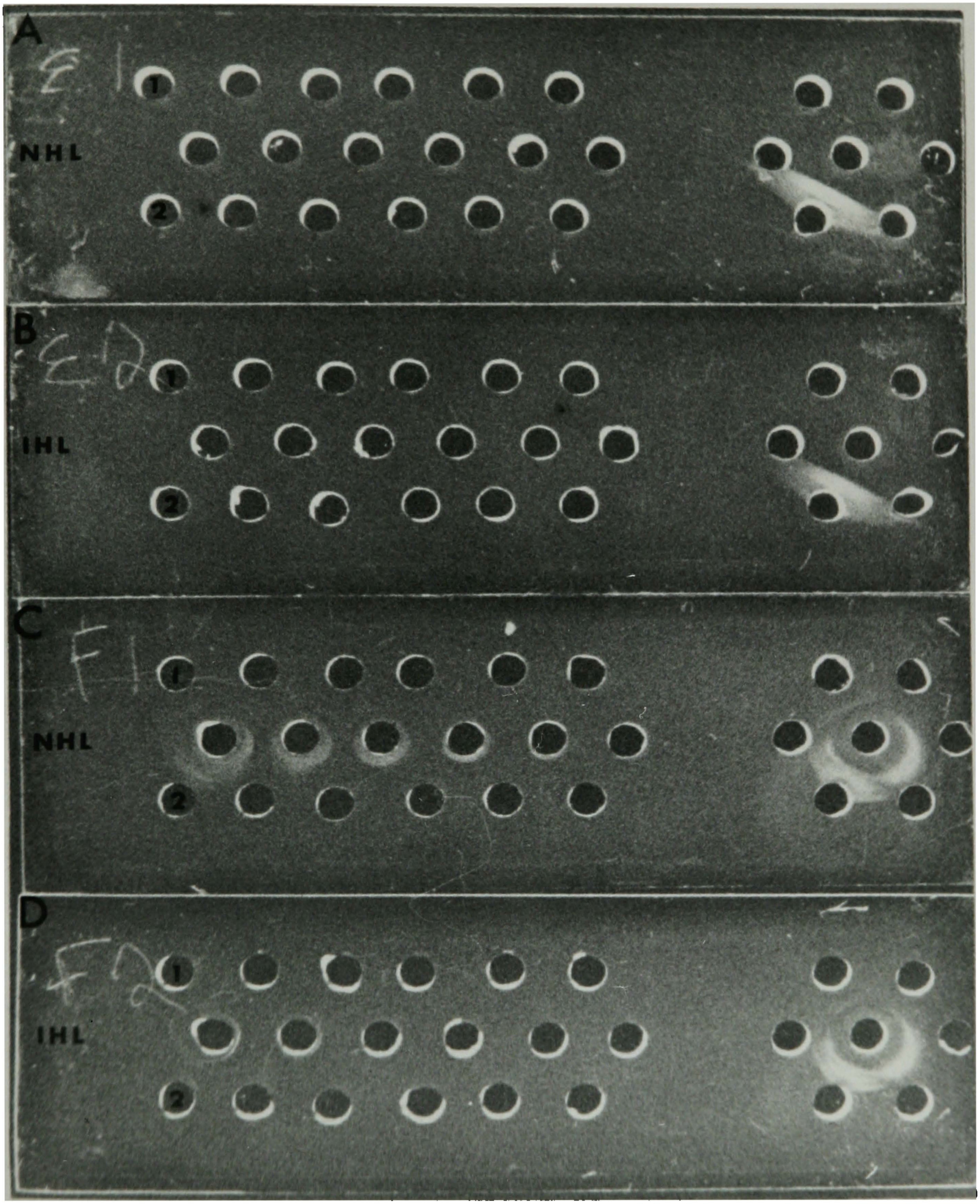
Bottom row: all wells 2 mg/ml PLA

Without

0.6 M KCl

Without

0.6 M KCl


A
E1
1
NHL
2
B
E2
1
IHL
2
C
F1
1
NHL
2
D
F2
1
IHL
2

reaction was stable at pH 5.0 in the presence of 0.6M KCL, removal of the salt led to a marked deterioration of the precipitin bands. The published pI's for a variety of mammalian immunoglobulins range from 4.4 to 7.0 (22). If these values are representative of the rabbit immunoglobulins employed in the present study, perhaps both HL and anti-HL molecules bore a net positive charge at pH 5.0, and the resulting electrostatic repulsion interfered with the formation of an antigen-antibody precipitate. Addition of salt to the environment might have served to swamp the charge effect and restore activity.

The final immunodiffusion gels (Figure 6) were run at pH 4.0. Once again, both NHL (A) and IHL (B) failed to react with BV and PLA in the presence of KCl, whereas the HL-anti-HL reaction in the control wells appeared as usual. Slides 6C and 6D showed the effect of KCl absence. Dense precipitate ringes formed around the wells that contained the lowest dilutions of NHL (C); an analogous but much fainter ring formed by IHL could be seen in the first well of D. Note that the control reaction was completely disrupted. In this instance, the precipitates around the HL wells more likely represented nonspecific reactions than the interaction of HL with BV or PLA. In particular, the ring around the HL control well appeared dense on all sides, not only on the portions that faced wells containing venom components. In addition, the control well containing rabbit anti-HL was surrounded by a diffuse zone of precipitate. At pH 4.0,

Fig. 6: Immunodiffusion of HL vs. BV and PLA.

1% Noble agar, 0.1 M citrate-phosphate; pH 4.0; Slides 6A to 6D

With

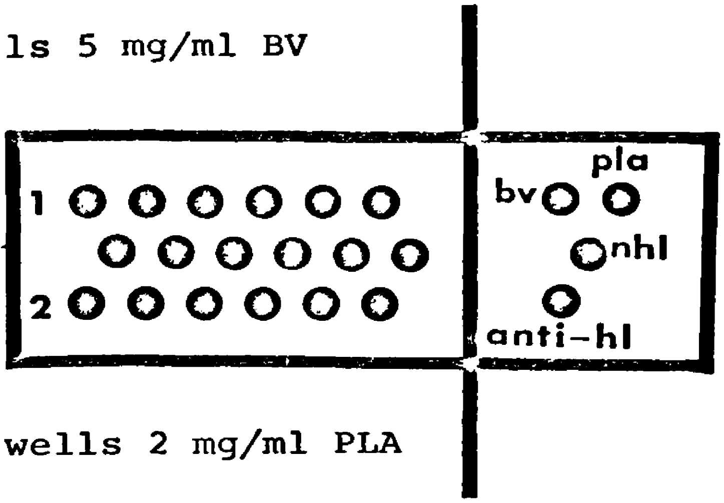
0.6 M KCl

With

0.6 M KCl

Top row: all wells 5 mg/ml BV

Center row: twofold serial dilutions HL in water; first well is HL 1:2 in glycerol



Controls

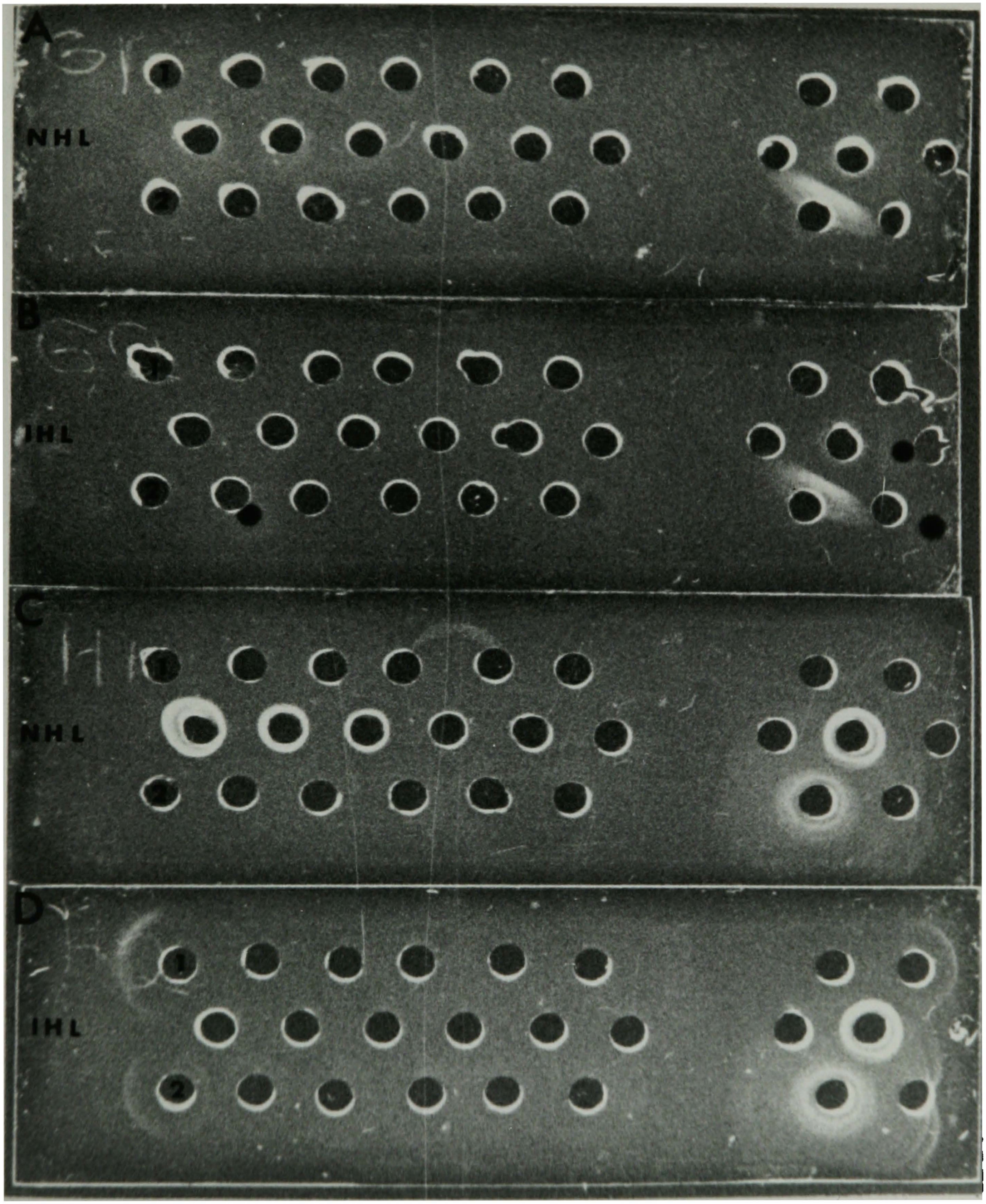
Bottom row: all wells 2 mg/ml PLA

Without

0.6 M KCl

Without

0.6 M KCl


A
Gr
1
NHL
2
B
Gr
IHL
C
Hr
1
NHL
2
D
Hr
1
IHL
2

both HL and rabbit serum proteins may have been so highly protonated that they interacted more strongly with the negatively charged agar substrate than with each other.

All lines formed by the reaction of HL with BV (other than lines attributed to the PLA component of BV) took up both the protein stain amido black and the lipid stain oil red O. Lines formed by the reaction of HL with PLA and the PLA component of BV took up neither. Significantly, the dense precipitates seen around the HL wells at pH 4.0 (Figure 6) stained black, thus supporting the contention that they were not formed via HL-PLA interaction. Bands formed by the reaction of HL with rabbit anti-HL took up only amido black.

The information gained from the immunodiffusion experiments may be summarized as follows: First, as evidenced by the behavior of the HL- rabbit anti-HL control, a high salt concentration tended to stabilize true antigen-antibody reactions at an acid pH. Second, both NHL and IHL could react independently with PLA and a separate but as yet unidentified BV component to form precipitin lines. The HL-PLA precipitate took up neither a lipid nor a protein stain, whereas the precipitate formed by HL and another BV component took both stains. Third, HL-BV precipitin formation was strongest at pH 9.0 in the presence of KCl; HL-PLA lines were most evident at pH 7.5 in the absence of KCl. This difference suggested that HL-PLA interaction may be based primarily on electrostatic attraction, whereas the reaction of HL with the other BV component might be more dependent on hydrophobic bonding. This problem will be dealt with in more detail in the Discussion. Fourth, formation of BV-HL precipitin lines was weak or nonexistent below pH 5.0 regardless of the ionic strength of the support medium. The reaction of a rabbit immunoglobulin with its corresponding antigen was stable down to pH 4.0 (below the pI of most antibodies (22)) in a high salt environment, but the reaction was disrupted in the absence of salt when the pH was less than or equal to 5.0.

Carbohydrate inhibition

Lectins are proteins or glycoproteins from plants or lower animals which bind specifically to certain sugar residues in carbohydrate moeities; lectin binding may usually be inhibited by simple sugars specific for the lectin. Glucose, galactose, mannose, fucose, N-acetylglucosamine, and sialic acid are among the most common constituents of lectin-binding sites (28).

A number of invertebrate species have been shown to produce circulating proteins with lectin-like activity (29, 30). The carbohydrate-inhibition tests were performed to determine if the reactions between HL and BV might be mediated by a lectin or lectins. The rationale was that each sugar tested should block precipitin formation by any lectin specific for that carbohydrate. Conversely, the mannose-binding lectin concanavalin A should interfere with any mannose-specific lectin by competing for mannose-containing binding sites. Since the immunodiffusion results described previously indicated that pH and ionicity influence precipitin formation, carbohydrate inhibition assays were carried out at two levels of pH and at two different KCl concentrations. Because IHL had proven less reactive in the earlier immunodiffusion tests, NHL was used for this experiment. The results are presented in Figure 7.

Slide A (pH 9.0, 0.6 M KCl) displayed, in column 1, a system of two continuous rings around the central HL well. The inner ring was due to the reaction of HL with PLA, and the outer ring to the reaction of HL with another BV component. Column 2 showed the ring formed by HL and purified PLA. The bands in column 1 and in column 2 formed by the reaction of NHL with BV and PLA combined with carbohydrates were comparable in intensity to the no-carbohydrate control bands seen in column 3 between the central HL well and positions 15 (BV) and 16 (PLA). The precipitin lines between the central HL well (column 3) and the wells containing concanavalin A (17,18,19) appeared intact. Therefore, neither concanavalin A nor any of the sugars tested inhibited precipitin formation. Slides B and C supported this contention. In slide B (pH 9.0, no KCl), some of the precipitin lines had moved away from the inner well; this was shown earlier to be an effect of low salt concentration. The PLA ring around the central well of slide C (column 1) was so close to the much stronger BV ring as to be nearly invisible. Nevertheless, the

Fig. 7:

Effect of carbohydrates on precipitin formation

Slide A: 1% Noble agar, 0.05 M Tris, 0.6 M KCL; pH 9

Slide B: 1% Noble agar, 0.05 M Tris; pH 9

Slide C: 1% Noble agar, 0.05 M Tris, 0.6 M KCl; pH 7.5

Numbering of wells for slides A and C is the same as that shown for slide B.

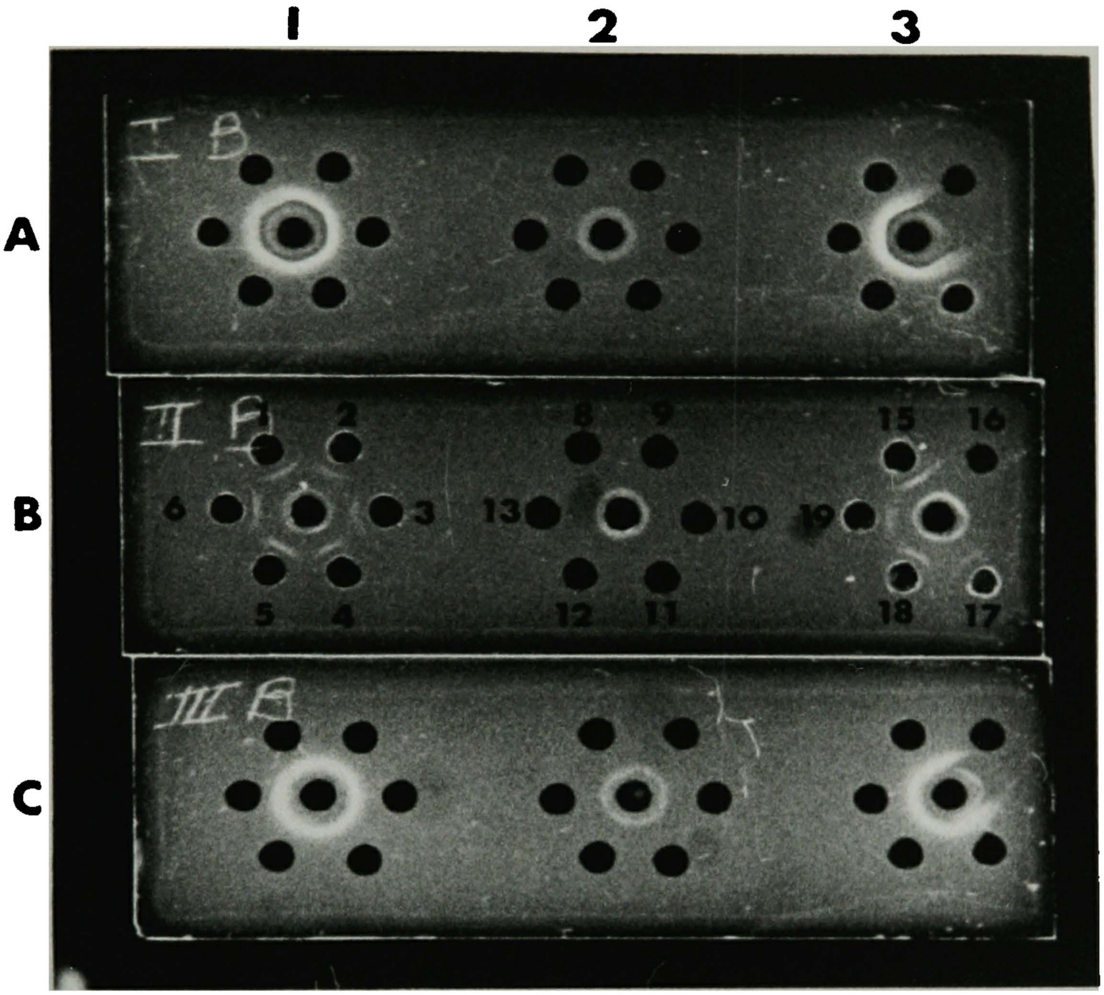
Column 1: central wells contain NHL 1:2 in glycerol. Wells 1 through 6 contain BV at 5 mg/ml final concentration, in addition to the following carbohydrates at a final concentration of 0.25 M:

1	D-galactose
2	D-glucose
3	D-mannose
4	alpha-L-fucose
5	alpha-L-rhamnose
6	N-acetyl-D-glucosamine

Column 2: same format as Column 1, with the substitution of PLA at a final concentration of 1 mg/ml for BV in the outer wells (8 through 13).

Column 3: central well contains NHL 1:2 in glycerol. Others contain (final concentrations):

15	BV at 5 mg/ml
16	PLA at 1 mg/ml
17	BV 5 mg/ml + concanavalin A 100 ug/ml
18	BV 5 mg/ml + concanavalin A 10 ug/ml
19	BV 5 mg/ml + concanavalin A 1 ug/ml


1
2
3
A
B
C
I B
II B
III B
1
2
8
9
15
16
6
3
13
10
19
5
4
12
11
18
17

uniformity of the precipitates shown on the last two slides again suggested that no inhibition had taken place. Therefore, it was unlikely that a lectin with specificity for mannose, or for any of the other carbohydrates assayed, was involved in the formation of BV-HL precipitates. In addition, the junction of continuity between the HL-PLA precipitate and the line formed by HL with the putative PLA component of whole BV (best seen in slide B, column 3, between the central HL well and wells 15 and 16) substantiated the assumption that the purified PLA used in these experiments and the PLA component of BV were identical.

Shortly after completion of the carbohydrate inhibition experiments, a report on an earlier study dealing with the behavior of various venoms in immunodiffusion assays was encountered. That paper (11) made several points relevant to the present investigation. First, melittin, the major component of BV (45 to 50% dry weight (33)), was capable of forming non-immune, lipid-containing precipitate lines when diffused against a variety of normal, non-immune mammalian sera in both agar and agarose gels. Second, bee and snake venoms often produced a "halo" effect around wells in agar gels, but not in agarose gels. Highly cationic venom components were thought to bind to the anionic macromolecules of agar, but not to the less highly-charged molecules of agarose. Thus, when investigating the interaction of charged molecules in diffusion gels, the choice of support media may influence the results. Third, heparin, a polyanionic macromolecule, has been shown to inhibit halo formation by snake venom in agar gels.

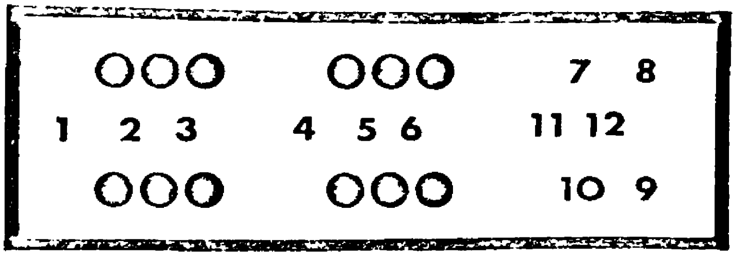
In order to compare the behavior of HL-BV and HL-PLA reactions in support matrices with different intrinsic charges and to determine if heparin reacts with BV in the same manner it reacts with snake venom, HL, heparin, BV, and PLA were diffused together in various ways in both agar and agarose gels at several levels of pH and ionic strength. Normal rabbit sera and rabbit anti-PLA were diffused against BV and against BV mixed with heparin so that nonspecific and true antigen-antibody precipitate formation could be compared. Results are presented in Figure 8.

The top rows in columns 1 and 2 of each slide contained BV, 5 mg/ml (aqueous). The bottom rows contained PLA, 2 mg/ml (aqueous). Wells in the central rows of column 1 contained serial two-fold dilutions of NHL in distilled water. Analogous wells in column 2 contained dilutions of NHL in 5 mg/ml sodium heparin (aqueous). The top rows of wells in column 3 contained normal rabbit serum while the bottom rows contained rabbit anti-PLA. Central wells in column 3 contained BV 5 mg/ml in sodium heparin 5 mg/ml; the wells to the left of center contained BV 5 mg/ml (aqueous).

In slide 8, column A (1% Noble agar in PBS; pH 7.2), faint precipitin lines formed between the HL in the central wells and BV in the top wells, as well as between HL and PLA in the bottom wells. As usual, there appeared to be

Fig. 8: Comparison of agar vs. agarose as gel support.

Top row: BV 5 mg/ml



Bottom row: PLA 2 mg/ml

Slide A: 1% Noble agar in PBS; pH 7.2

Slide B: 1% agarose in PBS; pH 7.2

Slide C: 1% Noble agar in 0.05 M Tris, 0.6 M KCl; pH 9

Slide D: 1% Noble agar in 0.05 M Tris, pH 9

Slide E: 1% agarose in veronal, pH 8.6

Wells 1 - 3: serial twofold dilutions of NHL in H_2O; first well is NHL 1:2 in H_2O.

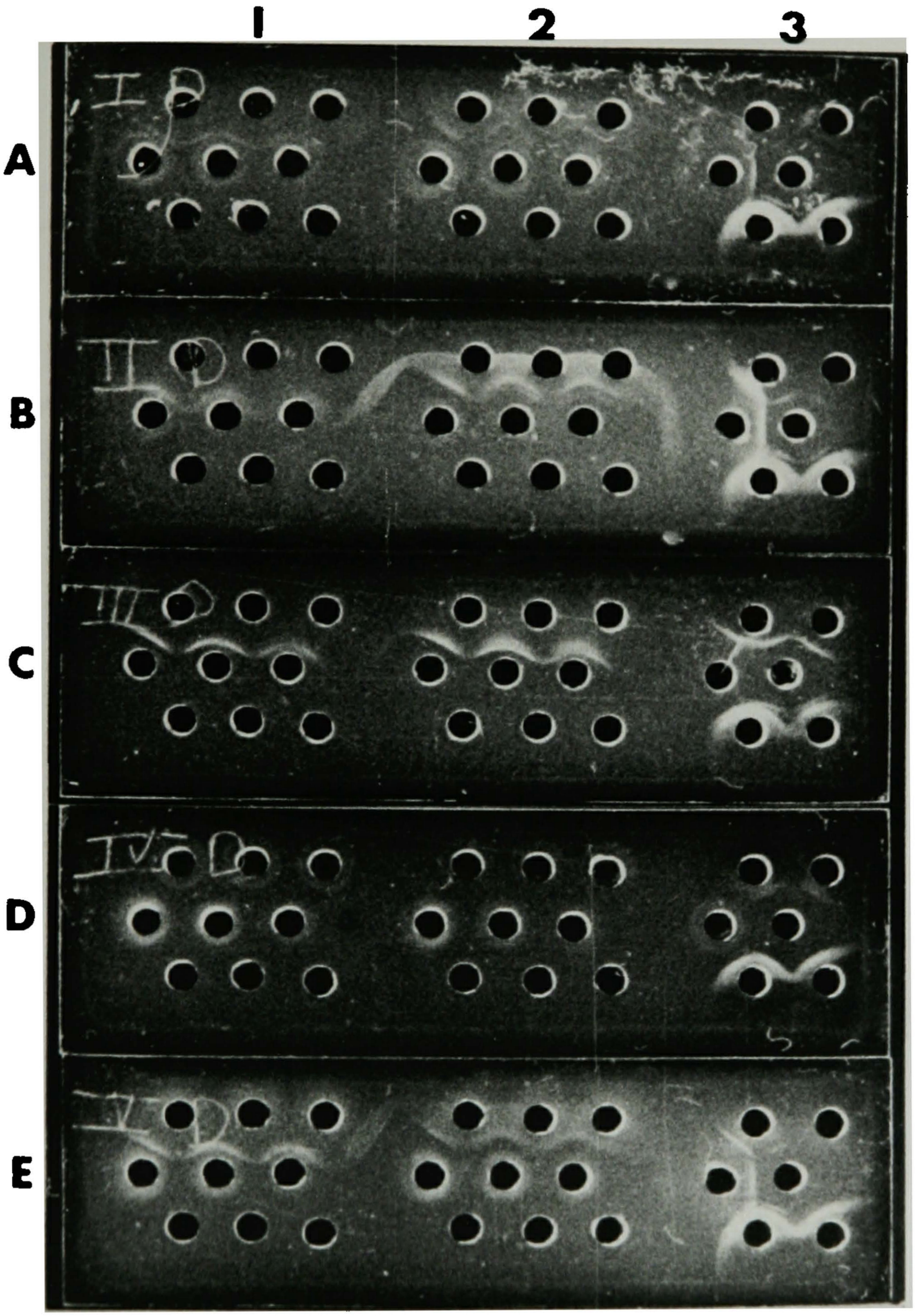
Wells 4 - 6: serial twofold dilutions of NHL in sodium heparin (aqueous) at 5 mg/ml final concentration; first well is NHL 1:2 in heparin.

Wells 7,8: normal rabbit serum

Wells 9,10: rabbit anti-PLA

Well 11: BV 5 mg/ml in H_2O

Well 12: BV 5 mg/ml in sodium heparin 5 mg/ml (aqueous)


1
2
3
A
B
C
D
E
I D
II D
III D
IV D
V D

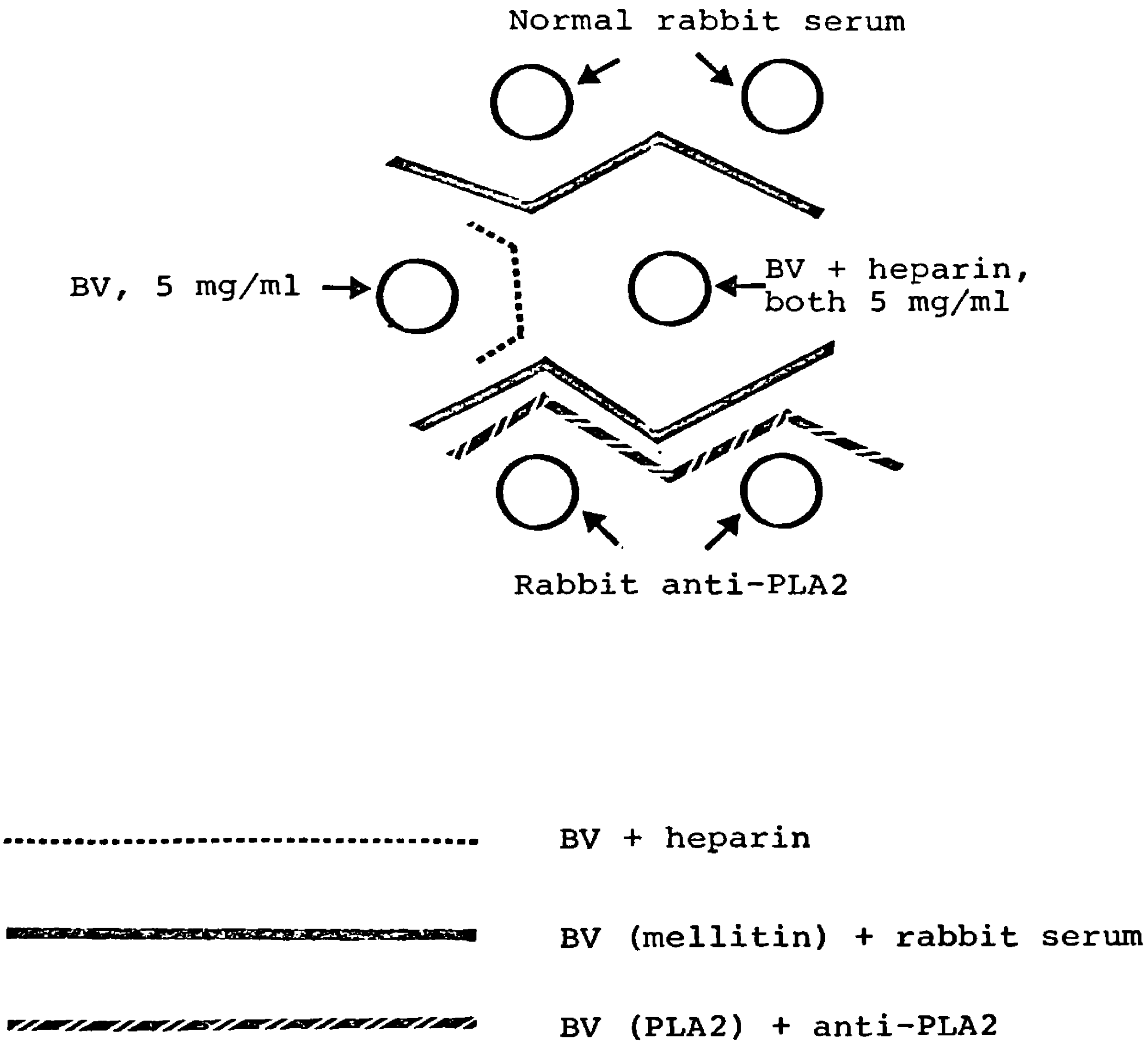
two systems of lines. There were precipitin rings closely surrounding the HL wells and formed by the reaction of HL with PLA, as well as separate lines formed by HL together with a non-PLA component of BV. For purposes of succinctness, this non-PLA component will be referred to as melittin from this point forward; the propriety of this designation will be made plain in a subsequent Results section.

In column 2 of slide A, the principal effect of the inclusion of heparin in the central HL wells may be viewed in two ways; first, as an upward displacement of the HL-melittin band seen in column 1 or second, as the appearance of a unique band due to the reaction of BV with heparin. Results shown on the remaining slides tended to support the latter interpretation. Heparin exerted no apparent effect on the reaction of HL with PLA.

The patterns seen in column 3 of slides A through E are more easily interpreted with the aid of the diagram presented in Figure 8B. In slide A, column 3, the most evident band was that formed between BV in the central row of wells and rabbit anti-PLA in the bottom row. Lines due to the reaction of melittin with NRS and with rabbit anti-PLA were only faintly discerned; a distinct line formed by the reaction of BV with heparin was intermediate in intensity.

The most striking feature of slide B (1% agarose in PBS; pH 7.2) was the emergence in column 2 of a dense,

Fig. 8B: composite diagram of precipitate patterns seen in column 3 of Fig. 8, slides A through E.


Normal rabbit serum
BV, 5 mg/ml
BV + heparin, both 5 mg/ml
Rabbit anti-PLA2
BV + heparin
BV (mellitin) + rabbit serum
BV (PLA2) + anti-PLA2

broad precipitin band. This band most likely was due to the reaction of BV in the upper wells with heparin in the central wells; this view was supported by the intense reaction between heparin and BV seen in column 3 of the same slide (between the two central wells) and by the presence of a faint but separate line attributable to HL-melittin interaction visible just below the BV-heparin band in column 2.

The enhancement of the BV-heparin reaction appeared to be dependent on the substitution of agarose for agar in the gel formulation. It was postulated that the negative charge intrinsic to agar might have competed with heparin (also negatively charged) for interaction with cationic molecules of BV; when relatively uncharged agarose was substituted for agar, BV cations could have interacted more freely with heparin, and precipitation of BV-heparin complexes might have ensued.

The results shown on slides C and D implied a role of pH in BV-heparin reactivity. Both slides contained 1% Noble agar in Tris buffer; pH 9.0; slide C included 0.6 M KCl. As noted under Immunodiffusion, a high salt concentration tended to favor formation of HL-melittin bands, whereas low salt enhanced the HL-PLA reaction.

Note that no obvious manifestation of heparin activity was seen in either slide. Both slides contained agar, but so did slide A, and a distinct BV-heparin band was visible in slide A, column 3, at pH 7.2. Therefore, the nonreactivity of heparin in slides C and D could not have been solely an effect of agar versus agarose or of ionic strength; pH must also have played a part. As the pH increased, so did the ratio of OH^- to H^+. Perhaps the additional negative ions, in a fashion similar to the anionic groups of agar, competed with heparin for for BV cations and, thus, inhibited BV-heparin precipitate formation.

Slide E presented the precipitin patterns formed in agarose with veronal buffer, pH 8.6. This slide was included to aid in interpreting the immunoelectrophoresis information to be presented in the following section. The results were encouraging. Agarose in veronal at pH 8.6 seemed to represent an optimal compromise in terms of pH, ionicity, and support matrix, with all of the patterns that were manifested in the preceding slides being visible to a reasonable extent.

When the slides in Figure 8 were stained for protein and lipid, bands formed between melittin and both normal and anti-PLA rabbit sera took up oil red O; those formed between BV and heparin took up amido black. Precipitates due to HL and whole BV stained with both amido black and oil red O; those formed between HL and PLA stained very faintly with amido black or not at all.

In conclusion, the results of this section may be summarized as follows. First, heparin was shown to react with whole BV, but not with PLA, to form a protein-containing precipitin line. This reaction was more evident in agarose than in agar, was neutralized by a high salt concentration, and appeared to be stronger at pH 7.2 than at pH 9.0. These observations were interpreted to indicate that BV and heparin formed lines of precipitate via electrostatic interaction. Second, a non-PLA component of BV (melittin) was shown to form a non-immune, lipid-containing complex when BV was diffused against both normal and anti-PLA rabbit sera. Third, in support of conclusions reached in a previous section, it was again noted that the formation of precipitin lines attributed to the reaction of melittin with hemolymph was enhanced by high ionic environments, whereas lines formed by HL and PLA were more evident at lower salt concentrations.

Immunoelectrophoresis

Results of the immunodiffusion tests demonstrated that the reaction of BV with NHL and IHL in diffusion gels gave rise to more than a single system of precipitin lines. The fact that BV could form precipitin lines when diffused against heparin or non-immune rabbit serum complicated the interpretation of the BV-HL interactions. In order to clarify the immunodiffusion results, immunoelectrophoresis was performed on various combinations of HL, BV, PLA, and rabbit sera. The results are presented in Figures 9 and 10. Electrophoresis was conducted in 1% agarose in veronal buffer, pH 8.6. Slide A was included as a control on the procedure and to serve as a reference in comparing systems of true antigen-antibody precipitin lines with those generated by the reaction of HL with BV and PLA.

Figure 9, slide B, shows the precipitates that resulted when normal and immune HL were electrophoresed and diffused against PLA. As usual with this lot of HL, NHL was more reactive than IHL. These precipitates stained diffusely with amido black. In slide C, where whole BV was diffused against electrophoresed HL, a system of precipitin bands common to both NHL and IHL appeared superimposed on the diffuse HL-PLA reaction. These bands stained faintly with both oil red O and amido black; they were attributed to the reaction of HL with melittin. An additional band present only in the NHL pattern (arrow) failed to stain; its identity was unknown.

In Figure 10, slide D, PLA and BV were electrophoresed and diffused against NRS. The sole band or system of bands that were produced stained with oil red O, as did the analogous band(s) in slide E. These bands were formed by the reaction of melittin with normal lipoidal serum components. Slide E also displayed a unique system of bands formed by the reaction of BV and PLA with rabbit anti-PLA. As usual, these bands took up the protein stain.

The IHL in Figure 10, slide F, failed to react to any great extent when diffused against BV and PLA. The suggestion of a very faint IHL-PLA precipitin line was seen to be hugging the outer edge of the IHL trough near the

Fig. 9: Immunoelectrophoresis of NHL and IHL

1% agarose in veronal buffer; pH 8.6

Slides A through C

NHL = normal hemolymph 1:2 in glycerol

IHL = immune hemolymph 1:2 in glycerol

PLA = phospholipase A_2, 2 mg/ml in H_2O

BV = bee venom, 5 mg/ml in H_2O

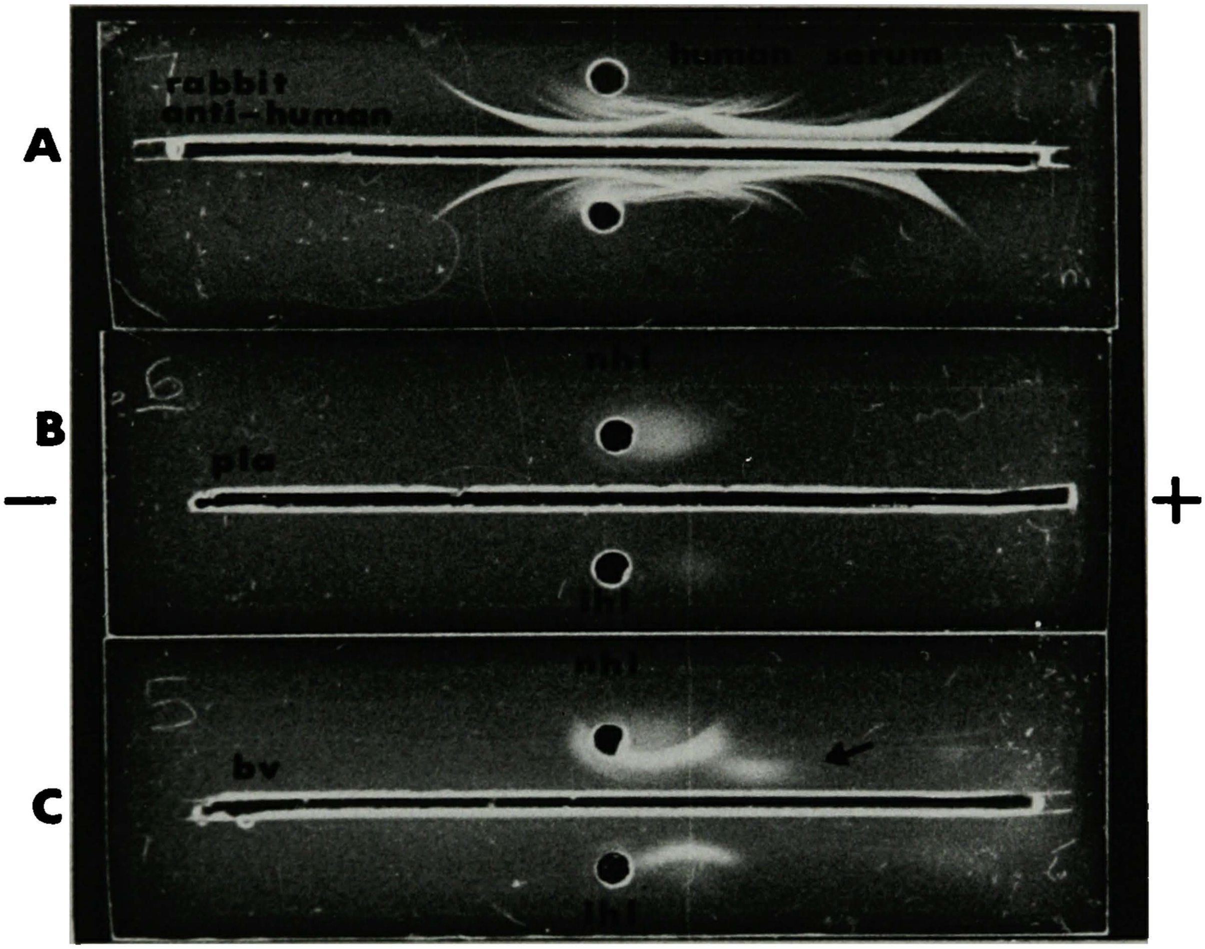

7
human serum
rabbit
anti-human
A
6
nhl
B
pla
—
+
lhl
5
nhl
bv
C
lhl

Fig. 10: Immunoelectrophoresis of BV and PLA

1% agarose in veronal buffer; pH 8.6

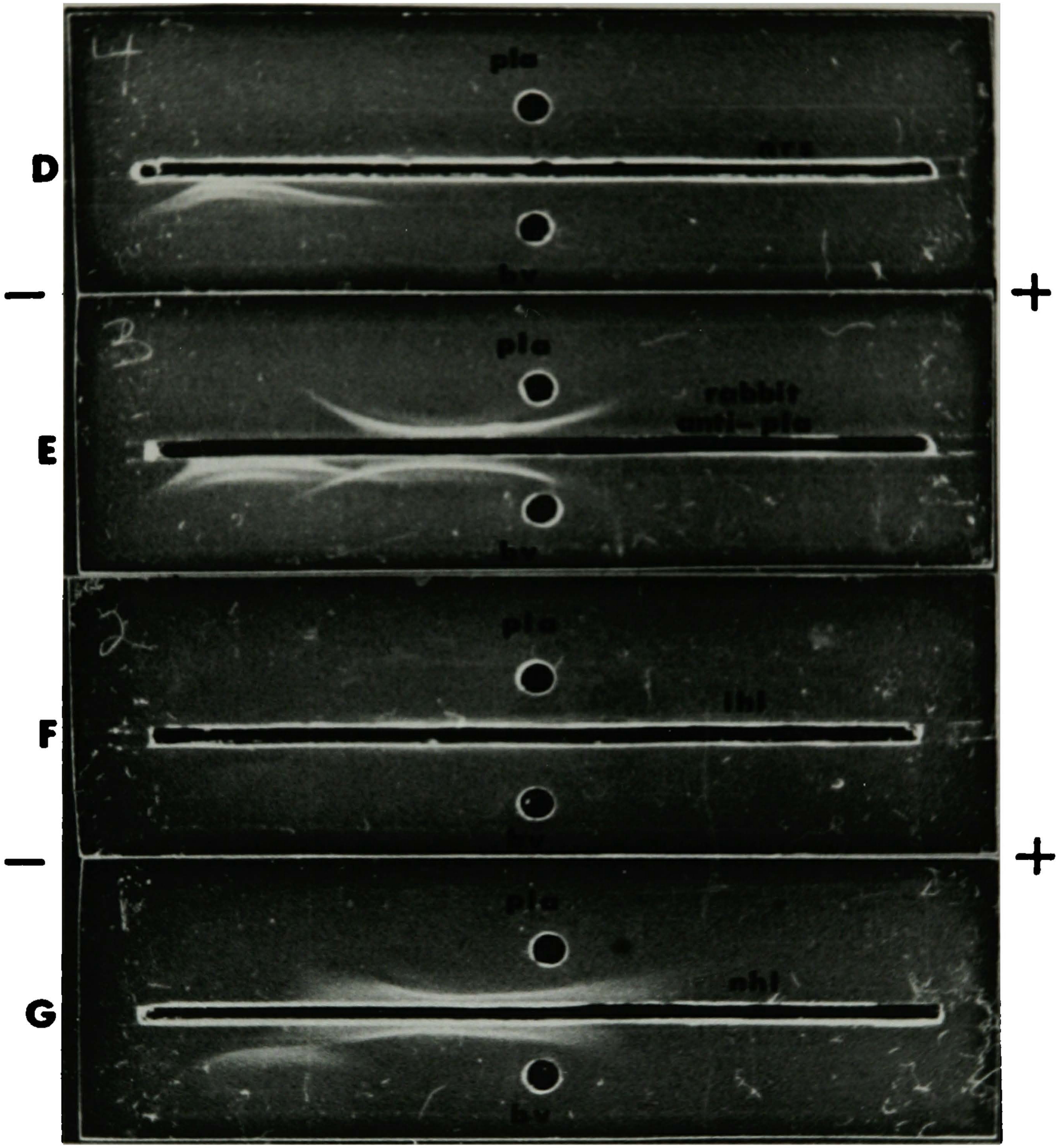
Slides D through G

NHL = normal hemolymph 1:2 in glycerol

IHL = immune hemolymph 1:2 in glycerol

PLA = phospholipase A_2, 2 mg/ml in H_2O

BV = bee venom, 5 mg/ml in H_2O


4
pla
ars
D
hv
−
+
3
pla
rabbit
anti-pla
E
hv
2
pla
ihl
F
hv
−
+
1
pla
nhl
G
hv

BV and PLA wells. Slide G was much more revealing, and looked remarkably like Slide E. The major bands present on both sides of the NHL trough failed to take up stain; this behavior was noted in HL-PLA lines formed in previous diffusion gels. The cathodal band unique to BV stained faintly with both amido black and oil red O. Its affinity for the lipid stain and its location as compared to the analogous bands in slides D and E justified its interpretation as a non-immune precipitate formed by the reaction of melittin with some component of HL.

Overall, the immunoelectrophoresis results clearly demonstrated that NHL was capable of combining independently with both PLA and melittin. The low reactivity of the IHL that was assayed precluded any statement about its ability to combine with melittin; it was shown to bind (albeit weakly) with PLA. Evidently the ability to form complexes with PLA was not restricted to HL from immunized cockroaches. This observation failed to support the idea that IHL contained a unique molecule generated in response to BV immunization, but it did not rule out the possibility that both immune and normal HL contained an endogenous PLA-binding substance that may have varied in concentration between normal and immunized animals.

Immunoprecipitation assay

As stated in the Introduction, PLA was chosen as the BV component most likely to have served as the "antigen" that stimulated the protective response in immunized cockroaches. One objective of this project was to devise an assay whereby the relative binding affinities of NHL and IHL for PLA might be compared. Since a supply of ^{125}I-PLA had been prepared for use in earlier experiments, the initial attempts at constructing such an assay were based on radioimmunoprecipitation. The general idea was to combine equal amounts of NHL or IHL with a standard amount of ^{125}I-PLA, allow the components to interact, then precipitate any HL-PLA complexes (either directly or after treatment with rabbit anti-HL), while leaving unbound ^{125}I-PLA in solution. After centrifugation and washing, comparison of the total radioactivity with the radioactivity that was precipitated would provide an index of relative PLA-binding ability. While straightforward in principle, the approach proved unsuccessful in practice. The central difficulty was the failure to find a precipitating agent that would selectively pull down HL without precipitating unbound ^{125}I-PLA. Various concentrations of common precipitating reagents (ammonium sulfate and polyethylene glycol) were tried without success. The protein A precipitation technique described in Materials and Methods promised to provide a way around the problem: the protein A moieties of Pansorbin would bind specifically to and precipitate only the rabbit anti-HL antibodies incorporated into the assay. These antibodies, with any attached HL or HL-PLA complexes, would be pulled out of solution, while any unbound PLA would be left behind. In order to confirm the efficacy of the system, positive controls (wherein anti-PLA Pansorbin was substituted for the anti-HL form) and two different non-specific binding controls (Pansorbin coated with NRS and Pansorbin alone) were included in each experiment. Several trials using various proportions of IHL, NHL, ^{125}I-PLA, and appropriately coated Pansorbin indicated that neither NHL nor IHL enhanced the precipitation of label as compared to the nonspecific binding controls (i.e., less than 20% of the label was precipitated, with no significant difference between the samples and the controls). Positive controls consistently precipitated greater than 50% of the added label.

The failure of the immunoprecipitation trials may well have been related to the instability of the HL-PLA complexes noted in immunodiffusion gels. Therefore, it was decided to approach the problem of devising a HL-PLA binding assay from an angle that did not involve the mechanism of precipitate formation.

Radial diffusion assay

Two of the peptide components of BV, melittin and PLA, have been shown to be capable of disrupting the cell membranes of several species of mammalian erythrocytes. Melittin is a "direct" hemolysin; it interacts directly with susceptible cells to cause hemolysis. PLA is termed an "indirect" hemolysin. It acts by cleaving its substrate (lecithin) into lysolecithin and a fatty acid; lysolecithin in turn attacks red cell membranes to cause hemolysis (31, 33, 40, 50).

Preliminary experimentation demonstrated that when approximately 10 µg of BV was added to 1 ml of a 1% suspension of sheep erythrocytes in PBS, the cells were totally hemolyzed over a period of several minutes. Addition of 20 µl of IHL or NHL to the cell suspension along with the BV slowed the hemolysis perceptibly; addition of a small amount of heparin blocked it completely. When the experiment was repeated under more closely controlled conditions in a recording spectrophotometer, the reduction in the rate of hemolysis was found to correlate with the amount of heparin, HL, or other inhibitor added. Because the various substances tested for inhibitory activity could only be assayed one at a time, the spectrophotometric technique proved tedious. Its modification to a radial diffusion method wherein the sheep cell suspension was immobilized in an agarose gel permitted several different inhibitors or lots of HL to be tested simultaneously at a number of different dilutions. It was felt that the assay might yield quantitative information on the hemolysis-inhibiting properties of immune as compared to normal HL, and that perhaps hemolysis-inhibition might correlate with protection against BV *in vivo*.

Since heparin had demonstrated the ability to bind to BV in the immunodiffusion experiments, it was the first substance other than HL to be checked for hemolysis-inhibiting activity. It was felt that heparin complexes with BV primarily via electrostatic attraction; therefore a number of polyionic compounds were evaluated to determine if they could react with BV in a similar fashion. It was predicted that if the charge interaction hypothesis was correct, polyanions, like heparin, should inhibit hemolysis, while polycations should have no effect. The concentrations of most of the inhibitors employed were chosen arbitrarily; no attempt was made to standardize molarity or charge density. Initial tests of NRS suggested that it possessed a slight anti-hemolytic property; therefore separate solutions of bovine serum albumin and bovine gamma globulin were assayed to determine if this inhibitory effect resided specifically in either of those fractions.

Results of the radial diffusion assay are presented in Figures 11 through 15. The assay yielded raw data in the form of zone diameters. The zones were measured 18 to 24 h after initiation, at which time diffusion had not yet reached an endpoint. In order to compare values from separate runs, all data were normalized. The diffusion zone diameter produced in each run by 12.5 µg of uninhibited BV was designated 100% activity, and the other zone sizes were expressed as a fraction (%) of that value. Since the initial concentrations of the components assayed varied or (in the case of serum and HL) were undefined, concentrations were expressed as the $\log_{10}$ of serial two-fold dilutions, with zero (the $\log_{10}$ of one) representing the 1:1 dilution of the initial stock. HL preserved in glycerol was considered to be full strength. All points plotted, with the exception of those for HL, represented the average of three separate runs. The SD value given for each component was the average of the SD values calculated for each set of three data points that comprised and individual line.

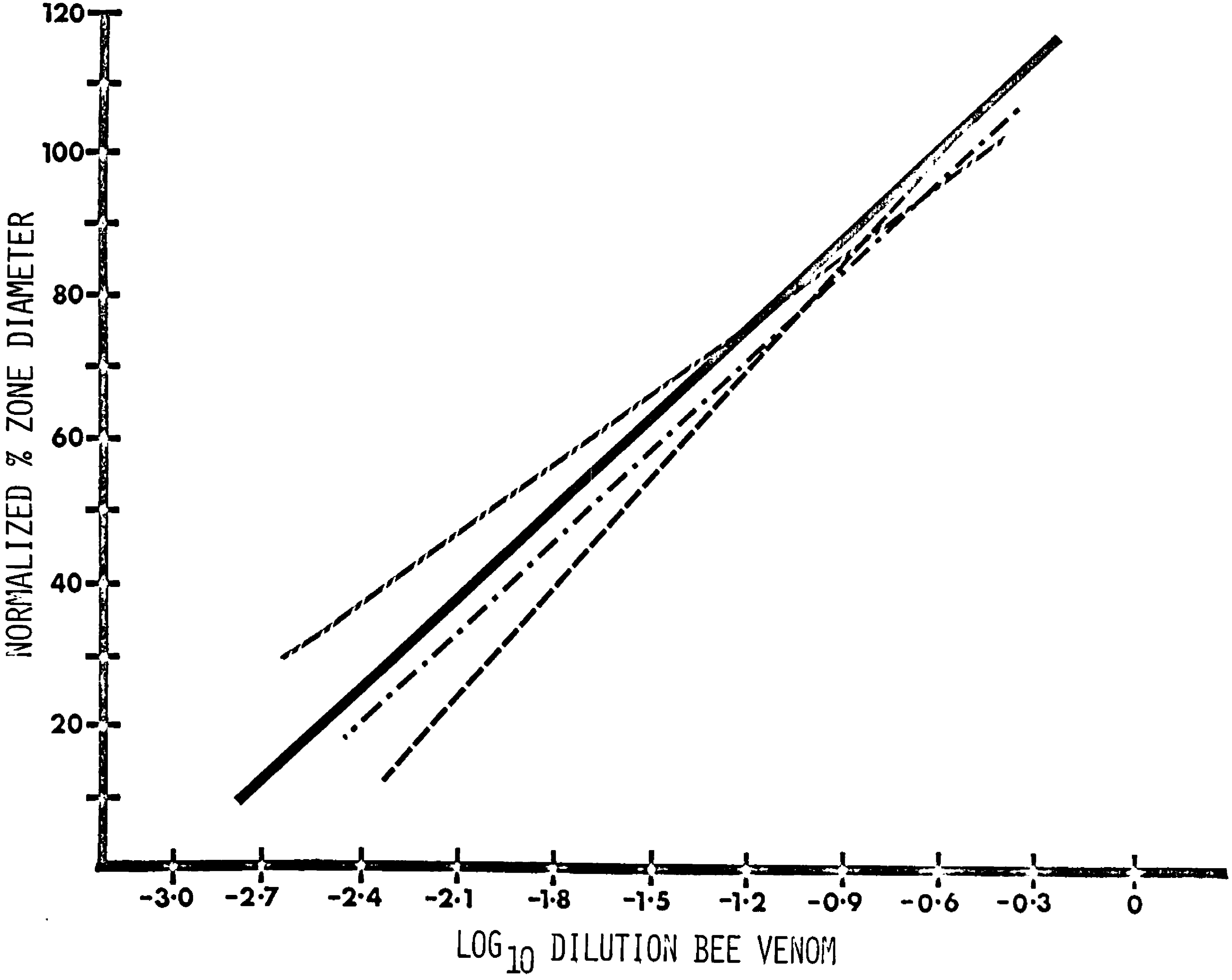
Figure 11 presents the graph obtained when the $\log_{10}$(concentration of serial two-fold dilutions) of BV added to a set of diffusion wells was plotted versus the resulting normalized hemolysis zone diameters. The linear plot was characteristic of most radial diffusion assays (20), and indicated that the amount of BV added to an assay well correlated with the resulting degree of hemolysis in a predictable and reproducible manner.

When dilutions of PLA (initial strength 2 mg/ml) were assayed in a similar fashion, no hemolysis was observed at any concentration. This result was not unexpected. PLA has demonstrated the capacity to utilize suspensions of red cell membrane "ghosts" as substrate, but the enzyme appeared to require exogenous Ca^{++} for activation (40). No calcium was incorporated into the cell suspension employed in this first set of assays; therefore, the hemolytic activity observed was due to the action of melittin alone.

The data in Figures 12 through 15 were obtained by plotting the $\log_{10}$(serial two-fold dilutions) of various

Fig. 11. Log_{10} dilutions of BV versus normalized % zone diameters. Solid line represents the average of three runs, derived from linear regression analysis. Correlation coefficient = 0.979.

Initial concentration of BV: 5000 µg/ml.


NORMALIZED % ZONE DIAMETER
120
100
80
60
40
20
-3·0
-2·7
-2·4
-2·1
-1·8
-1·5
-1·2
-0·9
-0·6
-0·3
0
LOG$_{10}$ DILUTION BEE VENOM

inhibitors versus the hemolytic zone diameters that resulted when 10 µl portions of the diluted inhibitors were combined with 12.5 µg of BV. Any variance from the 100% normalized zone diameter sggested inhibition or enhancement of hemolytic activity. The reproducibility of the degree of inhibition obtained among three replicate trials was excellent for some of the compounds tested but erratic for others; the SD value given for each compound (other than HL) should serve as a guide in interpreting the significance of the results.

Figure 12 presents the results obtained when rabbit serum and bovine serum components were tested for inhibitory activity. The deviation noted for bovine gamma globulin and normal rabbit serum is probably not significant. The inhibition caused by the two strongest concentrations of both bovine serum albumin and rabbit anti-PLA is questionable. Rabbit anti-PLA was not expected to prove any more inhibitory than NRS in this assay because all of the hemolytic activity being investigated was attributed to melittin rather than to PLA. The effect may have been spurious, or it may have been due to some inhibitory serum component that varied bewteen the immune and normal rabbits used in the experiment. Melittin is generally regarded as non-antigenic in mammals (50), therefore the inhibitory effect of the anti-PLA serum was not due to antibodies raised against melittin that may have contaminated the PLA vaccine.

Fig. 12. Log$_{10}$ dilutions of serum components in 1250 µg/ml BV versus normalized % zone diameter.

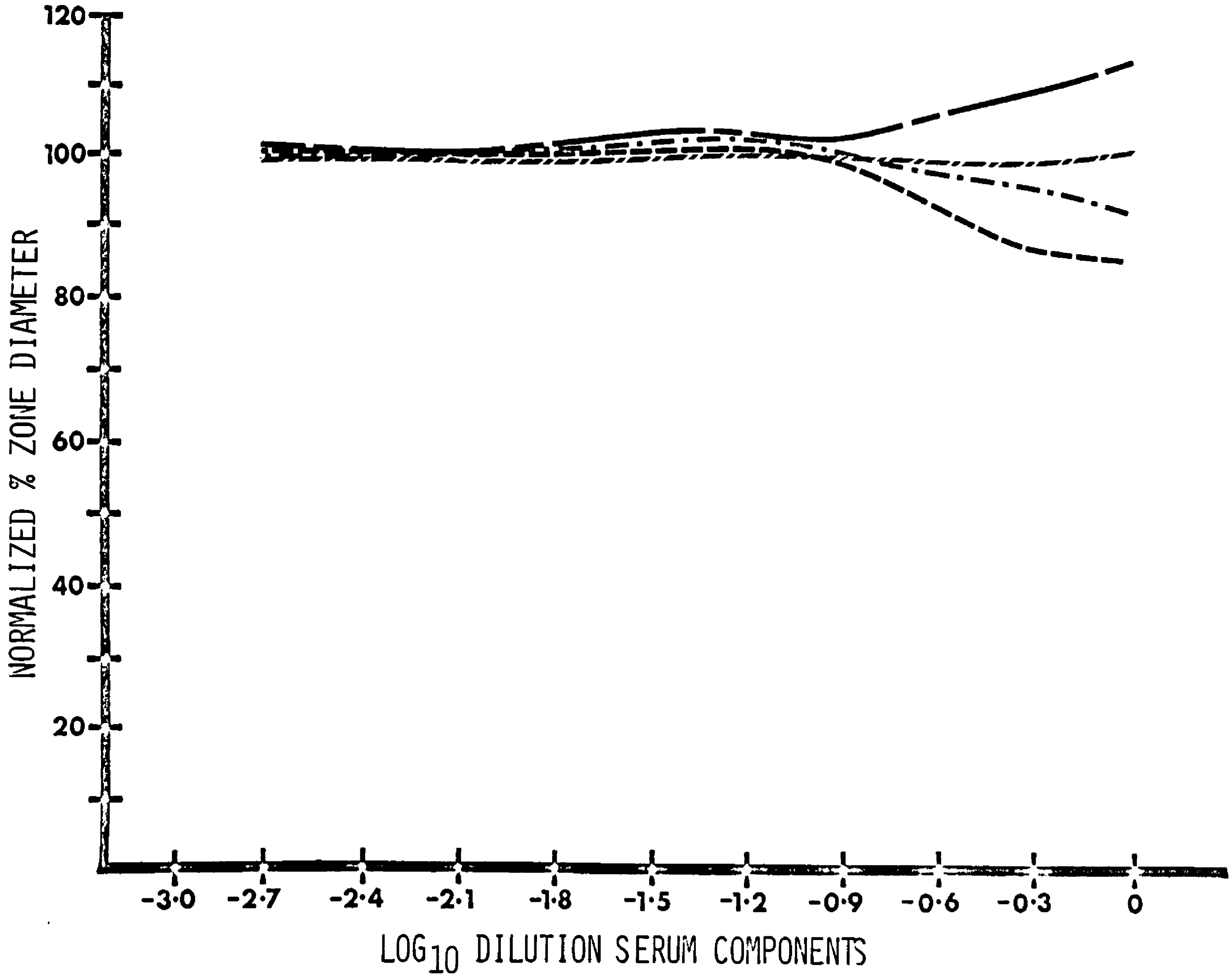
——— — —— = Bovine serum albumin, 20,000 µg/ml initial.
Standard deviation = 4.2

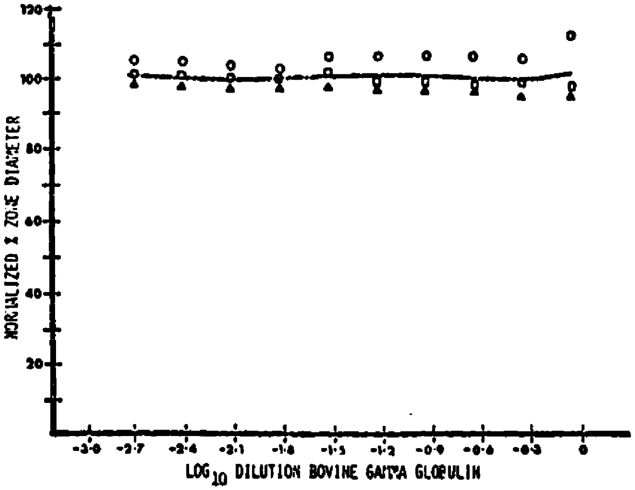
-⁄—⁄—⁄—⁄—⁄—- = Bovine gamma globulin, 12,500 µg/ml initial.
Standard deviation = 4.8

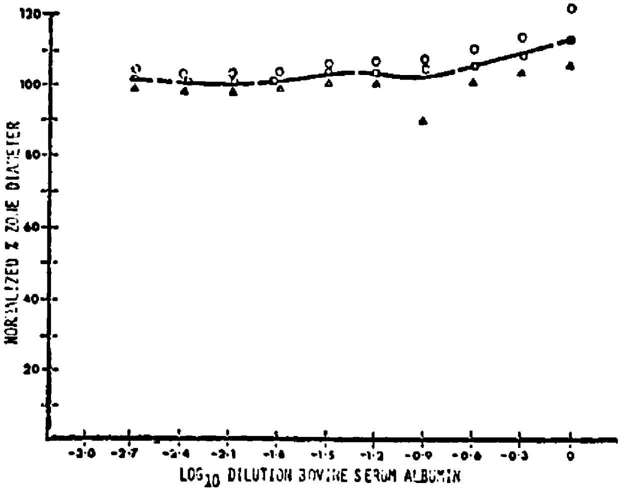
··—·—·—·· = Normal rabbit serum
Standard deviation = 4.3

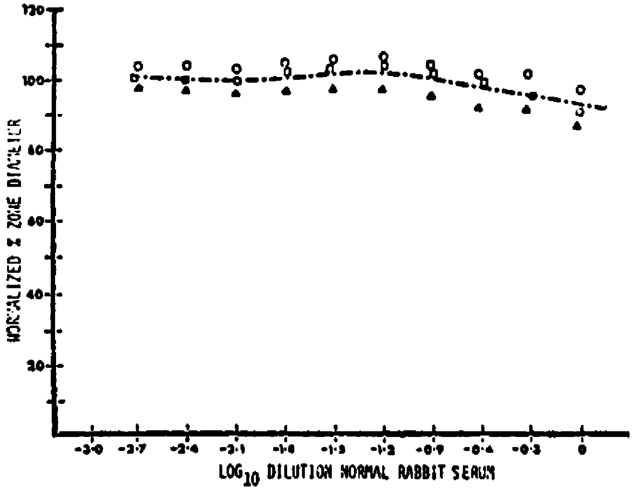
·————— = Rabbit anti-PLA
Standard deviation = 5.0

Fig 12B (p. 77): Individual graphs of data combined in Fig. 12, with all data points plotted.


120
100
80
60
40
20
NORMALIZED % ZONE DIAMETER
-3·0
-2·7
-2·4
-2·1
-1·8
-1·5
-1·2
-0·9
-0·6
-0·3
0
LOG10 DILUTION SERUM COMPONENTS


NORMALIZED % ZONE DIAMETER
120
100
80
60
40
20
-3.0
-2.7
-2.4
-2.1
-1.8
-1.5
-1.2
-0.9
-0.6
-0.3
0
LOG10 DILUTION BOVINE GAMMA GLOBULIN


NORMALIZED % ZONE DIAMETER
120
100
80
60
40
20
-3.0
-2.7
-2.4
-2.1
-1.8
-1.5
-1.2
-0.9
-0.6
-0.3
0
LOG10 DILUTION BOVINE SERUM ALBUMIN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20
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-2.7
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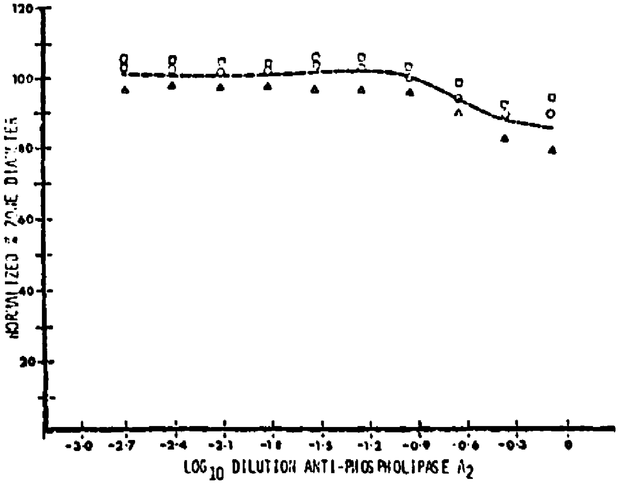
-1.5
-1.2
-0.9
-0.6
-0.3
0
LOG10 DILUTION NORMAL RABBIT SERUM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0
LOG10 DILUTION ANTI-PHOSPHOLIPASE A2

The apparent hemolysis-enhancing effect of bovine serum albumin was also unexpected. Albumin is the most electronegative of the serum proteins, (22) and, operating on the charge interaction hypothesis set forth previously, might be predicted to interfere with BV by binding to it and reducing its activity. Why albumin appeared to behave in the opposite fashion is unknown.

Figure 13 shows the plots obtained when protamine sulfate and lysozyme, both polycationic compounds, were tested for inhibitory activity. As predicted, neither diminished the degree of hemolysis at any concentration.

In contrast are the results in Figure 14. All of the polyanions tested exhibited marked inhibition of hemolysis. In addition, heparin and phosvitin both demonstrated a reproducible "prozone" effect, i.e., each of those inhibitors was more active at some lower concentration than it was at full strength. This behavior is typical of many true antigen-antibody reactions; it has now been shown to occur in less specific interactions as well.

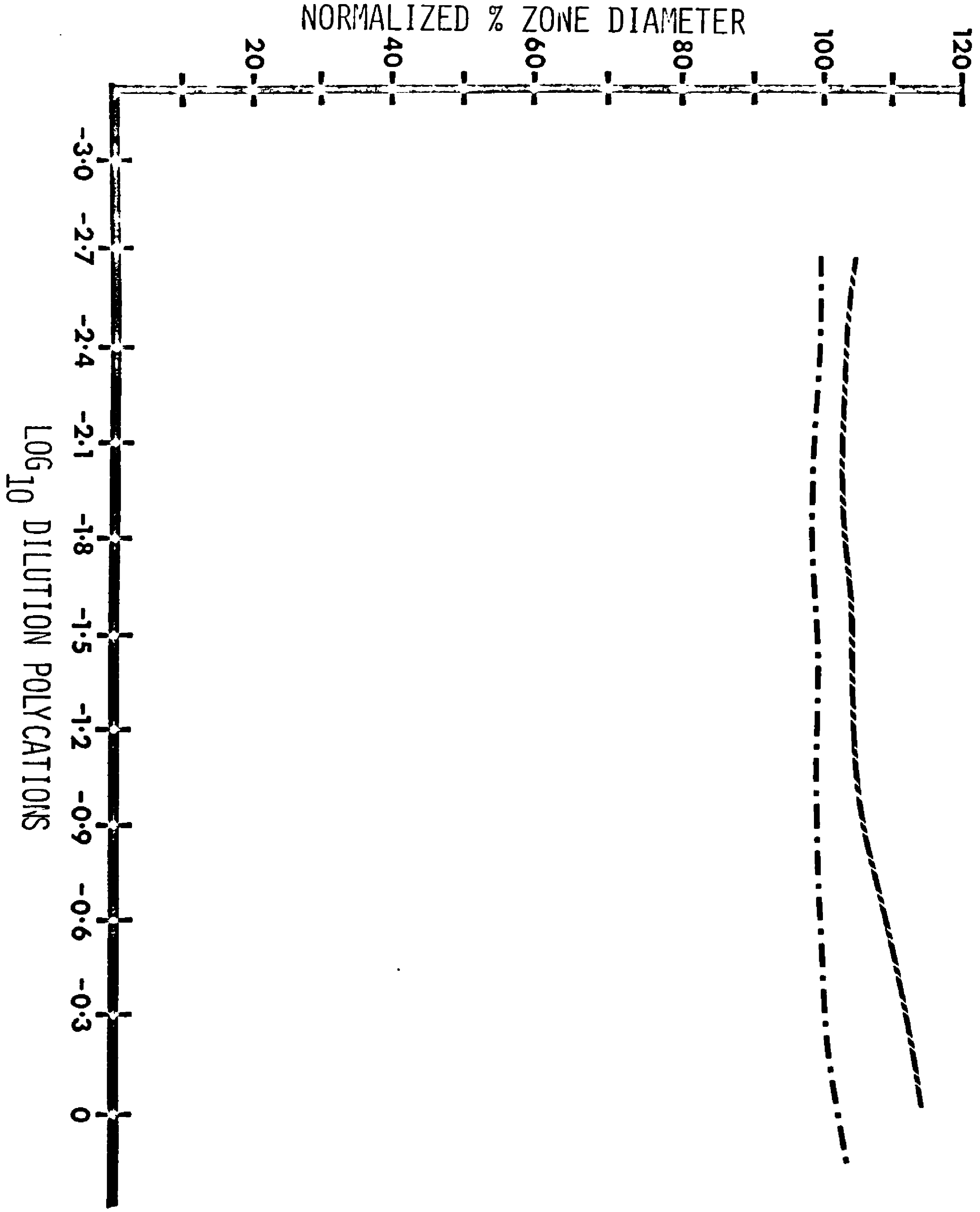
Figure 15 presents the results obtained when immune and normal HL were assayed for inhibitory activity. As was often the case in this investigation, insufficient HL was available to permit replicate studies; the data presented were derived from single runs. Both IHL and NHL at high concentrations appeared to inhibit hemolysis. NHL exhibited a slight prozone effect. Total protein content for three of the four lots of HL assayed is listed in

Fig. 13. Log$_{10}$ dilutions polycations in 1250 µg/ml BV versus normalized % zone diameter.

—·—·—·—·—· = Protamine sulfate, 2500 µg/ml initial.
Standard deviation = 4.4

—·—·—·—·· = Lysozyme, 2500 µg/ml initial.
Standard deviation = 4.9

Fig. 13B (p. 81): Individual graphs of data combined in Fig. 13, with all data points plotted.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-2·7
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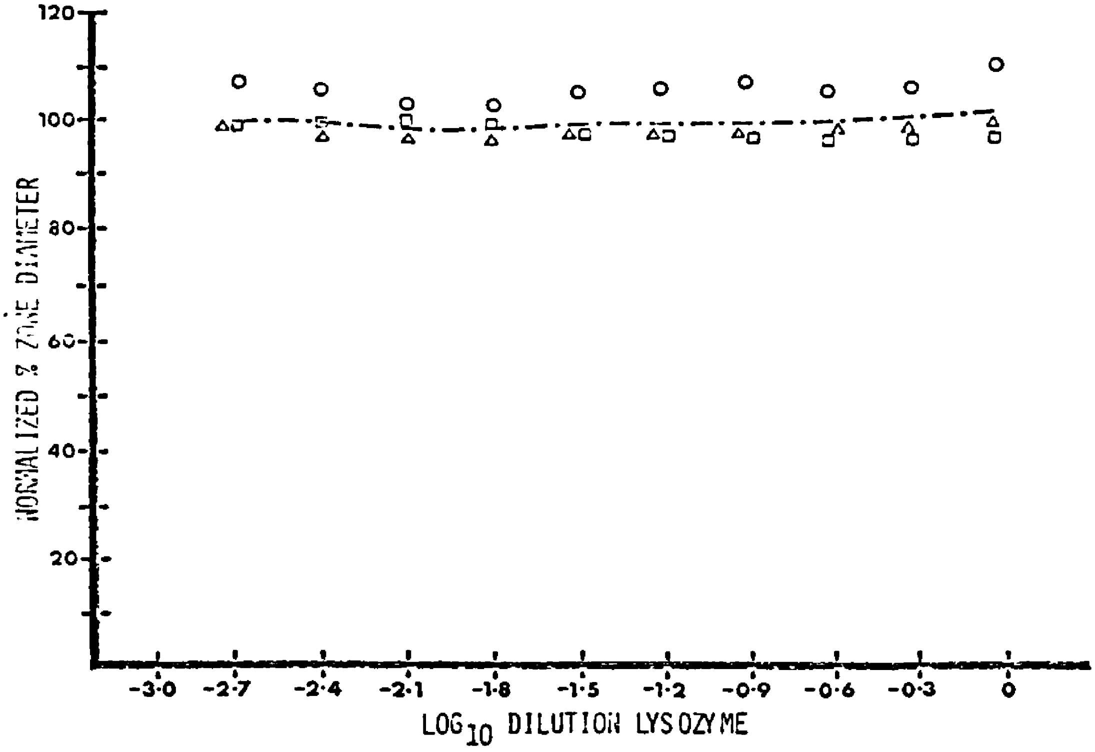
-0·3
0
LOG_{10} DILUTION POLYCATIONS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NORMALIZED % ZONE DIAMETER
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-2·7
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-1·5
-1·2
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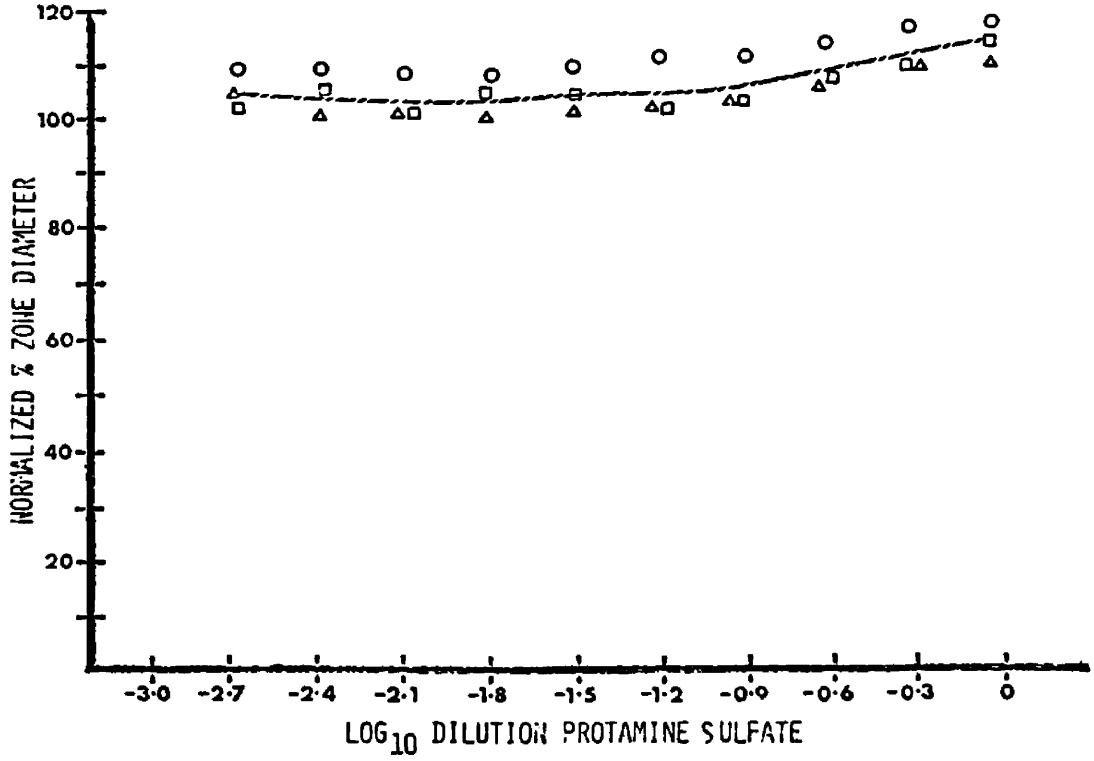
-0·3
0
LOG$_{10}$ DILUTION LYSOZYME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NORMALIZED % ZONE DIAMETER
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-2·7
-2·4
-2·1
-1·8
-1·5
-1·2
-0·9
-0·6
-0·3
0
LOG$_{10}$ DILUTION PROTAMINE SULFATE

Fig. 14. Log_{10} dilutions polyanions in 1250 µg/ml BV versus normalized % zone diameter.

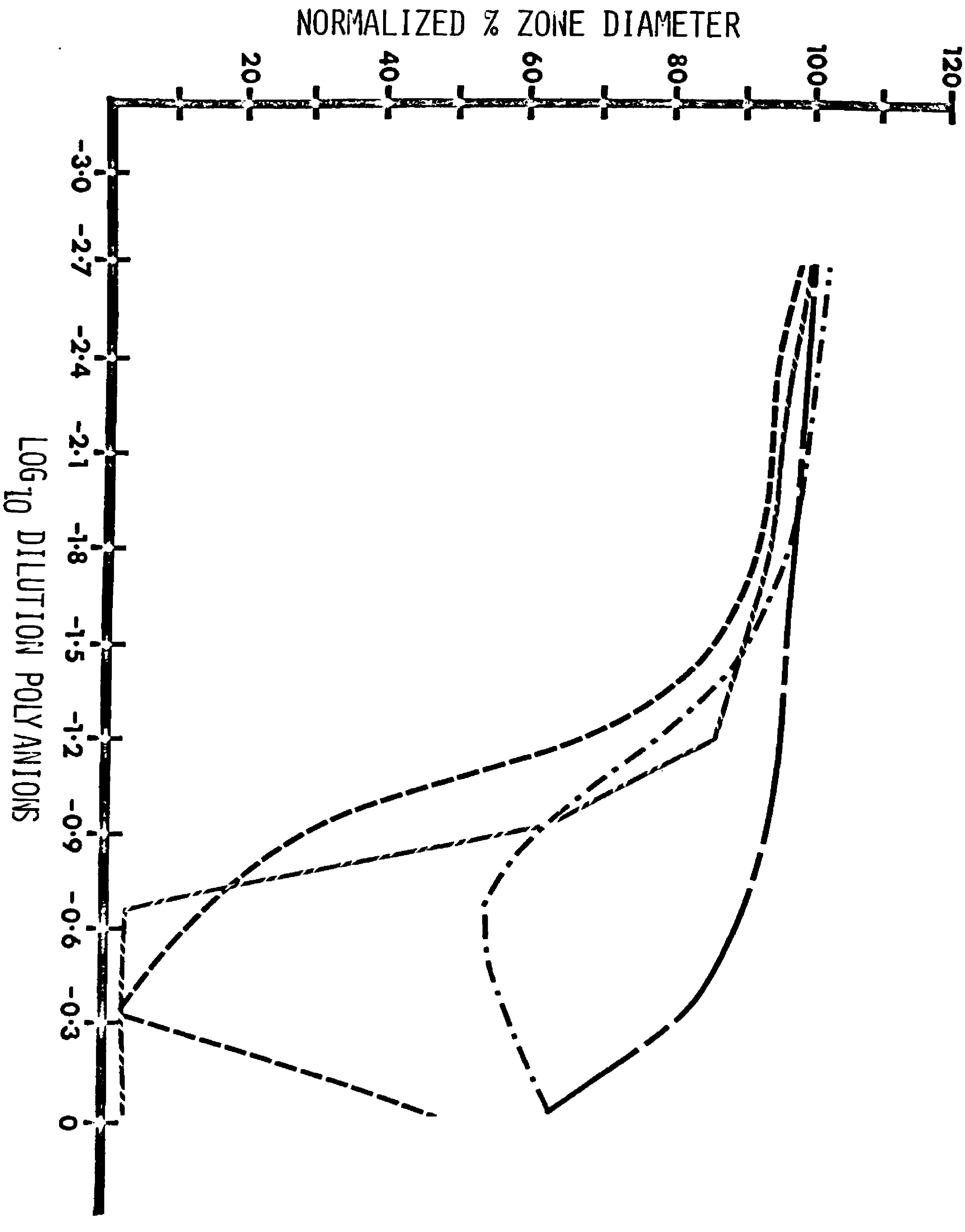
——— — ——— = Endotoxin (LPS), 2500 µg/ml initial.
Standard deviation = 2.3

·—·—·—·– = Phosvitin, 2500 µg/ml initial.
Standard deviation = 7.9

-—-—-—-—- = Sodium polyanethol sulfonate, 2500 µg/ml initial.
Standard deviation = 2.0

•———————• = Sodium heparin, 2500 µg/ml initial.
Standard deviation = 12

Fig. 14B (p. 84): Individual graphs of data combined in Fig. 14, with all data points plotted.



NORMALIZED % ZONE DIAMETER
20
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60
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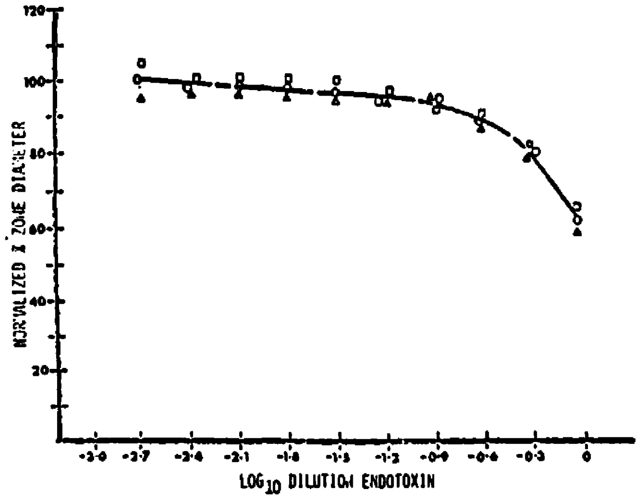
100
120
-3·0
-2·7
-2·4
-2·1
-1·8
-1·5
-1·2
-0·9
-0·6
-0·3
0
LOG$_{10}$ DILUTION POLYANIONS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NORMALIZED % ZONE DIAMETER
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-2.7
-2.4
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-1.5
-1.2
-0.9
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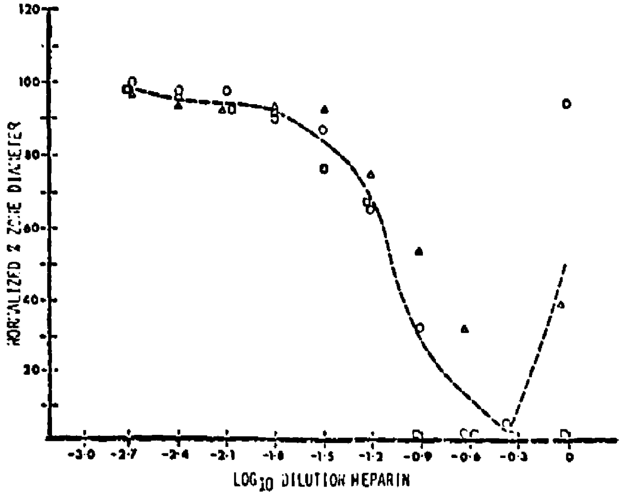
0
LOG$_{10}$ DILUTION ENDOTOXIN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NORMALIZED % ZONE DIAMETER
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-2.7
-2.4
-2.1
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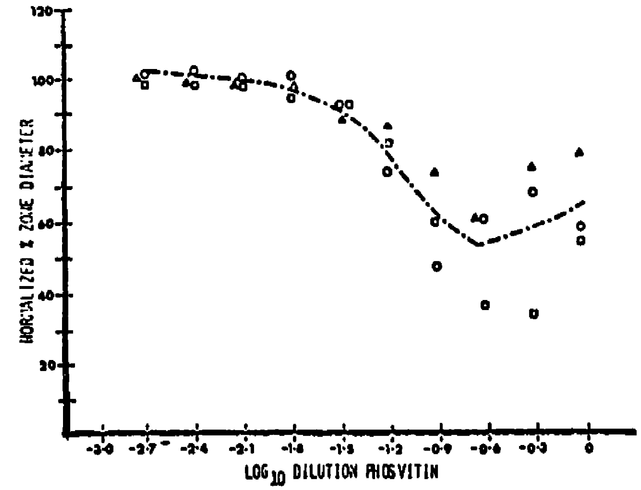
-1.5
-1.2
-0.9
-0.6
-0.3
0
LOG$_{10}$ DILUTION HEPARIN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NORMALIZED % ZONE DIAMETER
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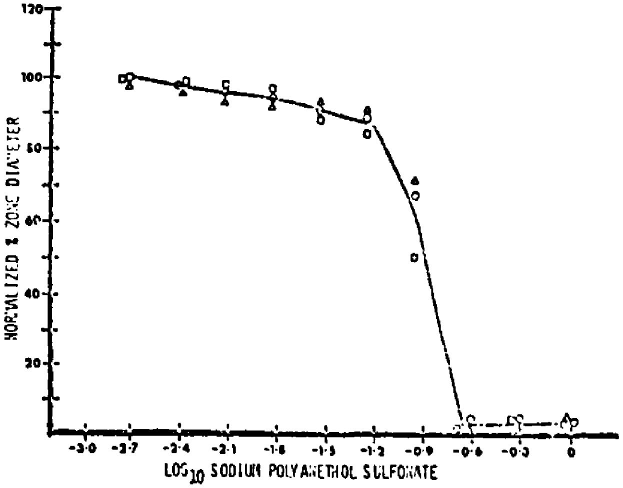
-2.7
-2.4
-2.1
-1.8
-1.5
-1.2
-0.9
-0.6
-0.3
0
LOG$_{10}$ DILUTION PHOSVITIN


120
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NORMALIZED % ZONE DIAMETER
-3.0
-2.7
-2.4
-2.1
-1.8
-1.5
-1.2
-0.9
-0.6
-0.3
0
LOG$_{10}$ SODIUM POLYANETHOL SULFONATE

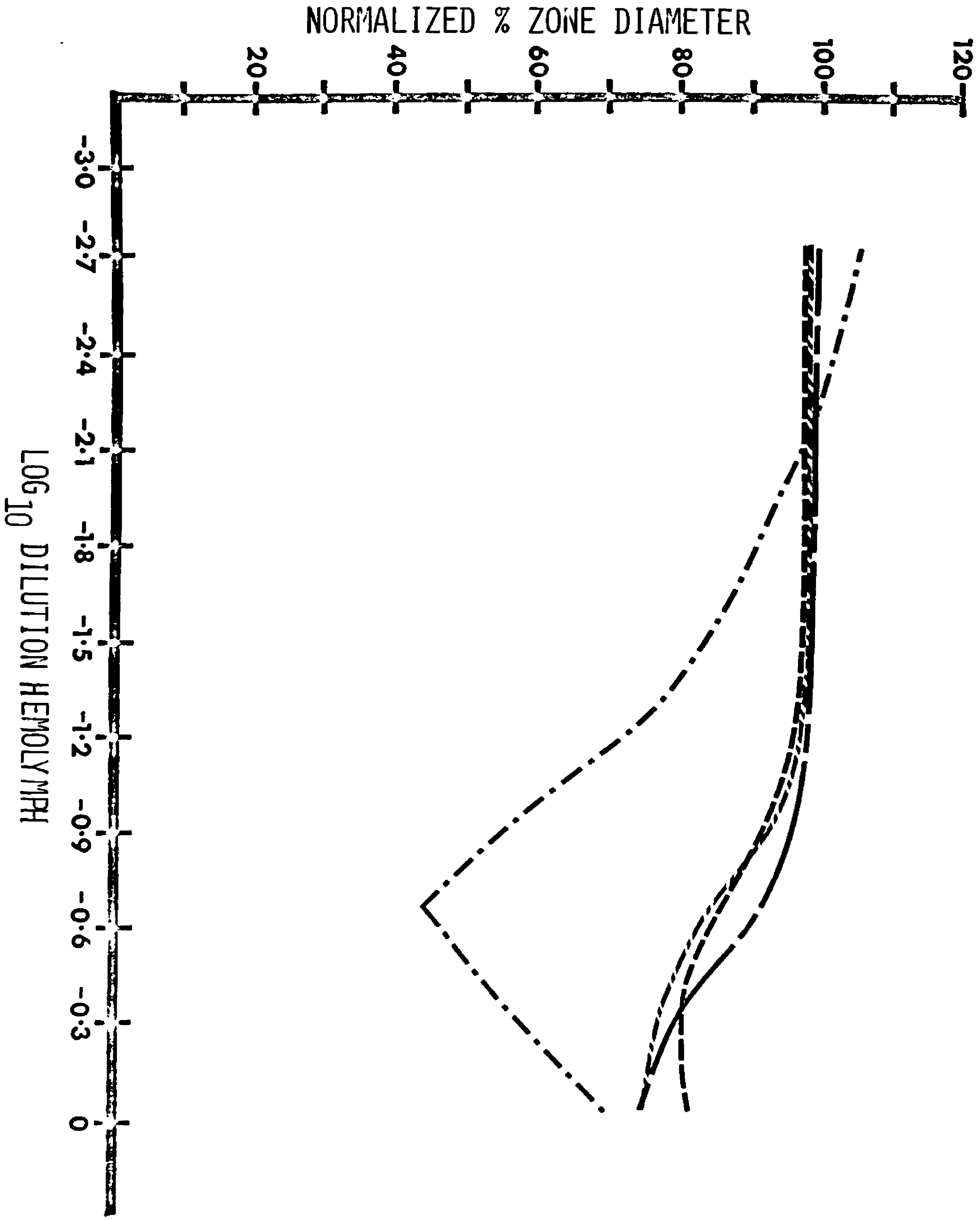
Fig. 15. Log_{10} dilutions hemolymph in 1250 µg/ml BV versus normalized % zone diameter.

·—·—·—·—· = NHL 3/19/82 Total protein: 10.4 mg/ml

— — — — — — — — — = NHL 5/4/82 Total protein: not determined

——— — ——— = IHL 5/4/82 Total protein: 10.5 mg/ml

—-—-—-—-—-- = IHL 6/2/82 Total protein: 13.5 mg/ml


NORMALIZED % ZONE DIAMETER
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100
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-3·0
-2·7
-2·4
-2·1
-1·8
-1·5
-1·2
-0·9
-0·6
-0·3
0
LOG_{10} DILUTION HEMOLYMPH

Figure 15; protein content did not appear to correlate with the degree of inhibition.

The following overall conclusions may be drawn from the radial diffusion assay results. First, PLA was inactive under the conditions of the assay, therefore all hemolysis observed was due to the action of melittin. Second, all (two) of the polycationic substances tested failed to inhibit the hemolytic activity of melittin, whereas all (four) of the polyanions tested did so. This substantiated the hypothesis that compounds which inhibited some of the binding and hemolytic activities of BV tended to do so via electrostatic interaction. Third, the effect of HL on melittin's hemolytic activity was analogous to that of polyanions; HL appeared to be slightly more inhibitory than NRS, and there seemed to be no significant difference in the degree of inhibition caused by IHL as compared to NHL. The inhibitory effect of HL did not appear to be related to its total protein content.

Inhibition of PLA activity

In order to assess the effect of polyions and HL on the hemolytic activity of PLA, the radial diffusion assay previously described was modified by the inclusion of $CaCl_2 \cdot 2H_2O$ (35 µg/ml) in the substrate as a source of Ca^{++}. All concentrations of PLA tested in the Ca^{++}-supplemented gels failed to cause hemolysis. However, when soy lecithin as well as Ca^{++} was incorporated into the gels, PLA-mediated hemolysis did occur, but it was superimposed on a generalized spontaneous hemolysis that rendered the plates difficult to read and hence unreliable. Therefore, attempts to quantify PLA activity with the radial diffusion procedure were abandoned. Two standard assays for PLA activity were modified to evaluate the effect that HL and other compounds might have on the enzyme.

The reaction mixture for the first assay consisted of sheep erythrocytes and soy lecithin suspended in a PBS-Ca^{++} buffer. When PLA was added to the system, it hydrolyzed lecithin to lysolecithin, which in turn caused lysis of the sheep cells. The reaction was terminated by the addition of ice-cold buffer, and the degree of hemolysis was read in a spectrophotometer and expressed as a percent of the total hemolysis obtained by lysing a control cell suspension with water. The amount of hemolysis reflected the activity of the enzyme present.

Various quantities of HL, rabbit sera, and polyionic compounds were evaluated for PLA-inhibiting capacity by adding them to portions of the reaction mixture along with a standard amount of PLA. The resulting hemolysis was compared to that generated by a non-inhibited PLA control.

As mentioned under Materials and Methods, the fact that the HL samples tested were preserved in glycerol caused some problems with the initial trials of this assay. When the HL-PLA reaction mixtures were diluted with cold buffer to terminate the reactions prior to spectrophotometric determination of hemolysis, complete lysis of the indicator cells resulted. The glycerol content of the HL-PLA reaction mixtures was calculated to be 2.2%. Human erythrocytes will generally withstand a sudden transition of 4% in the glycerol content of their suspending medium without undergoing osmotic lysis (19); sheep cells appeared to be more fragile. The problem was solved by incorporating 2.2% glycerol into the cold buffer used for dilution. Unfortunately, this complicated the assay by requiring that 2.2% glycerol be added to the reaction mixtures of all non-HL compounds tested. The general protocol for sample preparation is given in Table 1.

Table 2 presents the results of tests of polyionic compounds. Neither polyanions (heparin, sodium polyanethol sulfonate) nor polycations (protamine sulfate, lysozyme) inhibited PLA activity. The result for heparin had a large standard deviation; the apparent enhancement

Table 1: Sample preparation protocol for PLA hemolysis-inhibition assay

	Blank	Uninhibited control	100% hemolysis control	Inhibition tests: hemolymph	Inhibition tests: other
HL or other compound	-	-	-	10 µl	10 µl
50% glycerol in PBS	10 µl	10 µl	10 µl	-	10 µl
PBS-Ca	20 µl	10 µl	10 µl	10 µl	-
PLA in PBS 125 or 250 µg/ml	-	10 µl	10 µl	10 µl	10 µl

Table 2

Effect of polyionic compounds on PLA -mediated SRBC lysis[a]

Polyion	Hemolysis (%)	Standard deviation
uninhibited control	87	3
heparin	95	6
sodium polyanethol sulfonate	89	2
protamine sulfate	98	0.5
lysozyme	88	0

[a] All results represent the average of two replicate assays. 2.5 µg of PLA was employed in each assay.

of hemolysis was probably spurious. In addition, the value of 98% hemolysis obtained with protamine sulfate did not differ significantly from the uninhibited control.

In the assay just described, a standard amount of 2.5 ug of PLA was employed in each test. It was felt that decreasing the amount of enzyme might increase the sensitivity of the procedure; therefore, 1.25 ug of PLA was used in subsequent trials. The results obtained when HL and NRS were assessed for inhibitory activity are shown in Table 3. Again, no inhibition of PLA activity was seen. The value of 82% hemolysis obtained for one lot of NHL suggested a significant enhancement (p 0.05). To determine if some component innate to HL might be able to serve as a substrate for PLA, resulting in the production of excess lysolecithin and hence enhanced hemolysis, the assay was repeated with the omission of exogenous lecithin from the reaction mixtures. Various amounts of HL were tested in order to determine if the hemolysis-enhancing effect might be related to the HL protein content. Results are given in Table 4. Note that in this instance, no hemolysis was obtained (or expected) in the control tube (buffer blank), since no substrate of any kind was added to that tube. Two lots of IHL with protein contents of 5.3 and 6.5 mg/ml failed to cause hemolysis when 10 ul portions were tested. Increasing the volume of 6.5 mg/ml HL to 30 ul did result in a slight amount of hemolysis. The single lot of NHL assayed was high in protein

Table 3

Effect of HL and NRS on PLA -mediated SRBC lysis[a]

Component	Hemolysis (%)	Standard deviation
uninhibited control	54[b]	4
NHL 5/1/82	82	0
IHL 5/1/82	69	6
NHL 5/4/82	76	4
IHL 5/4/82	57	6
NRS	52	3

[a] HL and NRS results represent the average of 2 replications. 1.25 µg of PLA was employed in each assay.

[b] Control value represents average of 16 replications.

Table 4

Effect of utilizing HL or NRS as sole substrate on PLA-mediated SRBC lysis [a]

Component	Total protein of component (mg/ml)	Hemolysis (%)	Standard deviation
buffer [c] (blank)	-	0	0
IHL 5/4/82	5.3	0	0
NHL 6/2/82	12.5	54	5
NHL 6/2/82 1:2 in buffer	6.3	3	5
IHL 6/2/82	6.5	0	0
IHL 6/2/82 [d] 3X standard amount	6.5	16	6
NRS	38.5	0	0

[a] All results represent the average of 2 replications.

[b] Protein content estimated by UV absorption.

[c] Buffer: glycerol 1:2 in PBS.

[d] 30 µl HL used in place of 10 µl.

(12.5 mg/ml) and caused a marked amount of hemolysis. When it was diluted 1:2 in PBS and retested, its hemolysis-engendering capacity was essentially abolished. NRS (protein content 38.5 mg/ml) did not cause hemolysis when provided to PLA as sole substrate.

The overall conclusions drawn from the preceding experiments were as follows. First, neither NHL, IHL, NRS, nor any of the polyions tested proved capable of inhibiting the enzymatic activity of PLA in this assay. It was shown previously that the hemolytic action of melittin could be antagonized by HL and polyanionic compounds, which appeared to inhibit melittin by binding to it via electrostatic attraction. Therefore, the mechanisms by which HL forms precipitin lines with melittin and with PLA may be distinct. Second, some lots of HL appeared to enhance the ability of PLA to cause red cell lysis; this property may be related to the HL sample's content of indigenous phospholipids capable of serving as a PLA substrate. Third, the capacity of HL to serve as a substrate seemed to correlate with its total protein content, but the details of this relationship were obscure.

A second assay for inhibition of PLA activity by HL and rabbit sera was modified from a previously published colorimetric technique as described under Materials and Methods. Results are shown in Table 5. For HL samples 2, 3, and 5, the amount of inhibition detected appeared to be significant; in each case the 95% confidence range

Table 5

Colorimetric assay for inhibition of PLA activity by HL, NRS, and rabbit anti-PLA [a]

Sample no.	Component	Total protein of component[b] (mg/ml)	Hydrolysis of substrate (%)	Standard deviation
1	uninhibited control	-	30.4	0.4
2	NHL 5/4/82	not done	25.2	0
3	IHL 5/4/82	5.3	28.6	0.2
4	NHL 6/2/82 1:2 in buffer[c]	6.3	28.1	0.8
5	IHL 6/2/82	6.5	28.3	0.3
6	NRS	38.5	24.7	2.2
7	anti-PLA	43.3	17.8	0.9

[a] All results represent the average of 3 replications.

[b] Protein content estimated by UV absorption.

[c] Buffer: glycerol 1:2 in PBS.

associated with the mean percent hydrolysis of each test did not overlap the 95% range of the uninhibited control. However, neither immune nor normal HL proved as effective as normal rabbit serum in inhibiting the hydrolysis of lecithin by PLA, while rabbit anti-PLA cut back the enzyme's activity by nearly 50%. The inhibition caused by NRS was unexpected and unexplained. If, as suggested by the results described in Table 4, some fraction of HL was capable of serving as a substrate for PLA, the apparent inhibition by HL seen in Table 5 might actually have represented a manifestation of excess substrate, because the colorimetric reaction detected substrate remaining, not the enzymatic hydrolysis product lysolecithin. In any event, neither of the assays described indicated the presence of a PLA antagonist unique to IHL. Whatever inhibitory property may have been present seemed to have been common to both immune and normal cockroaches.

Radioimmunoprecipitation

In previous Results sections it was shown that PLA and HL could react to form precipitin lines in Ouchterlony diffusion gels; electrophoresis experiments demonstrated that ^{125}I-PLA appeared to bind to certain components of both immune and normal HL. The radioimmunoprecipitation procedure was performed in order to determine if a binding affinity for PLA resided in specific HL fractions. Briefly, samples of NHL, IHL, and NRS were labeled with ^{125}I and allowed to react with unlabeled PLA. Labeled-HL-PLA complexes were precipitated with rabbit anti-PLA conjugated to Pansorbin. The precipitates were washed free of unbound label, then solublized in SDS sample solublizing fluid and electrophoresed on an SDS-polyacrylamide gradient gel. Autoradiograms of the gel revealed the location of labeled HL and NRS proteins.

The results are presented in Figure 16. Samples 2, 3, and 4 in Figure 16A represented the electropherograms obtained when labeled NHL, IHL, and NRS respectively were precipitated with rabbit anti-PLA-coated Pansorbin. Samples 5 and 6 were nonspecific binding controls; labeled IHL and NRS were mixed with anti-PLA-coated Pansorbin, but no PLA was added to the system. Sample 7 was a positive control: labeled IHL was precipitated with rabbit anti-HL linked to Pansorbin. The dense bands common to samples 2 through 7 that were visible between the 66 and 45 K molecular weight markers in the stained gel (A) but not in the autoradiogram (B) were due to proteins that were liberated from the Pansorbin by the SDS solublizing treatment.

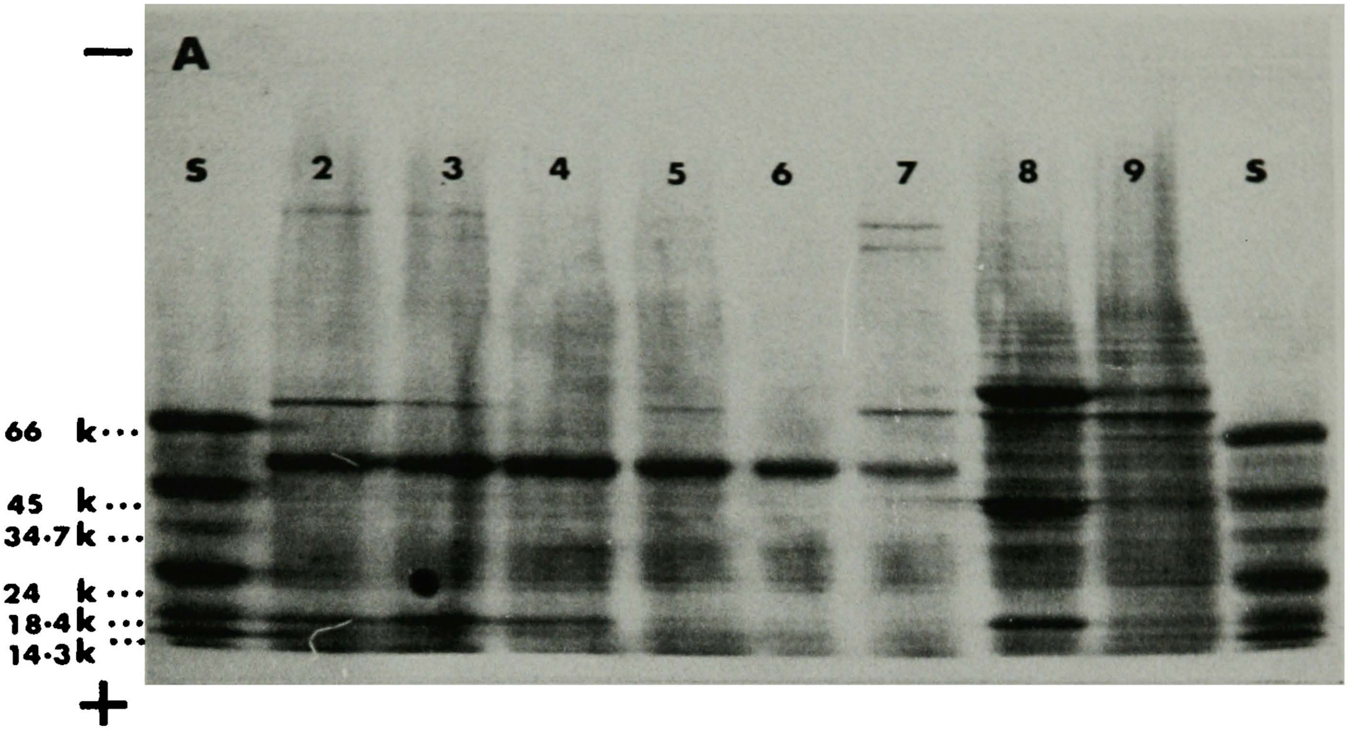
When the autoradiograms of samples 2 and 3 were compared with the negative control (sample 5), it was evident that the incorporation of PLA into the precipitation

Fig. 16: Results of radioimmunoprecipitation procedure

A: SDS-PAGE of samples derived from radioimmunoprecipitation. Polyacrylamide gradient ranges from approximately 5 to 18%. Tris-glycine buffer; pH 8.3.

- S: molecular weight standards
- 2: ^{125}I-NHL 6/14/82 + PLA + anti-PLA :protein A
- 3: ^{125}I-IHL 6/14/82 + PLA + anti-PLA :protein A
- 4: ^{125}I-NRS + PLA + anti-PLA :protein A
- 5: ^{125}I-IHL 6/14/82 + anti-PLA :protein A
- 6: ^{125}I-NRS + anti-PLA :protein A
- 7: ^{125}I-IHL + anti-HL:protein A
- 8: IHL 6/14/82 bled manually from 1 roach
- 9: IHL 6/14/82 obtained by usual centrifugation method

B: autoradiogram of gel described in A.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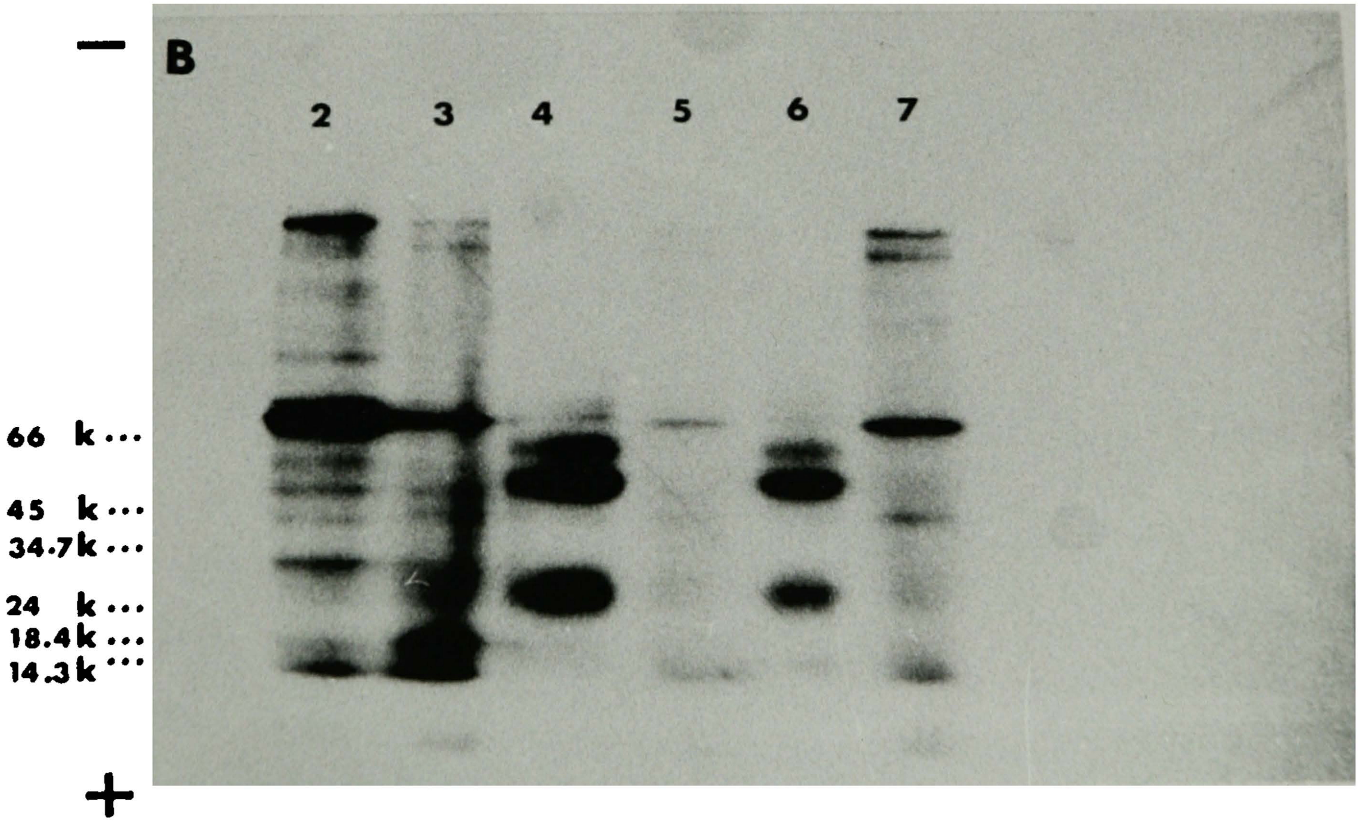
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system caused a variety of both immune and normal HL proteins to be pulled out of solution. In both cases, a heavy concentration of label was seen in the vicinity of the 66 K marker. A corresponding band was visible in all the HL samples shown in the stained gel. In addition, PLA had complexed with a high molecular weight NHL fraction (sample 2, cathodal end of pattern) and with a low molecular weight fraction of IHL (sample 3, anodal end), as well as to a lesser extent in both samples with various fractions of intermediate mobility. The appearance of the autoradiogram of sample 4 suggested that PLA bound to and furthered the precipitation of several fractions of labeled NRS. In comparison, the analogous nonspecific binding control (sample 6) exhibited a similar but somewhat fainter pattern. The intensity of the control banding was unexpected. It is possible that the anti-PLA coated Pansorbin used to precipitate the samples was not fully saturated with immunoglobulin, in which case the unoccupied protein A moeities of the Pansorbin could have bound to radiolabeled immunoglobulins in the NRS in both samples 4 and 6. This would have resulted in a precipitation of labeled NRS immunoglobulins independent of any effect of PLA. Significantly, the major radiation bands in samples 4 and 6 were found in the 45 to 66 K and 24 K molecular weight ranges; the published molecular weight values for isolated immunoglobulin heavy and light chains are 55 to 75 K and 23 K respectively (51).

Overall, the results of this experiment suggested that both normal and immune HL contained components capable of binding to PLA, and that of those components, a fraction with a molecular weight of approximately 66,000 daltons appeared to exhibit the highest affinity for the enzyme. It was not determined if this binding capacity was related to the protection against BV elicited in immunized cockroaches by BV toxoid.

Discussion

The original objectives of this project were to isolate and to characterize a substance from cockroach hemolymph which was capable of binding to a component of BV, and to devise an *in vitro* assay to quantify the difference in immune activity between normal cockroaches and insects which had been immunized with BV toxoid. However, the results of early experiments indicated that the binding interaction of BV with HL was more complex than was appreciated initially. Many of the subsequent experiments were conducted in an attempt to dissect and to clarify the nature of that interaction.

When HL samples were mixed with BV or with ^{125}I-labeled PLA and subjected to agarose or polyacrylamide gel electrophoresis, the results suggested that both normal and immune HL contained components capable of binding to BV and to the purified PLA derived from BV. When HL electropherograms were treated with the protein stain Coomassie Blue, the banding patterns produced by normal and immune HL were usually identical; no bands unique to either NHL or IHL were consistently observed. When ^{125}I-PLA was bound by the HL samples, it generally appeared to be associated with a protein band of intermediate mobility that was common to both immune and normal HL. Attempts to confirm the location of this band with an electroblotting technique proved unsuccessful.

It had been reported previously (Rheins, 36) that when IHL was diffused against BV in gel diffusion tests, a strong line of precipitate was obtained, whereas NHL yielded a faint line or failed to react entirely. Since the results of the electrophoresis experiments conducted for the present study indicated that both immune and normal HL could bind PLA, additional immunodiffusion tests were performed to determine if both immune and normal HL would demonstrate precipitate-forming activity. Because it was felt that PLA, with a pI of 10.5 (50) might bind to HL components via electrostatic attraction, the diffusion tests were carried out at several levels of pH; it was reasoned that changing the pH of their environment would alter the intrinsic charges of the BV and HL proteins, and would thus alter their binding properties. The original diffusion tests performed by Rheins were conducted in a gel containing 0.6 M KCl (36). The amount of salt in the solvent is known to affect the solubility of proteins (27), therefore, the immunodiffusion tests in the present study were run both with and without the inclusion of 0.6 M KCl. The information derived from these experiments indicated that both normal and immune HL could form lines of precipitate when diffused against BV. In the present study, NHL yielded a stronger precipitin reaction than did IHL. The immunodiffusion results also suggested that HL contained elements that were capable of combining independently with both PLA and with a separate BV component, and showed that HL failed to react with either whole BV or PLA at a pH less than or equal to 5.0. Attempts to inhibit the formation of the precipitates with various simple carbohydrates were unsuccessful, thus indicating that the HL component involved was not a lectin specific for any of the common sugars tested.

When HL proteins were separated by electrophoresis and diffused against whole BV, two distinct precipitin lines were observed. One of them was shown to be formed by the reaction of HL with PLA. The other proved analogous to the non-specific lipid-containing line formed by the diffusion of BV against electrophoretically separated normal rabbit serum. The venom component responsible for this nonspecific precipitate was postulated to be melittin.

The interaction of HL with melittin. With the nature of the two precipitation systems thus established, it was evident that the reaction of melittin with HL appeared to be strongest in the diffusion gels run at pH 9.0 in the presence of 0.6 M KCl, whereas the precipitates formed by the reaction of HL with PLA were most visible at pH 7.5 in the absence of KCl. The working hypothesis of HL-BV interaction set forth in the Introduction assumed that the components in question were bound together by ionic attraction. The results of the immunodiffusion experiments indicated that more than a single binding mechanism might be involved. Since the precipitates formed by the reaction of HL with melittin were enhanced in a high salt environment, it was felt that ionic attraction alone could not provide a reasonable explanation of their development. Melittin contains 26 amino acid residues. The asymmetric arrangement of basic and nonpolar amino acids within the molecule suggest that it can participate in both ionic and hydrophobic interactions (31, 50). In aqueous solutions, melittin has been shown to exist as an aggregate of four or more molecules (16). Apparently, it is energetically more favorable for the individually cationic melittin moeities to associate hydrophobically than it is for them to be dispersed by electrostatic repulsion. Fluorometric studies have demonstrated that when phospholipid micelles were added to an aqueous solution of melittin, conformational changes in one or both of the compounds took place which permitted hydrophobic bonding to occur between the fatty acid chains of the lipid and the nonpolar portions of melittin (31, 32). The addition of 0.15 M NaCl to this system greatly facilitated the interaction (31). The salt was thought to influence the components in a fashion such that their hydrophobic moeities became more exposed to water and hence more accessible to one another (54). It should be recalled that the melittin-HL immunodiffusion lines described in the present study were enhanced by the addition of KCl to the diffusion gels, and that the precipitates consistently reacted with the lipid stain oil red O. These observations, when considered together with the information on melittin-phospholipid reactions just presented, suggested that the interaction of melittin with HL observed in the immunodiffusion gels might have represented the hydrophobic association of melittin with phospholipids or phospholipoproteins innate to HL.

The interaction of melittin with polyanions. In a subsequent experiment, heparin was found to form a precipitate when diffusd against whole BV (but not PLA) in agarose gels. The structure of heparin is well characterized: it is a highly anionic sulfonated polysaccharide. The molecular weight of commercial preparations ranges from 10,000 to 15,000 daltons (28). The reaction of heparin with BV was noted to be more pronounced at pH 7.5 than at pH 9.0. The binding of BV cations to heparin was blocked by a high environmental salt concentration, and inhibited by the substitution of agar (another anionic polysaccharide) for agarose, a relatively uncharged agar derivative. These results were interpreted to indicate that heparin reacted with BV primarily, if not exclusively, by electrostatic attraction, and that PLA did not participate in the reaction. The results of the radial diffusion assay indicated that once again, melittin was the BV component involved. That diffusion assay had evolved from the observation that both HL and heparin could inhibit the hemolysis of sheep erythrocytes by BV; it was hoped that the inhibition of hemolysis might prove to correlate with the protection against BV observed in cockroaches that had been immunized with BV toxoid. The conditions of the assay (*i.e.*, no exogenous Ca^{++} or lecithin supplied) were such that the PLA component of the BV employed was inactive: it was felt that all of the hemolysis observed was attributable solely to the action of melittin. That contention was supported by the observation that purified PLA at a concentration of 2 mg/ml failed to cause hemolysis when tested, whereas whole BV which had been heated to approximately 97°C for 40 min produced a hemolytic zone equivalent to that formed by a sample of unheated venom of equal concentration. The results of the assay showed that the two polycationic compounds tested failed to block the hemolytic activity of melittin, whereas all of the polyanions evaluated proved inhibitory. Both immune and normal HL displayed an inhibitory capacity that was slightly greated than that seen with NRS, but less marked than the effect caused by most of the polyanions. The inhibitory property of HL did not appear to be related to its total protein content. The polyanions tested were quite varied in their molecular structure. Heparin has been described previously. Phosvitin is a polyphosphorylated egg yolk protein of 21,000 daltons in molecular weight (41). Endotoxin from Gram negative bacteria (LPS) is a more complex molecule. The lipid moeity of LPS from Salmonella sp. is a glycophospholipid consisting of a chain of Beta-1,6-D-glucosamine disaccharide units, connected by 1,4'-pyrophosphate bridges and with all the OH groups substituted. One of the substituents is a polysaccharide; the others are long-chain fatty acids. Because there are five fatty acids and two phosphates per disaccharide, this material behaves as an amphipathic molecule (8). Sodium polyanetholsulfonate is a polymer formed by the sulfonated derivative of 1-methoxy-4-propenylbenzene (41). The obvious quality shared among these four compounds is their polyanionicity. It seems probable that their melittin-inhibiting capacity resides in that property. It was stated that melittin presumably exists in aqueous solutions as a cationic aggregate of several molecules (31). Although the lysis of red cell membranes by melittin is believed to be a consequence of hydrophobic interaction (31, 32), it seems plausible that the initial contact of a melittin aggregate with the negatively charged (28) erythrocyte surface might be through ionic attraction. Anionic macromolecules might inhibit erythrocyte lysis by providing melittin with alternate binding sites. Proteins can only behave as polyanions at a pH above their isoelectric points. Perhaps this is why no immunodiffusion lines attributable to the interaction of melittin with HL were observed at a pH less than or equal to 5.0. Although ultimately the formation of a precipitate might have been a consequence of hydrophobic bonding, the initial contact of the HL and melittin molecules might be dependent on ionic attraction, therefore, complex formation would have been inhibited at a low pH where both HL and melittin were positively charged.

The interaction of HL and polyionic compounds with PLA. The reaction of melittin with heparin has been presented as a model of molecular interaction based on ionic attraction. By contrast, the reaction of melittin with HL appears to be an example of hydrophobic bonding. The immunodiffusion precipitates formed by the interaction of HL and PLA were found to be strongest at a pH of 7.2 in the absence of 0.6 M KCl. Unlike the precipitates formed by the reaction of heparin with melittin, they were not strongly affected by the substitution of agar for agarose as a gel support, nor were they significantly disrupted by 0.6 M KCl. Whereas precipitates formed by the reaction of HL with melittin consistently took up the lipid stain oil red O and appeared to be enhanced by the addition of 0.6 M KCL, the HL-PLA precipitates did neither. Therefore, the available evidence suggested that HL-PLA precipitate formation could not easily be classified as being a consequence of hydrophobic or electrostatic interactions. In order to investigate further the interaction of PLA with HL and with polyanions, two different assays for the inhibition of PLA activity were performed. The first of these employed soy lecithin as a substrate. When a standard amount of PLA was added to a suspension of soy lecithin and sheep erythrocytes, the enzyme cleaved its substrate into lysolecithin and fatty acids; the lysolecithin in turn caused hemolysis of the indicator cells, and the degree of hemolysis provided an index of PLA activity. When polyanions or polycations were added to a sheep cell-lecithin suspension along with the PLA, no inhibition of hemolysis was observed. When several lots of immune and normal HL were tested for PLA-inhibiting capacity, they appeared to slightly enhance the activity of the enzyme. A follow-up experiment indicated that HL was capable of serving as a substrate for PLA; other investigators have found that human serum, egg yolk emulsion, and brain tissue homogenates would serve as well (40). The results of the first set of PLA assays were confirmed with a second technique, wherein a colorimetric reaction replaced the indicator function of sheep erythrocytes. Though the results of the latter assay at first seemed to suggest a significant inhibition of PLA activity by HL, closer inspection of the assay system revealed that the apparent inhibition was more likely an artifact arising from the capacity of HL to serve as a PLA substrate.

When considered together with the previously described behavior of HL-PLA precipitates in immunodiffusion gels, the demonstrated inability of HL and of polyanions to inhibit the enzymatic activity of PLA failed to support the hypothesis that HL and PLA interact via electrostatic attraction. In retrospect, the simplicity of the working hypothesis may have been optimistic. The formation of a HL-PLA complex, like many enzyme-substrate interactions, might have represented a constellation of mechanisms involving electrostatic forces, hydrogen bonds, van der Waals bonding, the polarity of the reacting molecules, and their degree of geometric complementarity (1, 47). The nature of the HL-PLA interaction is yet to be elucidated.

Significance of the radioimmunoprecipitation results. Although consideration of the available data failed to provide a definitive explanation for the mode of interaction of PLA with cockroach hemolymph, it did reinforce the assertion that some kind of HL-PLA binding was taking place. The results of the radioimmunoprecipitation procedure supported that claim; they indicated that both normal and immune HL possessed a protein fraction in the 66,000 dalton molecular weight region that demonstrated an apparent affinity for PLA. The fraction was not unique to the hemolymph of immunized insects, and its relationship to the *in vivo* protection of immunized cockroaches against BV remained uncertain.

Conclusions and suggestions for future research. The HL of normal cockroaches and of insects immunized with a BV toxoid was shown to contain components capable of binding to two distinct fractions of whole BV, melittin and PLA. This binding was not inhibited by any of several common sugars tested, and hence could not be attributed to a lectin specific for any of those sugars. The formation of the HL-melittin complex was believed to represent a form of hydrophobic bonding. The mechanism of HL-PLA binding was not resolved. The hemolytic activity of melittin was shown to be inhibited by both immune and normal HL, and by a variety of polyanionic macromolecules. Polycations had no inhibitory effect. It was felt that cationic melittin might initially approach the negatively charged surface of erythrocytes due to electrostatic attraction, and that polyanions might inhibit melittin by providing alternate ionic binding sites. It was shown that the enzymatic activity of PLA was not inhibited by polyanions, polycations, or HL. HL demonstrated some capacity to serve as substrate for the enzyme. Results of a radioimmunoprecipitation experiment suggested that both IHL and NHL may contain a protein of approximately 66,000 daltons in molecular weight that exhibits a binding affinity for PLA.

None of the experimental data derived from this project provided any clear or consistent evidence of any difference in the behavior or composition of NHL as compared to IHL, and it is uncertain as to whether any of the binding phenomena discussed correlate with the immune response demonstrated in cockroaches by Karp and Rheins. It might be that the protective factor generated by immunized cockroaches in response to the BV vaccine is not a unique molecule synthesized de novo in the immunized animals, but rather a normal HL component capable of binding to the BV toxoid. The rise in titer of this component observed following vaccination might represent a slight overproduction of the factor as the insect's homeostatic mechanisms attempt to replace HL components that had complexed with the vaccine.

Since heparin, which is normally synthesized in a variety of mammalian tissues (28), has been shown to inhibit the lytic action of melittin, it might be worthwhile to assay immune and normal HL for heparin content. Cholesterol is known to block the hemolytic activity of PLA by combining with lysolecithin to form an inactive complex (40, 50). A determination of HL cholesterol levels might prove enlightening.

The observation that HL contains a protein with PLA binding affinity warrants further investigation. The fact that this protein has a molecular weight of about 66 K (on the order of an immunoglobulin heavy chain (51)) is intriguing. Much uncertainty exists as to whether or not precursors of the mammalian immune system may be present in invertebrates (25, 30). A comparison of the HL factor's structure with the amino acid sequences of antibody subunits or of T-cell receptor molecules might be revealing. Perhaps the construction of a PLA affinity column would permit the isolation of a quantity of the compound sufficient for detailed analysis. The related problem concerning the nature of HL-PLA precipitate formation might be clarified if the behavior of the system were to be examined in diffusion gels at a pH greater than or equal to 10.5 (*i.e.*, at or above the pI of PLA). Ethanol is known to interfere with hydrophobic bonding in aqueous systems (31). It would be interesting to examine the behavior of PLA-HL precipitates in ethanol-containing gels.

BV is obviously a complex antigen, and one that is not well-characterized. It might prove useful to examine the response of cockroaches immunized with a homogenous antigen such as diphtheria toxin, which has been shown to be active against all eukaryotic cells tested, including yeasts, protozoans, and the cells of higher plants and mammals (8). Another candidate might be the crystallizable protein toxin of *Bacillus thuringiensis*, which is known to be lethal to a variety of insects in the order *Orthoptera* (6, 43).

Chloramine-T labeling of phospholipase A_2 with ^{125}I

1. Place 100 µg of phospholipase A_2 in 100 µl PBS into a glass reaction vial with 50 µl of 0.5 M sodium phosphate, pH 7.5.

2. Add approx 400 µCi of ^{125}I sodium iodide (New England Nuclear, Boston, MA).

3. Start the reaction by adding 25 µl of 3.5 mg/ml Chloramine-T (Sigma) in 0.05 M phosphate buffer, pH 7.5. Mix the components and allow them to react for 60 sec.

4. Stop the reaction by adding 25 µl of 3.5 mg/ml potassium metabisulfite in 0.05 M phosphate buffer, pH 7.5.

5. Remove unreacted ^{125}I by passing the reaction mixture over a column of Sephadex G-25. Elute with 10 mM tricine, pH 8.0. Pool the fractions exhibiting the most radioactivity, and store at 4°C.

Preparation of diazobenzyloxymethyl paper for electroblotting

1. Trim ten pieces of # 589 paper (Schleicher and Schuell, Keene, NH) to 10 X 14 cm (sufficient for gels run in standard vertical electrophoresis chambers).

2. Lay the papers in a single layer on glass plates, and cover each with 2.5 ml of a solution containing 2 g of 1-m-((nitrobenzyloxy) methyl) pyridinium chloride (Sigma) and 1 g sodium acetate (trihydrate) in 25 ml distilled deionized water.

3. Allow the papers to air dry, then heat at 130 - 135°C for 35 min.

4. Wash twice with distilled water and twice with acetone.

5. Air dry, place in a boilable plastic bag (Oster Corp., Milwaukee, WI), add 200 ml of 20% sodium dithionite (aqueous), heat seal, and heat in a water bath at 60°C for 30 min with occasional agitation.

6. Wash papers thoroughly with distilled water until the odor of dithionite is no longer detected (this is best done under a fume hood). Wash once in 30% acetic acid, then again in four changes of distilled water. Air dry. Store at 4°C with dessicant.

7. In order to convert the papers to the active diazo form, soak them in 64 mg of sodium nitrite dissolved in 216 ml cold distilled water and 24 ml concentrated HCl at 4°C for 30 min with agitation. Wash five times in cold distilled water. Use within 15 min.

Tris-gelatin buffer (TGB) for electroblotting procedure

5X concentrated stock:

0.25 M Tris
0.75 M NaCl
5 mM disodium EDTA
0.25% Nonidet P-40 (Sigma)

Working strength:

To 200 ml of 5X stock add 750 ml distilled deionized water plus 50 ml water in which 2.5 g gelatin have been dissolved. Adjust pH to 7.5. Store at 4 C.

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